이학박사 학위논문

Targeting EGFR Signaling Pathway in NSCLC and Malignant Glioma Cells for Radiosensitization

비소세포성 폐암과 악성 교종 세포종에서 EGFR 신호전달계 조절을 통한 방사선 감수성 증강작용에 관한 연구

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협동과정 종양생물학 전공

최 은 정

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Targeting EGFR Signaling Pathway in NSCLC and Malignant Glioma Cells for Radiosensitization

by

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Targeting EGFR Signaling Pathway in NSCLC and Malignant Glioma Cells for Radiosensitization

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ABSTRACT

Targeting EGFR Signaling Pathway in NSCLC and Malignant Glioma Cells for Radiosensitization

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Epidermal growth factor receptor (EGFR) signalings play important roles in the pathogenesis of lung cancer and malignant glioma, and therefore, molecular inhibition of the EGFR signaling pathway represents a promising strategy to enhance the anti-tumor activity of radiation. However, therapeutic resistance has emerged as an important clinical issue. Therefore, we investigated whether inhibitors targeting downstream molecules in pathways associated with EGFR signaling would radiosensitize a panel of non-small cell lung cancer (NSCLC) and malignant glioma cell lines showing activated EGFR.

In the first part, we evaluated the efficacy of targeting EGFR-associated downstream signaling in NSCLC with activated EGFR, mutant K-RAS, or both. Specific inhibition of K-RAS attenuated downstream signaling and increased radiosensitivity of A549 and H460 cells, while inhibition of EGFR did not. A549 cells having a K-RAS mutation at codon V12 were radiosensitized by small interfering RNA (siRNA) targeting this codon. H460 cells harboring mutation at codon V61 were radiosensitized by siRNA targeting this codon. K-RAS siRNA did not radiosensitize H1299 cells possessing wild-type K-RAS. Inhibition

of the phosphoinositide 3-kinase (PI3K)-AKT-mammalian target of rapamycin pathway led to significant radiosensitization of the two cell lines, while selective inhibition of extracellular signal-regulated kinase signaling did not. Inhibitors targeting PI3K-AKT-mTOR pathway also abrogated G_2 arrest following irradiation and induced delay of γH2AX foci formation. A dual inhibitor of Class I PI3K and mTOR increased radiosensitivity of A549 and H460 cells effectively. Inhibition of PI3K-AKT signaling was associated with down-regulation of p-DNA-PK, respectively. While apoptosis was the major mode of cell death when the cells were pretreated with LY294002 or AKT inhibitor VIII, the cells were pretreated by rapamycin or PI103 showed mixed mode of cell death including autophagy.

In the second part, we evaluated whether targeting EGFR-associated signaling would radiosensitize EGFR-activated glioma cells and further increase the radiosensitizing effect of temozolomide (TMZ). Although the result of radiotherapy (RT) for high grade glioma has significantly improved by concurrent and adjuvant TMZ, glioblastoma multiform (GBM) still has a dismal prognosis. Therefore, we tried to identify the effective targeting strategies to improve the therapeutic outcome of concurrent RT and TMZ. We first investigated the efficacy of targeting downstream molecules in EGFR-associated signaling pathway, such as Src, PI3K-AKT-mTOR, and STAT3, in glioma cell lines U251 and T98G. Next, we tested the effect of ligand-independent modulation using an HSP90 inhibitor and epigenetic modulation using a histone deacetylase (HDAC) inhibitor. U251 cells showing a high proportion of methylated methyl guanine transferase (MGMT) were highly responsive to the radiosensitizing effect of TMZ. Treatment with a Src inhibitor, PP2; a dual inhibitor of Class I PI3K and mTOR, PI103; a STAT3 inhibitor, Cpd188; a HSP90 inhibitor, 17-

DMAG; or a HDAC inhibitor, LBH589, further increased the cytotoxic effect of RT plus TMZ in this cell line. Conversely, T98G cells showing a high proportion of unmethylated MGMT had a lower response to the radiosensitizing effect of TMZ although treatment with PI103, 17-DMAG, or LBH-589 increased the cytotoxic effect of radiotherapy plus TMZ. The mechanism of enhanced radiosensitizing effects of TMZ was multifactorial, involving impaired DNA damage repair and induction of autophagy or apoptosis.

Taken together, these results suggest possible mechanisms for counteracting EGFR prosurvival signaling implicated in radioresistance of NSCLC and malignant glioma cells, and offer a potential strategy for overcoming resistance to EGFR inhibitors combined with irradiation.

Keywords: **Non-small cell lung cancer (NSCLC), Glioblastoma multiform (GBM), Epidermal growth factor receptor (EGFR), K-RAS, Temozolomide (TMZ), Radiosensitization**

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General Introduction

1. EGFR in cancer

The epidermal growth factor receptor (EGFR) is a member of the EGF receptor tyrosine kinase family, which consists of the EGFR (ErbB1/HER1), HER2/neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). EGFR is a 170-kD RTK that is composed of an extracellular binding domain, a transmembrane lipophilic segment, and an intracellular domain that has protein kinase activity[1]. After binding of ligand, EGFR can homodimerize or heterodimerize with another member of the ErbB family, causing activation of the intracellular tyrosine kinase domain and receptor transphosphorylation. The newly formed phosphotyrosine residues act as docking sites for various adaptor molecules that consequently activate a number of intracellular signaling cascades, that, in case of constitutive activation of the pathway, leads to cell proliferation, inhibition of apoptosis, angiogenesis, and invasion/metastasis, resulting in tumor growth and progression[2]. Aberrant expression or activity of the EGFR has been identified as an important biological factor in many human epithelial cancers including head and neck squamous cell carcinoma (HNSCC), non-small cell lung cancer (NSCLC), colorectal cancer (CRC), breast, and brain cancer. In particular, peptides of the EGFR are freguently overexpressed in NSCLC[3]. Additionally, constitutively active form of EGFR termed EGFRvIII, which is commonly found in glioblastoma multiforme (GBM). EGFR is amplified in 50% of all GBM cases, and 40% of these cases express EGFRvIII. GBM cell lines expressing EGFRvIII are resistant to gefitinib and, therefore, require higher drug doses and prolonged exposure to decrease the activity of EGFRvIII[4]. In cancer, EGFR is frequently mutated, activated, and

over-expressed, and is linked to aggressive biology of the tumors[5]. Therefore, EGFR is associated with therapeutic resistance and poor clinical outcome. Consequently, EGFR is an important target of anti-cancer therapy. However, EGFR-targeted therapy has demonstrated only modest effects on most cancer types.

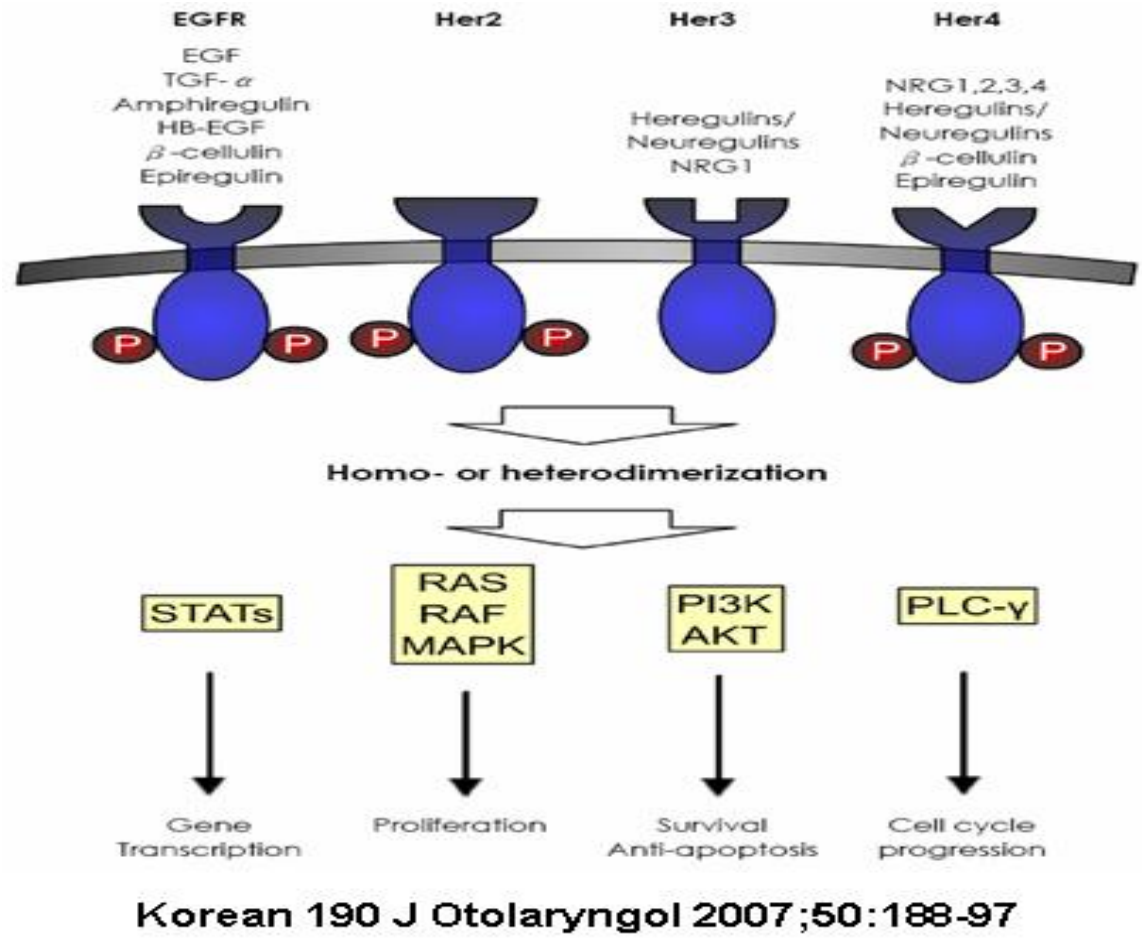


Figure 1. The ErbB receptors .

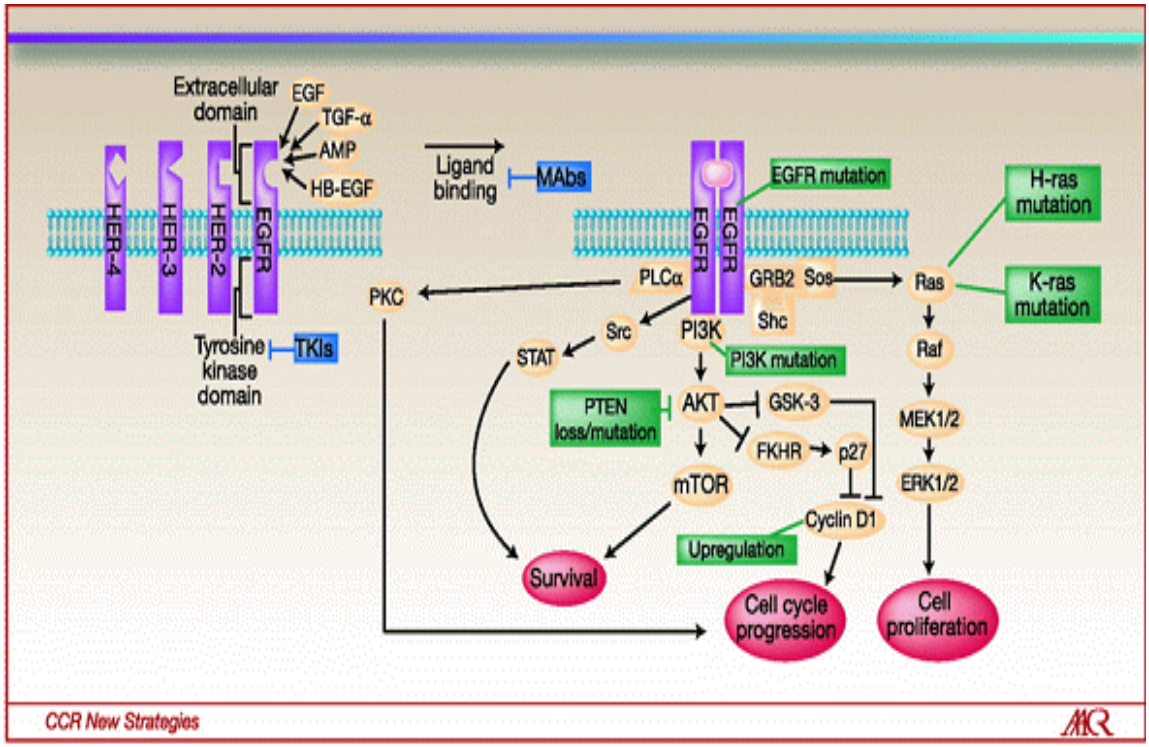
Binding of specific ligands to the extracellular domain leads to the activation of several intracellular signaling pathways.

2. Mechanisms of Resistance to EGFR Targeted Therapy

2-1. Alternative mechanisms for activating downstream signaling

EGFR has distinct downstream signaling pathways that have been reported to play important roles in radiation resistance. These downstream pathways are illustrated in Fig.2. The three primary signaling pathways activated by EGFR include the RAS/RAF/MEK/ERK, PI3K/AKT and PLCγ/PKC axes; however, SRC tyrosine kinases and STAT activation have also been well documented[6]. Tumor cell proliferation, and survival, can be promoted through activation of these pathways. Several preclinical studies have shown that activation of downstream signaling, especially the PI3K pathway, is sufficient to confer resistance to EGFR TKIs. Most laboratory models of acquired resistance show continued activation of the PI3K pathway despite TKI treatment[7, 8]. Activation of PI3K/AKT signaling by an ectopically expressed p110a-activating mutant (PIK3CA) confers an EGFR mutant cancer resistant to TKIs[9]. PI3K phosphorylates phosphatidylinositol (4,5)-disphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which serves as a docking site for AKT where it is activated by PDK1 and PDK2. Phosphatase and tensin homolog (PTEN) dephosphorylates PIP3 back to PIP2. The PTEN gene is mutated in 15~40% of primary GBM cases and mutations in or loss of *PTEN* expression may serve as a marker of primary resistance to gefitinib and erlotinib[10, 11]. From the mutation analyses of large numbers of NSCLC samples, several features of mutations of EGFR and related genes are now becoming apparent and they may be correlated to sensitivity and resistance to TKIs therapy. K-RAS gene mutation, which occurs in 20% to 30% of NSCLCs, has been reported to be associated with poor response

to EGFR TKIs[12, 13]. RAS signals through multiple effector pathways, including the RAF/mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK MAPK and PI3K-AKT signaling cascades[14]. Even though aberrant oncogenic signaling driven by EGFR mutation and amplification plays an important role in NSCLC and GBM pathogenesis, the responsible downstream signalings remain less clear. Therefore, defining the underlying mechanism of therapeutic resistance in NSCLC and GBM is an essential step in developing a viable therapeutic approach to overcome this issue.



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AACR Clinical Cancer Research

Figure 2. EGFR downstream signaling pathway activation.

2-2. Nuclear EGFR and resistance to radiation therapy

There is strong evidence suggesting that EGFR plays a key role in contributing to radiation resistance of cancers. Recent reports reveal a novel link between EGFR signaling and the repair of radiation-induced DNA double-strand breaks (DSBs) [15]. Radiation therapy induces EGFR nuclear translocation and is used to create DNA damage and apoptosis in a variety of cancer types. 5DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is a key enzyme in the non-homologous end joining (NHEJ) pathway of DSB repair[16]. Nuclear EGFR's ability to associate and stabilize DNA-PKcs in the nucleus upon radiation treatment suggests that it plays a role in radiation resistance. Therefore, the activation of DNAPKcs by EGFR results in proficient repair of DSBs and provides an explanation for the increase in radioresistance conferred by EGFR. In the past couple of years, a number of reports have reconfirmed this link between EGFR signaling and DSB repair; many of these reports indicate that signaling through the PI3K–AKT or MAPK pathways might also impinge on DNA-PK activation rather than the direct interaction between EGFR and DNA-PK initially proposed by the group of Rodemann[17, 18]. Activated AKT prevents apoptosis by inhibiting proapoptotic factors such as BAD and procaspase-9 and stimulates cell proliferation by activating mammalian target of rapamycin[19, 20]. Dampening of PI3K-AKT signaling using small molecule inhibitors resulted in impaired DSB repair in and breast cancer cells, and this results in radiation sensitivity[21, 22]. Therefore, DSB repair is an established determinant of radiosensitivity; inhibition of critical components such as DNA-PK, genetically or pharmacologically, consistently induces radiosensitization[23]. In this study, we evaluated whether targeting EGFR-associated signaling would radiosensitize EGFR-activated NSCLC and glioma cells through the impaired DSB repair.

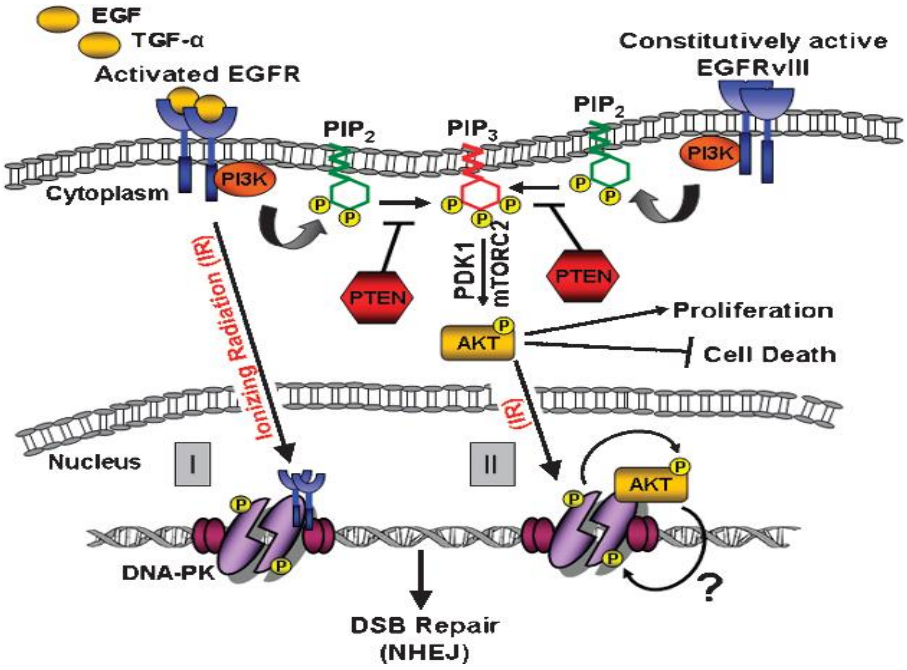


Figure 3. EGFR signaling and NHEJ.

The binding of ligands to EGFR activates the following pathways: (1) PI3K–AKT-1 pathway, Ras/RAF/MAPK/extracellular signal–regulated (ERK) pathway, and [24] signal transducer and activation of transcription (STAT) pathway (only the PI3K–AKT-1 pathway is shown for simplicity). EGFRvIII, a common deletion mutant that lacks the ligand-binding extracellular domain, is constitutively active and signals preferentially through the PI3K–AKT-1 pathway. In this pathway, activated PI3K phosphorylates phosphatidylinositol-4,5-biphosphate (PIP2) generating phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 anchors AKT-1 to the plasma membrane, where it is phosphorylated by mammalian target of rapamycin complex 2 (mTORC2) and 3-phosphoinositide–dependent kinase 1 (PDK1). Activated AKT-1 phosphorylates a variety of downstream targets that enhance proliferation and inhibit cell death. The PTEN tumor

suppressor negatively regulates PI3K–AKT-1 signaling by reversing PIP3 back to PIP2. Two models have been proposed to explain the connection between EGFR and NHEJ. In one scenario, wt EGFR translocates into the nucleus in response to IR, interacts with DNA-PKcs (DNA-dependent protein kinase, catalytic subunit), and stimulates its DNA repair activity (I). In another scenario, AKT-1 translocates into the nucleus in response to IR and interacts with DNA-PKcs (II). Phosphorylation of AKT-1 by DNA-PKcs promotes survival (curved arrow). Reciprocal phosphorylation of DNA-PKcs by AKT-1 might promote DSB repair through NHEJ[25] **(**Neoplasia 2010;12(9): 675-84).

Purpose

This article consists of two parts. Purpose of each part is as follows.

Part I : To investigate whether inhibitors targeting pathways downstream of EGFR signaling would cause radiosensitization in NSCLC having activated EGFR, mutant K-RAS, or both.

Part II : To evaluate whether targeting EGFR-associated Signaling would radiosensitize glioma cells with activated EGFR and mutated PTEN and further increase the radiosensitizing effect of temozolomide (TMZ).

Part I

Targeting EGFR-KRAS-PI3K/Akt/mTOR Signaling Pathway in Lung Cancer Cells: Implication in Radiation Response

1. Introduction

Non-small cell lung cancer (NSCLC) is a leading cause of cancer-related death worldwide[26]. Despite the intensive research carried out on this field and therapeutic advances, the overall prognosis of these patients remains unsatisfactory, with a 5-year overall survival rate of less than 15%[27]. Efforts to improve the survival of these patients is currently focused on the new target-based therapies directed against key signaling pathways involved in lung cancer growth and malignant progression. NSCLC specimens have been reported to exhibit mutations in multiple oncogenes and tumor suppressors, including epidermal growth factor receptor (EGFR), K-RAS, and tumor protein 53(TP53). Among these, EGFR, a receptor tyrosine kinase, is expressed in the majority of NSCLC[34]. EGFR signaling pathway is central to cell proliferation and survival. Ligand activation of the EGFR by epidermal growth factor, transforming growth factor-α, or other ligands leads to activation of several prosurvival signaling pathways, including the mitogen-activated protein kinase, phosphoinositide 3- kinase (PI3K)/AKT, and signal transducers and activators of transcription signaling cascades [28]. NSCLC expresses EGFR and its ligands, which together play important roles in the pathogenesis of lung cancer[29], and therefore,

molecular inhibition of the EGFR signaling pathway represents a promising cancer treatment strategy. Several studies have shown solid preclinical and clinical evidence supporting the potential value of targeting EGFR signaling to enhance the antitumor activity of ionizing radiation[30-32]. However, therapeutic resistance resulting from several factors, including activation of downstream pathways or alternative survival pathways, as well as molecular resistance mechanisms, has emerged as an important issue in the clinic[33-35].

Defining the underlying mechanism of therapeutic resistance is an essential step in developing a viable therapeutic approach to overcome this issue. Because radiotherapy has been an integral part of the comprehensive lung cancer treatment regimen, we aimed to define the pathways downstream of the EGFR implicated in the radiation response and identify potential therapeutic target(s) for overcoming resistance to EGFR-targeted therapy combined with radiation. Therefore, we hypothesized that inhibitors that target signaling pathways downstream of the EGFR could result in radiosensitization of a panel of lung cancer cells expressing activated EGFR, mutant K-RAS, or both. We also investigated the responsible downstream pathways that resulted in radioresisitance in NSCLC showing activated EGFR, mutant K-RAS, or both to overcome resistance to EGFR-targeted therapy in combination with radiation.

2. Materials and Methods

2-1. Cell culture

Three lung cancer cell lines were selected based on the known status of EGFR, K-RAS, and TP53 expression. A549 cells are known to express activated EGFR as a result of constitutive upregulation of autocrine/paracrine secretion of EGFR ligands, particularly ARG, and a resultant increase in p-EGFR. A549 cells also harbor a K-RAS mutation at codon V12. H460 cells posess a K-RAS mutation at codon V61. Both A549 and H460 cells have wild-type TP53 alleles. H1299 cells harbor deleted TP53 and wild-type K-RAS alleles. Cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and grown in DMEM (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum. Cells were maintained at 37^{o} C in an atmosphere of 95% air/ 5% CO_2.

2-2. Pharmacologic inhibitors

LY294002 and Rapamycin were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). UO126, PD98059, AKT inhibitor VIII and PI103 (the pyridinylfuranopyrimidine inhibitor) were obtained from Calbiochem® (Darmstadt, Germany). 3-methyladenine and z-VAD were obtained from Sigma (St. Louis, MO). Inhibitors were dissolved as concentrated stock solutions in DMSO, stored at -20^{o}C, and diluted at the time of use in culture medium. Control cells were treated with medium containing an equal concentration of drug carrier, DMSO.

2-3. RNA interference

Totals of 1 to 2 x 10^5 cells were plated into each well of six well tissue culture plates. The next day (when the cells were 40 - 50 % confluent), the culture medium was changed with antibiotics free medium. EGFR siRNA (5'- AAG AUC AUA AUU CCU CUG C - 3') ,K-RAS siRNA targeting the whole sequence (5'- UAG GUA CAU CUU CAG AGU C-3'), siRNA targeting codon V12 (5'- GGC CCC UGC CCG GUU CCC-3'), or V61 (5'-GCA GGU CAU GAG GAG UAC AG-3') were used. Nonspecific siRNA with GC content similar to that of the EGFR and K-RAS siRNAs was used for control (Bioneer®, Daejeon, Korea). Each siRNA in reduced serum medium (OPTIMEM, Life Technologies) was transfected into cells using Lipofectamine 2000 (Invitrogen®, Carlsbad, CA) according to the manufacturer's protocol. Forty-eight hours following transfection, cells were trypsinized, diluted to the appropriate cell density and plated in dishes for colony formation. Lysates from these cultures were screened for EGFR or K-RAS expression by Western blot analysis.

2-4. Clonogenic assays

A specified number of cells were seeded into each well of 6-well culture plates. As indicated, prior to irradiation cells were treated with each inhibitor followed by incubation at 37℃ for 10 to 14 days. Colonies were fixed with methanol and stained with 0.5% crystal violet; the number of colonies containing at least 50 cells was determined and surviving fraction was calculated. Radiation survival data were fitted to a linear-quadratic model using Kaleidagraph version 3.51 (Synergy Software, Reading, PA). Each point on the survival curves represents the mean surviving fraction from at least three dishes. Sensitizer enhancement ratio (SER) was calculated as the ratio of the isoeffective dose at

surviving fraction 0.5 and surviving fraction 0.05 in the absence of each inhibitor to that in the presence of each inhibitor.

2-5. Western analysis

Cells were washed, scraped, and resuspended in lysis buffer (iNtRON Biotechnology, Seoul, Korea). Proteins were solubilized by sonication, and equal amounts of protein were separated on SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Membranes were blocked in PBS containing 0.1 % Tween 20 and 5 % powdered milk and probed with primary antibody directed against p-EGFR (Tyr 1068), EGFR, p-Akt (Ser 473), Akt, p-ERK (Tyr202/204), ERK, p-$p70^{S6K}$ (Thr421/Ser424) and 70^{S6K}, p-DNA-PKs (Thr2609), Rad51, and anti-LC3 at 1:1,000 dilution respectively (Cell Signalling Technology, Inc.). Monoclonal anti-K-RAS, anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:500 and 1:5,000 dilution, respectively. Membranes were washed, and incubated with secondary antibody consisting of peroxidase-conjugated goat anti-rabbit or mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:2,000 dilution.

2-6. Flow cytometric analysis

Cells were harvested at the indicated times and fixed in 1 mL of 80% ethanol (1×10^6-2×10^6 cells per sample). Cells were then washed twice with PBS and incubated in dark for 30 minutes at 37°C in 1 mL of PBS containing 5 μg/mL propidium iodide (Molecular Probes, Eugene, OR) and 0.1% RNase A (Sigma). At least 1×10^4 events were counted. Flow cytometric analysis was done with a FACScan flow cytometer (Becton Dickinson,

Franklin Lakes, NJ). At least 1×10^4 events were counted. To evaluate for nonviable apoptotic cells after treatment, the proportion of cells of each treatment group with less than G_1 DNA content was assessed via fluorescence-activated cell sorting, which was done in the absence of gating to include all cells and minimize bias.

2-7. Immunocytochemistry

Cells were grown and treated on chamber slide. At specified times after treatment with each inhibitor, cover slips were rinsed, cells fixed in 4 % paraformaldehyde, and permeablized in methanol for 20 min. Cells were subsequently washed and blocked in PBS with 2 % bovine serum albumin for 1 hour. Primary antibody for γH2AX (Cell Signaling) was applied to the cells overnight. Secondary AlexaFluor488-conjugated donkey-anti-goat antibody (Molecular Probes, Eugene, OR) was applied for 1 hour. DAPI nuclear counter stain was applied at 1 μg/mL for 5 min. Slides were examined on a scope Axio Imager A1 fluorescent microscope. Images were captured and acquired using AxioCam MR c5 and acquisition software AxioVision v.4.4 (Carl Zeiss, Gottingen, Germany).

3. Results

3-1. Specific inhibition of EGFR or K-RAS using RNA interference.

The two most commonly mutated oncogenes in lung cancer encode for EGFR and K-RAS[36]. To determine the effect of targeting EGFR-K-RAS signaling during the radiation response, A549 cells expressing activated EGFR and K-RAS mutated at codon V12 were transfected with siRNAs specific for either EGFR or K-RAS. Inhibition of EGFR did not diminish the expression of p-AKT or p-ERK and did not result in significant radiosensitization [SER at surviving fraction of 0.5 (SER0.5), 1.0; (Figure 4A)]. In contrast, inhibition of K-RAS reduced p-AKT and p-ERK expression and significantly increased the radiosensitivity of A549 cells (SER0.5, 1.5), as shown in the cell survival curve Figure 4B. Similar results were obtained using H460 cells expressing K-RAS mutated at codon 61 (Figure 4C and 4D).

Next, we wanted to determine whether this effect was specific for the oncogenic mutant form of K-RAS. Figure 4E indicates that specific inhibition of mutant K-RAS at codon V12 resulted in the radiosensitization of A549 cells, although the degree of radiosensitization was slightly less than that induced by siRNA targeting of whole RAS (SER0.5, 1.3). The siRNAs specifically targeting mutant K-RAS at codon V61 potentiated the radiation-induced cell killing of H460 cells (Figure 4F). H1299 cells expressing mutant TP53 and wild-type K-RAS were not radiosensitized by siRNAs targeting wild-type K-RAS (Figure 4G) or mutant K-RAS at codon V12 (Figure 4H).

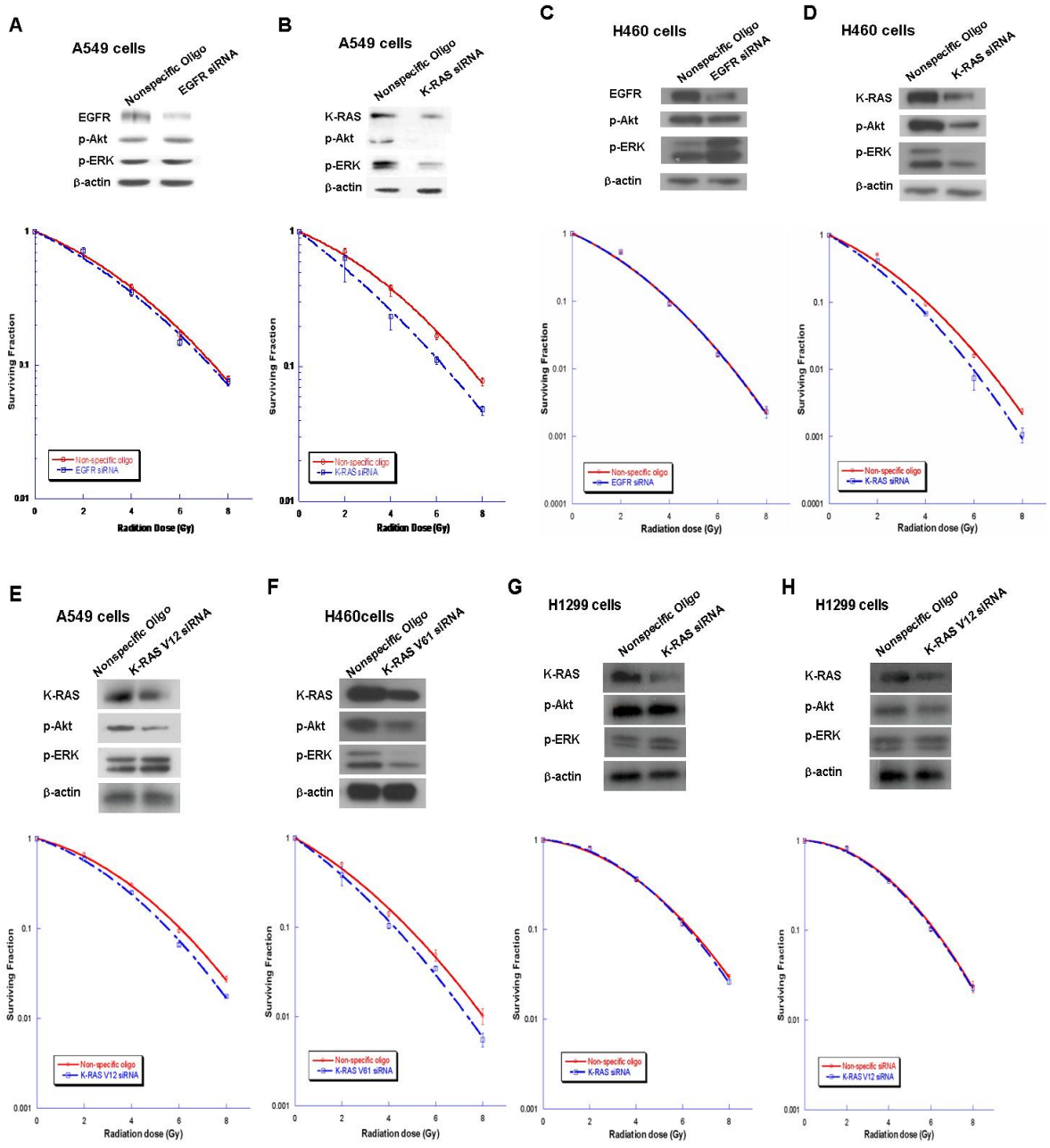


Figure 4. Specific inhibition of EGFR or K-RAS signaling in A549 cells.

Specific inhibition of EGFR or K-RAS signaling. A and B, A549 cells were transfected with siRNAs targeting either EGFR or whole K-RAS. C and D, H460 cells were transfected with siRNAs targeting either EGFR or whole K-RAS. E, siRNAs targeting

mutant K-RAS at codon V12 were transfected into A549 cells. F, siRNAs targeting mutant K-RAS at codon V61 were transfected into H460 cells. G and H, H1299 cells expressing wild-type K-RAS were tranfected with siRNA targeting either whole K-RAS or mutant K-RAS at codon V12. Points on the survival curves represent mean surviving fractions from experiments performed in triplicate.

3-2. Pharmacologic inhibition of ERK signaling did not increase A549 radiosensitivity

Having shown the involvement of K-RAS signaling in the radiation response of A549 and H460 cells, we wanted to identify the downstream effectors that play important roles following radiation. As shown by the survival curves in Figure 5A and Figure 5C, pretreatment with 20 μmol/L PD98059, an inhibitor of c-RAF and mitogen-activated protein/ERK kinase kinase, did not cause radiosensitization (SER0.5, 1.1). The selective mitogen-activated protein/ERK kinase inhibitor UO126 (10 μmol/L) also did not have a noticeable radiosensitizing effect on A549 or H460 cells (SER0.5, 1.0), although ERK phosphorylation was visibly reduced.

3-3. Pharmacologic inhibition of PI3K-AKT-mTOR signaling increased A549 radiosensitivity

Pretreatment with the PI3K inhibitor LY294002 (10 μmol/L) resulted in the significant reduction of p-AKT and decreased radiation survival in A549 cells (SER0.5, 1.5; Figure 5B). As expected, pretreatment with the AKT inhibitor VIII (200 nmol/L) caused significant reduction in p-AKT levels and decreased radiation survival slightly (SER0.5, 1.3). Because inhibition of mTOR could allow avoidance of possible side effects associated with inhibition of molecules with broader biological functions, we tested whether rapamycin would cause radiosensitivity in A549 cells. Pretreatment with rapamycin (0.1 μmol/L) caused a dramatic reduction in p-p70S6K levels and significantly increased radiosensitivity in these cells (SER0.5, 1.4). Similar results were seen with H460 cells (Figure 5D).

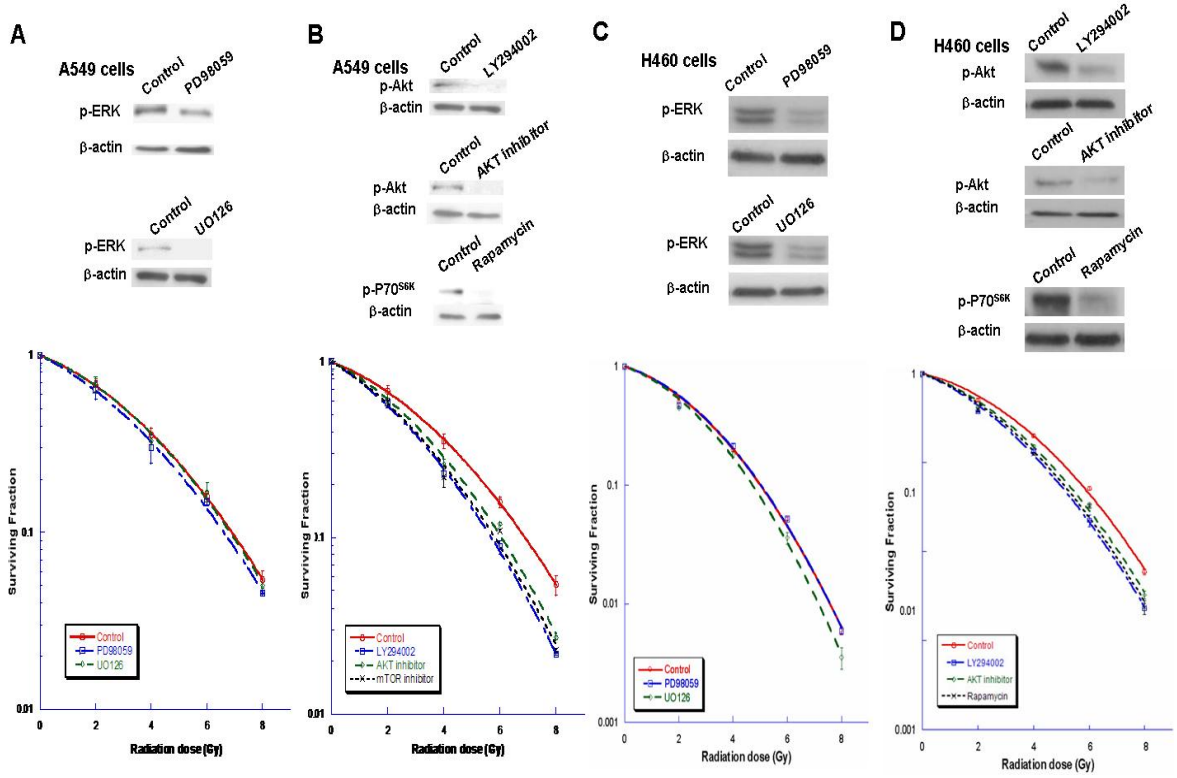


Figure 5. Pharmacologic inhibition of PI3K-AKT-mTOR signaling increased A549 radiosensitivity.

A and C, A549 and H460 cells were pretreated with PD98059 or UO126, respectively, and subjected to Western analysis using antibodies specific for p-ERK, p-AKT, and β-actin. Points on survival curves represent mean surviving fractions from experiments done in triplicate. B and D, A549 and H460 cells were pretreated with LY294002 or the AKT inhibitor VIII or rapamycin, then subjected to Western analysis using antibodies against p-Akt, p-p70^{S6K}, and β-actin. Points on survival curves, mean surviving fractions from experiments done in triplicate.

3-4. Effects of combined inhibitor and radiation treatment on cell cycle progression

To investigate possible mechanisms of the observed radiosensitization, we analyzed changes in cell cycle progression after pretreatment with each of the inhibitors. A549 cells were pretreated with each inhibitor and then irradiated with 6 Gy. Eight hours after irradiation, cells were harvested, fixed, stained with propidium iodide, and then analyzed by flow cytometry. As expected, A549 cells showed a typical G2-M delay after irradiation alone (Figure 6A). This effect was abrogated significantly by pretreatment with LY294002, AKT inhibitor VIII, or rapamycin. In contrast, abrogation of G2 arrest was not observed when cells were treated with PD98059 or UO126.

3-5. Inhibition of PI3K-AKT-mTOR signaling induced prolongation of γH2AX foci

We determined that resolution of γH2AX foci, a well known marker of DNA double strand break damage and repair , occurred rapidly after treatment with 6 Gy IR. Consistent with the results discussed above, pretreatment with PI3K-AKT-mTOR pathway inhibitors caused marked prolongation of radiation-induced γH2AX foci formation, indicating delayed DNA damage repair compared with mock-treated control cells, 5 hours following irradiation with 6 Gy (Figure 6B).

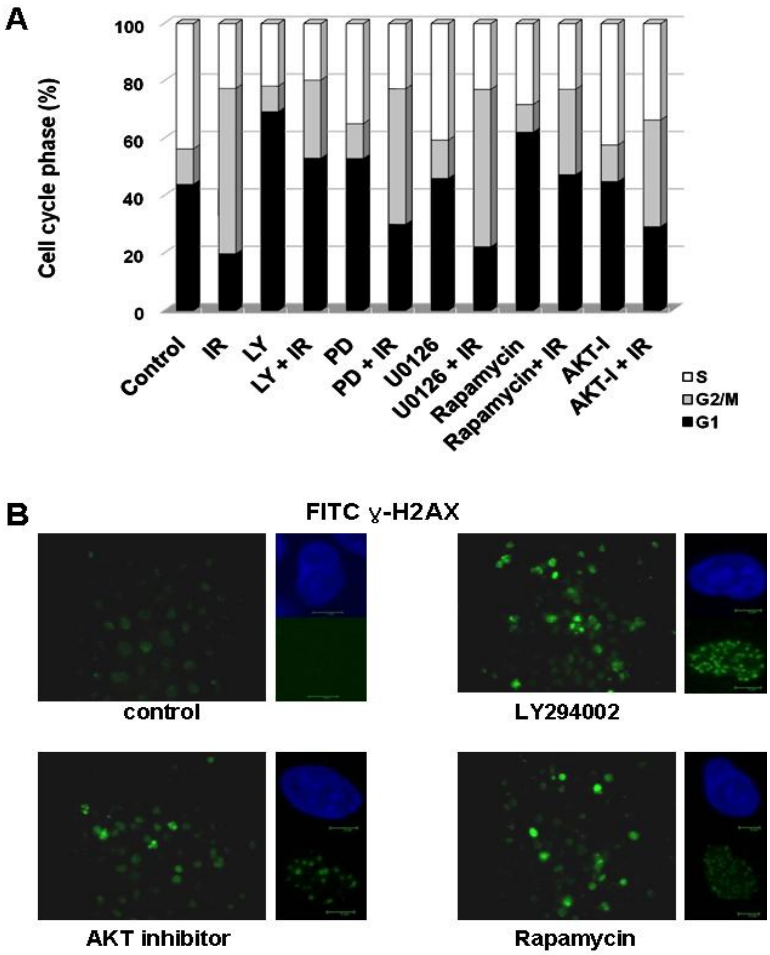


Figure 6. Changes in cell cycle progression and γH2AX foci formation following inhibition of PI3K-AKT-mTOR signaling.

Changes in cell cycle progression and γH2AX foci formation following inhibition of PI3K-AKT-mTOR signaling. A, A549 cells were treated with the indicated inhibitors before irradiation (IR) with 6 Gy for 8 h. Subsequently, cells were stained with propidium iodide and then analyzed by flow cytometry for the different cell cycle stages. B, A549 cells were pretreated with LY294002, the AKT inhibitor VIII, or rapamycin before assessment of γH2AX foci formation. Mock-treated control cells were analyzed 5 h following irradiation with 6 Gy.

3-6. A dual inhibitor of Class I PI3K and mTOR signaling led to effective radiosensitization in A549 cells

PI-103 is a dual inhibitor that targets class I PI3K and mTOR signaling. Pretreatment with 0.4 μmol/L PI-103 markedly reduced p-AKT and p-$p70^{S6K}$ protein levels and caused radiosensitization of A549 and H460 cells (SER0.5, 1.4 and 1.25; Figure 7A and Figure 7B). This sensitizing effect was associated with prolongation of γH2AX foci 5 hours following irradiation with 6 Gy in the presence of PI-103 (Figure 7C).

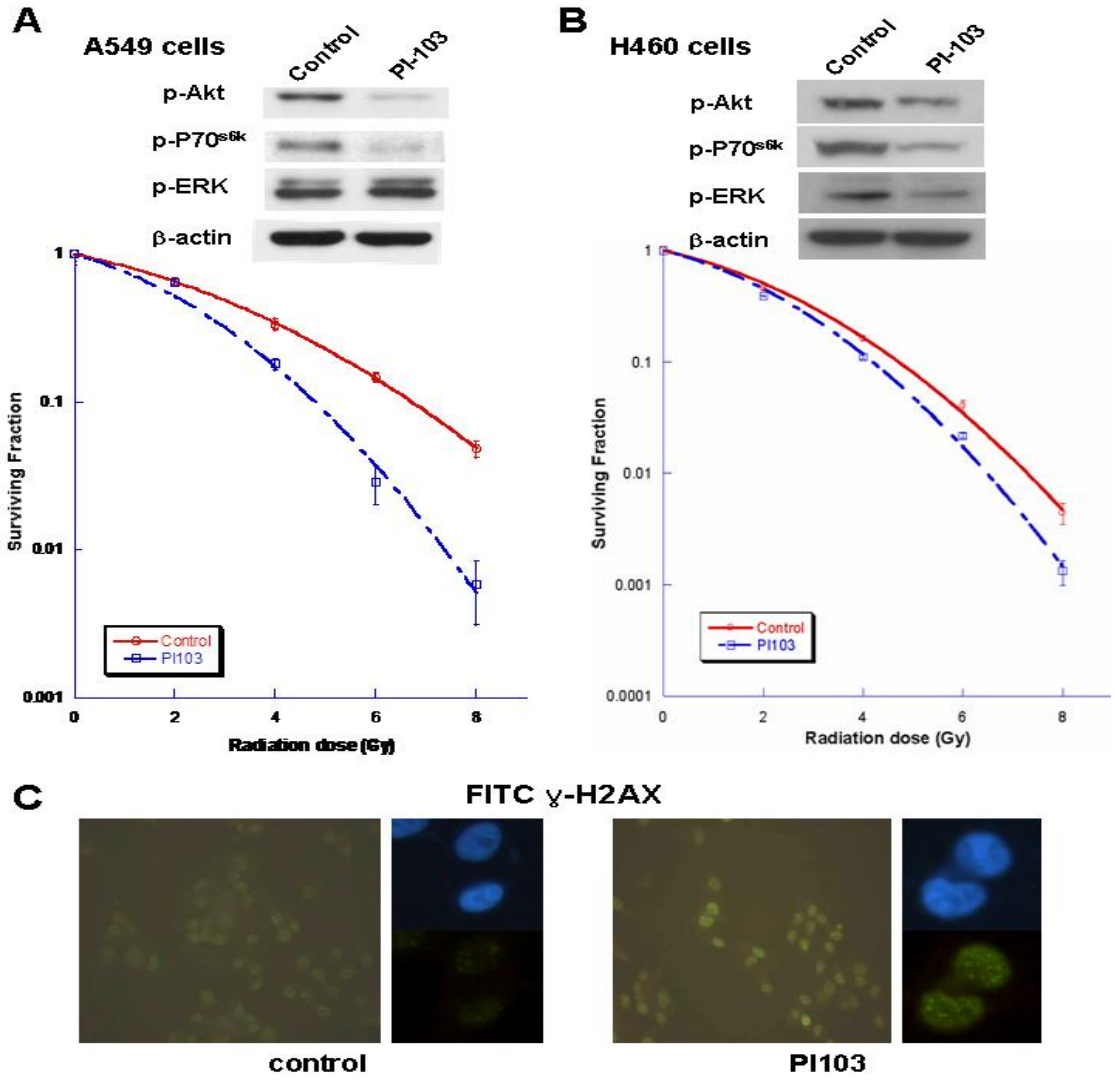


Figure 7. A dual inhibitor of Class I PI3K and m-TOR signaling resulted in effective radiosensitization of A549 cells.

A dual inhibitor of class I PI3K and mTOR signaling induced effective radiosensitization. A and B, A549 and H460 cells were pretreated with PI-103 and subjected to Western analysis using antibodies specific for p-AKT, p-p70^{S6K}, p-ERK, and β-actin. Points on survival curves, mean surviving fractions from experiments done in triplicate. Each experiment was also repeated thrice with similar results. C, pretreatment with PI-103 caused marked prolongation of radiation-induced γH2AX foci formation.

3-7. Marker for DNA damage repair

Because treatment with the inhibitors targeting PI3K-AKT-mTOR signaling consistently resulted in the prolongation of γH2AX foci, we wished to identify the molecule involved in this process. Pretreatment with the PI3K inhibitor, LY294002, the AKT inhibitor VIII, or a dual inhibitor, PI-103 was associated with decreased DNA-PK phosphorylation at Thr 2609 (Figure 8A). Similar data were obtained for H460 cells (Figure 8B).

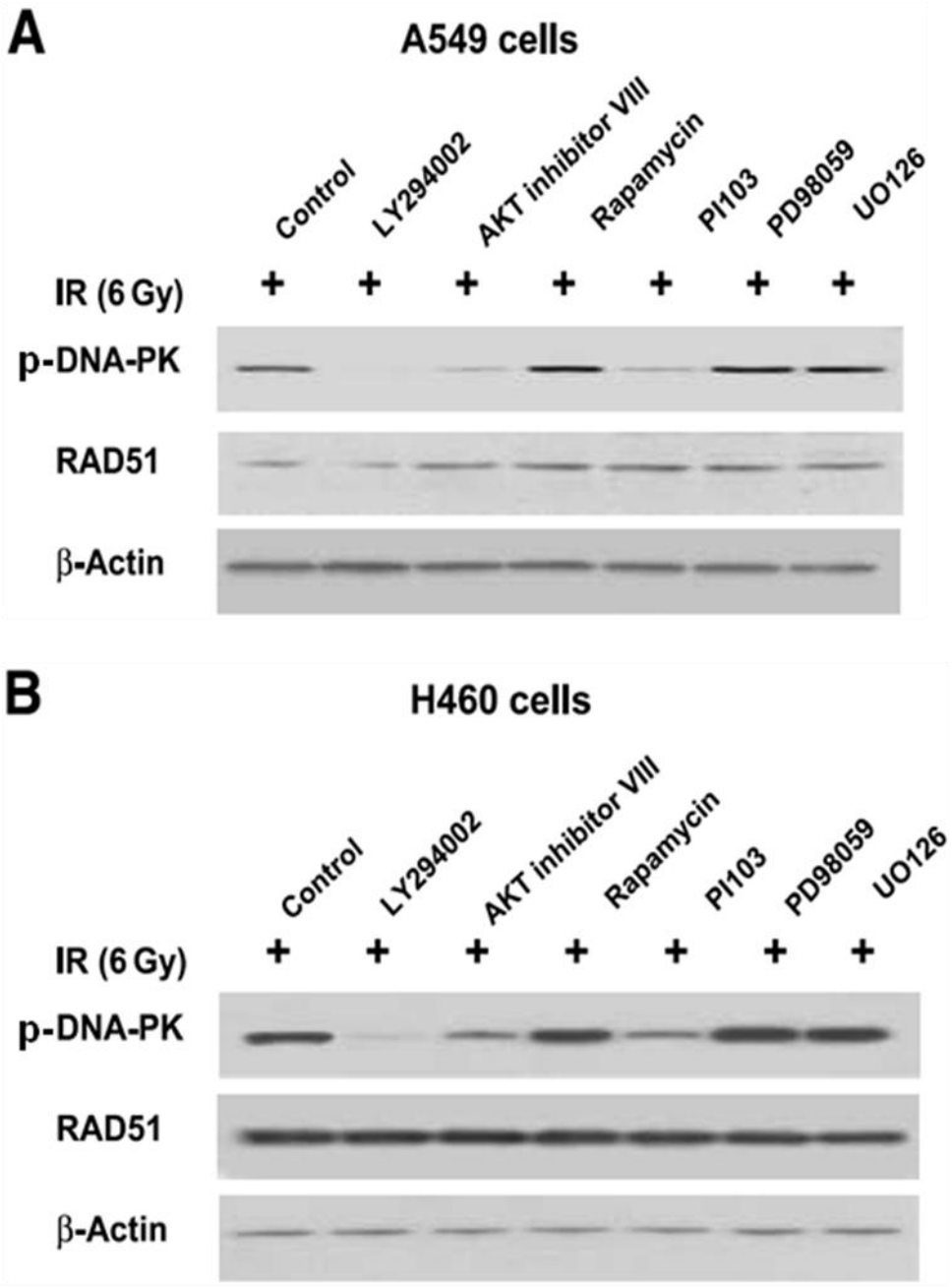


Figure 8. Marker for DNA damage repair.

Marker of DNA damage repair. Pretreatment of A549 (A) or H460 (B) cells with PI3K inhibitor LY294002 or AKT inhibitor VIII, or a dual inhibitor, PI-103, was associated with decreased DNA-PK phosphorylation at Thr2609.

3-8. Mode of cell death

Pretreatment with z-VAD, an inhibitor of caspase, rescued cells from the radiosensitizing effect induced by LY294002 or AKT inhibitor VIII, rapamycin, and PI-103 (Figure 9A, top). Pretreatment with 3-methyladenine, a known inhibitor of autophagy, rescued cells from radiosensitization induced by rapamycin or PI-103 (Figure 9B, bottom). Rapamycin or PI-103 increased punctate fluorescence or lysosomal localization of LysoTracker within 24 hours of treatment (Figure 9B). To elucidate the mechanism underlying autophagy in A549 cells, we examined the effect of these inhibitors on the conversion of LC3. Although pretreatment with rapamycin induced upregulation of LC3-II only, pretreatment with PI-103 resulted in markedly increased LC3-I (18 kDa) as well as LC3-II (16 kDa) expression in 24 hours. Upregulated levels of LC3 returned to basal level following treatment with 3-MA (Figure 9C), which is known to target the class III PI3K, although competition with its kinase domain, and inhibit the early stage of autophagic vesicle formation[37].

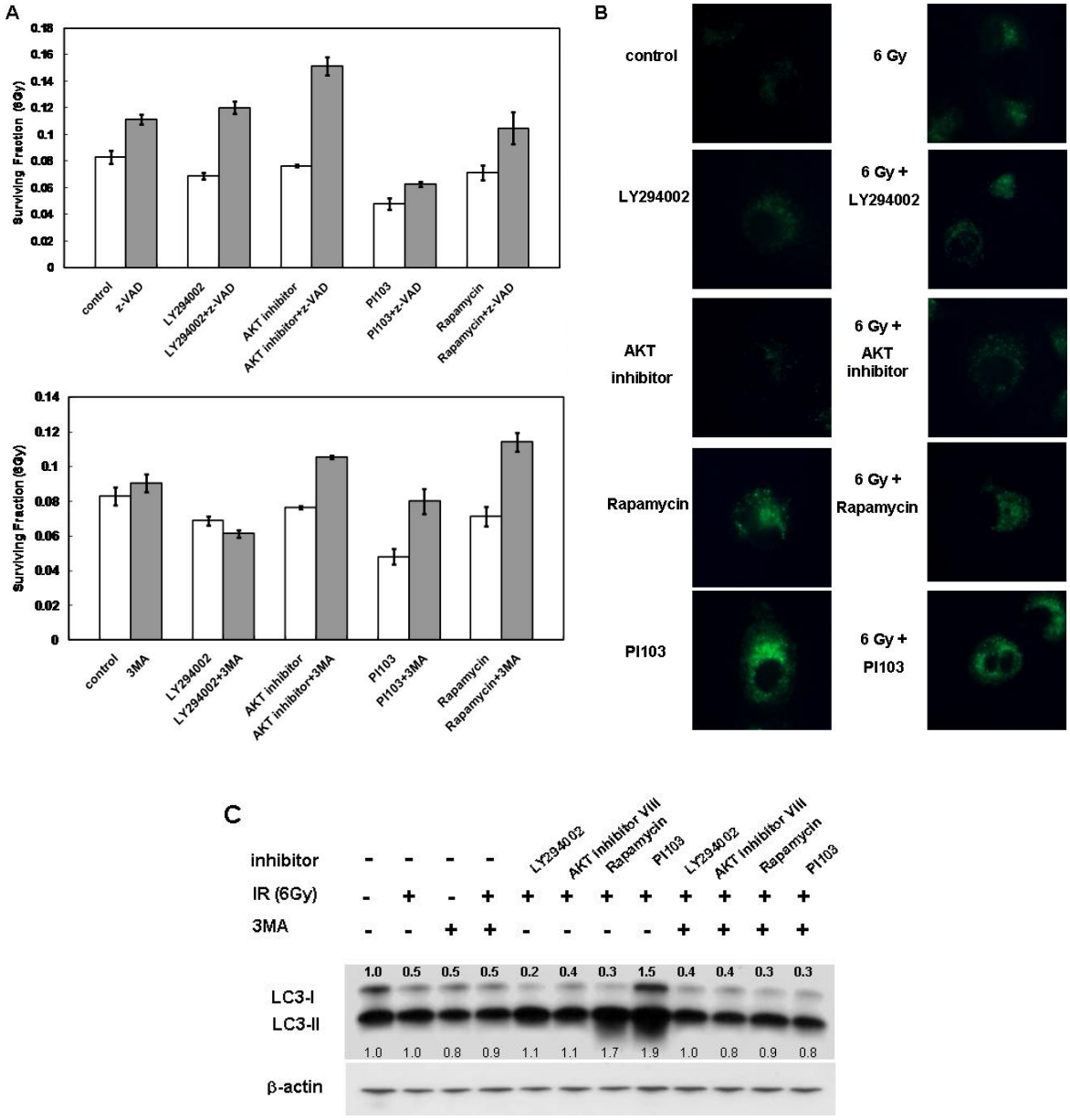


Figure 9. Mode of cell death.

Mode of cell death. A, pretreatment with z-VAD (20 μmol/L), an inhibitor of caspase, rescued cells from the radiosensitizing effect of LY294002 or AKT inhibitor VIII,

rapamycin, and PI-103 (top). Pretreatment with 3-methyladenine (250 μmol/L), a known inhibitor of autophagy, rescued cells from radiosensitization induced by rapamycin or PI-103 (bottom). B, rapamycin or PI-103 increased punctate fluorescence or lysosomal localization of LysoTracker in 24 h. C, whereas pretreatment with rapamycin induced upregulation of LC3-II only, pretreatment with PI-103 resulted in markedly increased LC3-I (18 kDa) and LC3-II (16 kDa) expression in 24 h. These upregulated levels of LC3 returned to basal level following treatment with 3-MA.

3-9.Toxicity towards normal human fibroblasts

The surviving fraction at 2 Gy (SF2) of normal human fibroblasts was not significantly decreased by the indicated siRNAs or inhibitors, excluding LY294002 (Figure10).

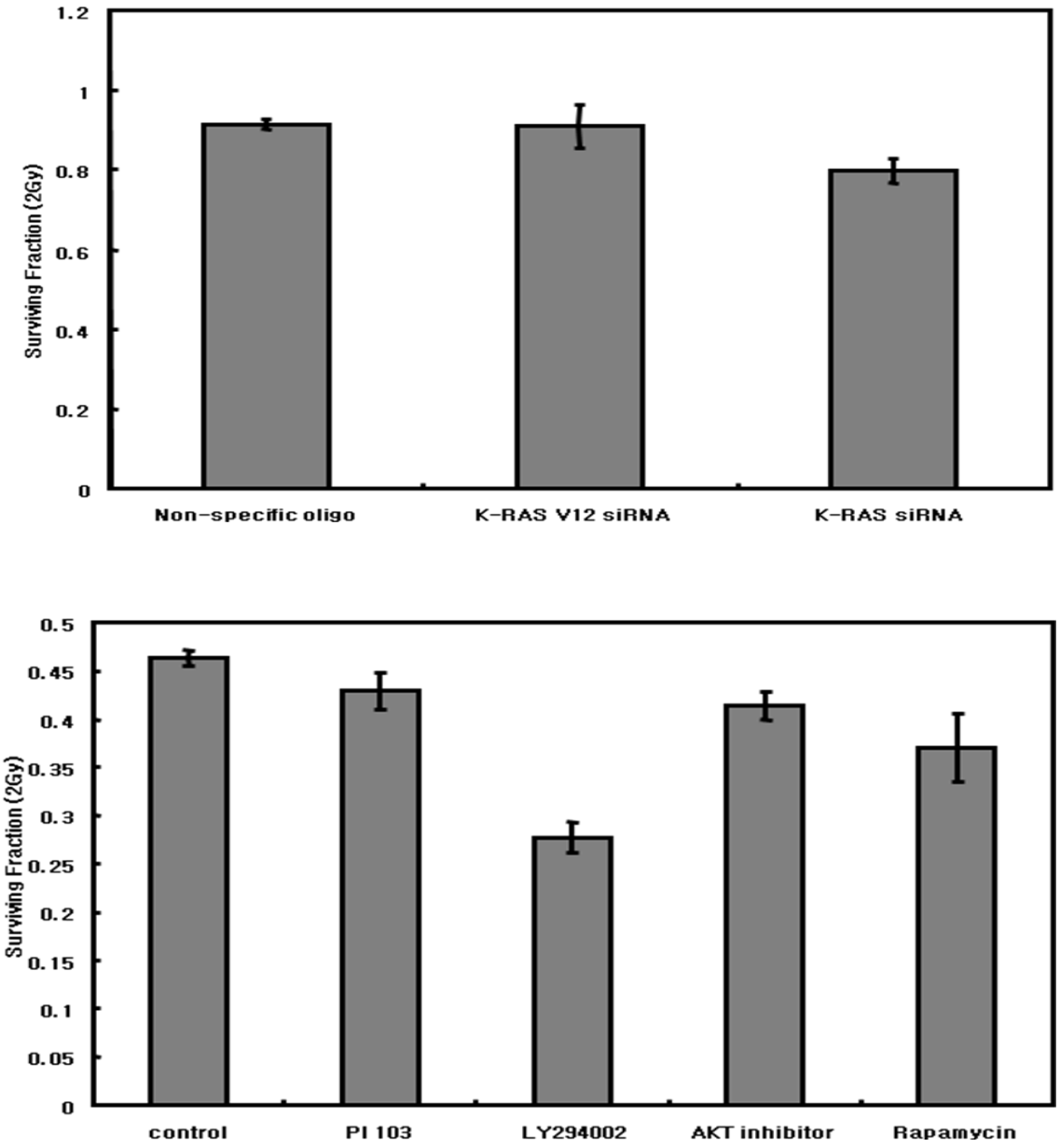


Figure 10. Toxicity towards normal human fibroblasts.

Effect of the treatment with the indicated siRNAs or inhibitors on the growth of normal fibroblasts. Surviving fraction at 2 Gy (SF2) is shown for normal human fibroblasts treated with the indicated siRNAs or inhibitors compared with control. Each value represents the mean surviving fraction calculated from cells treated in triplicate.

4. Discussion

The EGFR and its ligands are frequently expressed in NSCLC. The EGFR signaling pathway has been shown to play an important role in the pathogenesis of NSCLC, making novel agents targeted against this pathway effective in patients with advanced stage disease. In this regard, several anti-EGFR agents are in advanced phase of clinical development. One method of inhibiting this pathway is through antibodies, such as cetuximab and panitumumab, which bind to the extracellular domain of the EGFR. The mechanisms by which these antibodies work include prevention of ligand binding, induction of receptor internalization, and antibody-dependent cell-mediated cytotoxicity[38]. Because lung cancer tissues have been shown to express the EGFR, clinical use of antibodies against this receptor is a rational approach in the treatment of NSCLC. In preclinical studies, cetuximab, in combination with chemotherapy and/or radiation therapy, inhibited the growth of lung cancer cell lines and mouse xenografts[39, 40]. Although the Radiation Therapy Oncology Group trial reported promising results in applying radiation therapy in combination with cetuximab in treating head and neck cancer, thus far, no clinical trials have shown significant differences in outcome with the addition of cetuximab to chemotherapy/radiotherapy in NSCLC treatment. Interestingly, most studies investigating cetuximab have not shown a clear association between EGFR expression and the therapeutic response[41]. Moreover, whether negative predictors of responses such as those resulting from K-RAS mutations in colorectal cancer[42, 43] could be applied to lung cancer must be confirmed.

A different approach to modulating the EGFR signaling pathway in NSCLC is the use of small-molecule EGFR tyrosine kinase inhibitors, which include erlotinib and gefitinib. In combination with chemotherapy, treatment with neither erlotinib nor gefitinib resulted in improved survival compared with chemotherapy alone[44, 45]. Mutations of the tyrosine kinase domain of EGFR frequently occur as a ΔE746-E750 deletion in exon 19 and as an L858R replacement in exon 21 in adenocarcinoma of NSCLC patients who exhibit dramatic tumor sensitivity to EGFR tyrosine kinase inhibitor[46]. Das et al. showed that NSCLC cell lines harboring tyrosine kinase domain mutations exhibit marked sensitivity to irradiation, as a result of delayed DNA repair kinetics, defective radiation-induced arrest during DNA synthesis or mitosis, and pronounced increases in apoptosis or the occurrence of micronuclei[47]. On the other hand, the ONCOBELL study reported a poorer response rate in patients with increased EGFR copy number and p-AKT positivity, which indicate activated PI3K-AKT signaling[48].

We observed that inhibition of K-RAS, but not of EGFR, using RNA interference increased the radiosensitivity of A549 and H460 cells. These results suggest that targeting the EGFR alone is not an effective strategy for modulating the radiation response in NSCLC cells, as for other cancer cells possessing constitutive activation of downstream signaling due to mutations in K-RAS. Recent reports indicated that PI3K-mediated activation of AKT in K-RAS mutant human tumor cells in response to EGFR ligand binding or radiation was independent of a direct K-RAS function but dependent on increased production of EGFR ligands mediated by upregulated K-RAS/ERK signaling. This provided new insight into the importance of K-RAS mutation in the context of PI3K/AKT-mediated radioresistance in EGFR-activated tumors[49]. Another study showed

that cell lines possessing activating PI3K mutations or loss of PTEN expression were more resistant to cetuximab compared to wild type PI3K or PTEN-expressing lines. Furthermore, PI3K mutant/PTEN null and RAS/BRAF mutant cell lines are more highly resistant to cetuximab compared to those without dual mutations or PTEN loss, indicating that constitutive and simultaneous activation of the RAS and PI3K pathways confers maximal resistance to this agent[50].

PI3K activity generates specific inositol lipids that have been implicated in regulating cell proliferation, differentiation, survival, and angiogenesis[51, 52]. A previous report demonstrated that inhibition of PI3K signaling increases radiosensitivity of EGFR-activated head and neck cancer[53]. Activated in a PI3K-dependent manner by a variety of stimuli through growth factor receptors, AKT is a serine/threonine kinase that plays an important role in cell survival. We previously reported that inhibition of AKT1 using RNAi increased the radiosensitivity of EGFR- or RAS-activated cell lines[54]. In support of data presented here, a recent study showed that treatment with either the AKT inhibitor API or AKT1 siRNAs inhibits repair of DNA double-strand breaks in EGFR-activated lung cancer cell lines, as measured by the γH2AX foci assay[55]. Bozulic et al. recently demonstrated that AKT activation following DNA damage required PDK1 and DNA-PKcs, and activated AKT1 increased survival in vivo following DNA damage[56]. Pretreatment of PI3K or AKT inhibitor showed predominantly apoptotic mode of cell death.

Mammalian target of rapamycin (mTOR) is also an important downstream component of the PI3K-AKT signaling pathway. mTOR inhibitors can effectively block growth and survival signals by inactivating downstream effectors such as $p70^{S6K}$ and 4E-binding protein 1[57, 58]. mTOR represents an attractive target because its inhibition could avoid

possible side effects associated with inhibition of upstream PI3K/AKT signaling molecules with broader biological functions, including glucose signaling[59]. In the present study, we showed that rapamycin increased radiosensitivity primarily due to the impairment of DNA damage repair and also resulted in apoptosis and autophagy as final modes of cell death.

To increase tumor cell toxicity while leaving normal cells unaffected, targeting more than one component of a tumor-specific signaling pathway could be beneficial. Inhibition of PI3K using LY294002 lacks specificity and has shown unacceptable toxicity in preclinical studies. Previous studies showed that specific inhibition of Class I PI3K using RNAi enhanced the radiosensitivity of tumor cells possessing activated PI3K signaling resulting from EGFR overexpression or mutation of the RAS oncogene[54]. The pyridinylfuranopyrimidine inhibitor PI-103 is a dual inhibitor that targets class I PI3K and mTOR signaling and also shows minimal toxicity to normal cells[60]. We observed effective radiosensitization of A549 cells following pretreatment with PI-103 which was associated with persistence of γH2AX foci formation, down-regulation of p-DNA-PK, and leads the mixed mode of cell death including autophgy.

Taken together, this study revealed that targeting the PI3K-AKT-mTOR signaling pathway could be a viable approach to simultaneously counteracting EGFR and K-RAS prosurvival signaling, and an alternative strategy to overcome therapeutic resistance of currently available EGFR inhibitors used in combination with irradiation.

Part II

Enhancement of the Radiosensitizing Effect of Temozolomide: Targeting EGFR-associated Signaling in Malignant Glioma Cells

1. Introduction

Glioblastoma multiforme (GBM) is the most common malignant primary brain tumor in adults and is among the most aggressive of all human tumors. Radiation therapy has long been the standard adjuvant approach following the maximum possible resection, and it remains the primary treatment modality in unresectable GBM[61, 62]. Recent data from a randomized phase III clinical trial by the European Organization for Research and Treatment of Cancer/National Cancer Institute of Canada (EORTC 26981-22981/NCIC CE.3) suggest that concurrent and adjuvant temozolomide (TMZ) combined with radiation therapy results in significantly improved outcome in patients with GBM. However, despite this improvement the majority of patients with GBM relapse soon after treatment and the 2-year survival rate is only 26%[63].

A possible explanation for this poor prognosis can be that GBM is very heterogenic tumor in its pathology and genetic alterations[64]. Methylguanyl methyltransferease (MGMT) was the first molecular marker to serve as both a prognostic factor and a target for personalized therapy[65], and therapeutic resistance in MGMT-unmethylated tumors has emerged as an important clinical issue. Several other molecular biomarkers that regulate tumor growth, proliferation, and survival are being investigated as potential targets in the

management of GBM. One of the most common genetic alterations in primary GBM is over-expression of epidermal growth factor receptor (EGFR), which is observed in more than 50% of GBMs. Over-expression of EGFR and/or its constitutively activated variant EGFRvIII is associated with tumorigenesis and more aggressive phenotypes, such as, invasiveness and therapeutic resistance in GBM[5]. Preclinical data suggest that over-expression of EGFR confers radiation resistance on malignant glioma and that blocking EGFR restores radiosensitivity. However, the results of EGFR-targeted therapy trials for GBM, including gefitinib and erlotinib, have been disappointing due to diverse mechanisms of therapeutic resistance[66]. Emerging evidence indicates an important role that PTEN plays in predicting GBM response to EGFR-targeted therapy[11]. Aberrant PI3K/Akt/mTOR pathway has been shown to contribute to the resistant phenotype[67]. Also, several studies have reported that EGFR/EGFRvIII's cross-talk with the oncogenic transcription factor STAT3 or Src potentially lead to GBM resistance to anti-EGFR therapy[5,68]. In addition, the heat shock protein 90 (HSP90) is also recently shown to play a key role in maintaining the active conformation of EGFR mutants and preventing receptor down-regulation[69]. All these aberrant signal transductions associated with EGFR signaling pathways might be related to resistance to RT and poor prognosis in GBM patients.

In the present study, we have focused on the EGFR-associated signaling network, such as Src, PI3K-Akt-mTOR, and STAT3, to identify effective targeting strategies to improve therapeutic outcome when radiotherapy and TMZ are used concurrently to treat GBM. We also tested the effect of ligand-independent modulation using an HSP90 inhibitor and epigenetic modulation using a histone deacetylase (HDAC) inhibitor, focusing on targeting pro-survival signaling from EGFR.

2. Materials and Methods

2-1. Cell culture

The human GBM cell lines U251, U87, and T98G used in this study were obtained from the American Type Culture Collection (ATCC). All ATCC cell lines were authenticated by the company routine Cell Biology Program and were used within 6 months of receipt for this study. Cells were maintained and cultured according to standard techniques at 37℃ in 5% (v/v) CO2 using culture medium recommended by the supplier. In all experiments, the different cell populations were first cultured in DMEM media containing 10% fetal bovine serum. Glioblastoma cells were then seeded into 6 well plates in 10% fetal bovine serum and on the first day of treatment the media were replaced with vehicle control or each drug with or without TMZ in DMEM media without fetal bovine serum. The media treated with drugs were replaced with DMEM media containing 10% fetal bovine serum after 24 hr.

2-2. Pharmacologic inhibitors

PI103 (a pyridinylfuranopyrimidine inhibitor and a dual inhibitor of Class I PI3K and mTOR) and 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2, a Src inhibitor), were obtained from Calbiochem® (Darmstadt, Germany). Rapamycin was obtained from Cell Signaling Technology, Inc (Beverly, MA, USA). 4-((3-(Carboxymethylsulfanyl)-4-hydroxy-1-naphthyl)sulfamoyl)benzoicacid (Cpd188, a STAT3 inhibitor), was purchased from Calbiochem® (Darmstadt, Germany). 17-Desmethoxy-17-N, N-dimethylaminoethylamino-geldanamycin, HCl, 17-N,N-Dimethylaminoethylamino-17-demethoxy-geldanamycin, HCl (17-DMAG) was obtained

from Calbiochem® and Panobinostat (LBH589) was obtained from Selleck Chemicals LLC (Houston, TX, USA). Inhibitors were prepared as concentrated stock solutions in DMSO, stored at -20℃, and diluted in culture medium at the time of use. Control cells were treated with medium containing the same concentration of the drug carrier, DMSO.

2-3. Clonogenic assays

A specified number of cells was seeded into each well of 6-well culture plates. As indicated, prior to irradiation cells were treated with TMZ (25μM) with or without the inhibitors PP2 (10μM), PI103 (0.4μM), rapamycin (100nM), Cpd188 (10μM), 17-DMAG (25nM), and LBH589 (20nM). Clonogenic assays were performed as previously described [70].

2-4. Western blot analysis

Cells were washed, scraped, and resuspended in lysis buffer (iNtRON Biotechnology, Seoul, Korea). Proteins were solubilized by sonication and equal amounts of protein were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA). Membranes were blocked in PBS containing 0.1% Tween 20 and 5% powdered milk and probed with primary antibody directed against p-EGFR (Tyr1068), p-Akt (Ser473), p-ERK (Tyr202/204), p-p70S6K (Thr421/Ser424), p-Src (Thy416), HSP70, HSP90, p-DNA-PKs (Thr2609), Rad51, caspase-3, LC3, MMP-2, E-cadherin ,and EphA2 (Cell Signaling Technology, Inc.) at 1:1000 dilutions. Primary antibodies against MGMT (Abcam, Cambridge, UK) and Acetyl Histone H3 (Millipore Corp.) were used at a dilution of 1:1000. Antibodies against P-STAT3, VEGF and β-actin

(Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at dilutions of 1:500 and 1:5000, respectively. Membranes were washed and incubated with peroxidase-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at a dilution of 1:2500.

2-5. Immunocytochemistry

Immunocytochemistry was performed as previously described[70].

2-6. Caspase-3/7 Assay

Cells (3×10^4 per well) were seeded in a 96-well plate with 200 µl culture medium. Cells were treated with TMZ with or without each inhibitor prior to irradiation. Casapse-3/7 activity was measured as per manufacturer's instructions (Invitrogen).

2-7. Annexin V-FITC/Propidium Iodide (PI) double-staining

Apoptosis was demonstrated using Annexin V-FITC/Propidium Iodide (PI) double-staining. Cells were seeded in 8-well chamber slides, treated with each inhibitor with or without TMZ and double-stained with Annexin V-FITC and propidium iodide according to the manufacturer's instruction[71] and then analyzed under a fluorescence microscope (Carl Zeiss).

2-8. Cellular Senescence-Associated β-Galactosidase Assay

Cellular senescence was examined by detecting the activity of β-galactosidase. Tumor cells were seeded in 8-well chamber slides, treated with reagents, and then stained using

Senescence β-Galactosidase Staining Kit (Cell Signaling Technology) according to the manufacturer's instruction. Cells were examined using a light microscope.

2-9. Statistical Analysis

These results are expressed as the mean ± SD of three independent experiments. Data from these experiments were analyzed by Student's *t* test (SPSS12.0 software). Significant differences were established at $P<0.05$.

3. Results

3-1. Specific inhibition of EGFR using RNA interference

First, we evaluated p-EGFR, MGMT expression levels in a panel of glioma cell lines. U251 and T98G showed similar levels of p-EGFR expression. U251 and U87 cells showed low levels of MGMT expression, which indicated a high level of methylated MGMT, compared with T98G. In addition, U251 cells especially showed high levels of p-STAT3 expression among a panel of glioma cell lines (Fig. 11A). To determine the effect of targeting EGFR signaling during the radiation response, U251 cells and T98G cells were transfected with either EGFR-specific siRNA or nonspecific siRNA. Specific inhibition of EGFR did not attenuate signaling through downstream mechanisms such as p-Akt, p-ERK, or p-STAT3 (Fig. 11B), and did not result in significant radiosensitization (sensitizer enhancement ratio at surviving fraction of 0.5 [SER0.5], 1.0) (Fig. 11C).

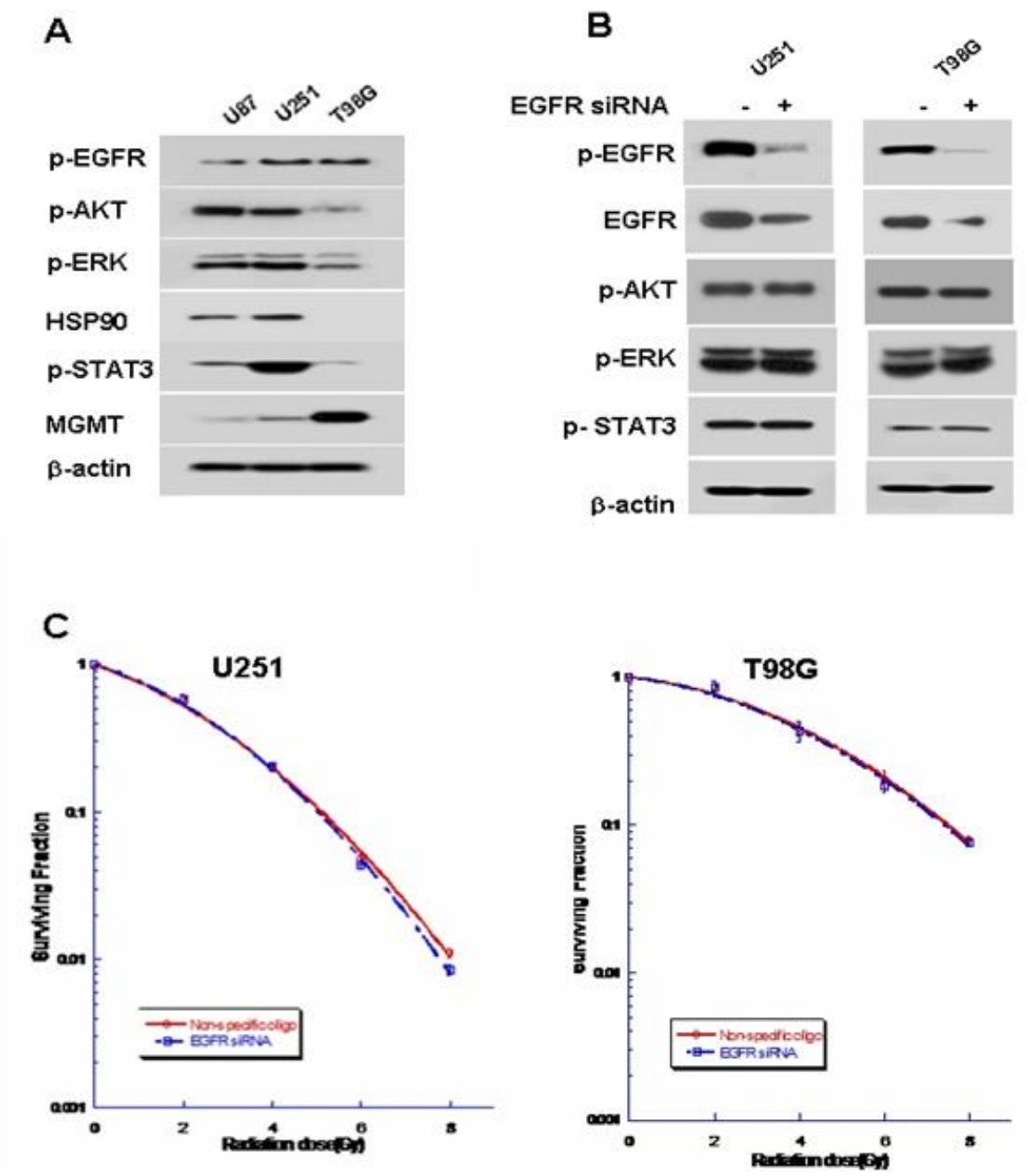


Figure 11. Specific inhibition of EGFR does not result in radiosensitization of U251 and T98G cells.

A. Forty-eight hours after serum starvation, western blot analysis showed low levels of MGMT expression in U87 and U251 cells, and a high level of MGMT expression in T98G cells. U251 and T98G showed similar levels of p-EGFR expression. U251 cells especially showed high levels of p-STAT3 expression among a panel of glioma cell lines.

B. Western blot analysis of U251 and T98G cells transfected with EGFR-specific or nonspecific siRNA.

C. Cells were plated for colony formation assay 48 h after transfection with EGFR-specific or nonspecific siRNA and irradiated as indicated. Points on survival curves represent mean surviving fractions from experiments performed in triplicate.

3-2. Targeting downstream signaling: Src, PI3K/mTOR, and STAT3

Even though aberrant oncogenic signaling driven by EGFR mutation and amplification plays an important role in glioblastoma pathogenesis, the responsible downstream signalings remain less clear. Therefore, we evaluated whether targeting downstream signaling of EGFR would radiosensitize EGFR-activated glioma cells and further increase the radiosensitizing effect of TMZ. Supplementary Tables 1 and 2 show the sensitizer enhancement ratio (SER) for each inhibitor alone and combined with TMZ in U251 and T98G cells. To determine whether the Src inhibitor PP2 would enhance the cytotoxic effect of radiotherapy plus TMZ, U251 cells were pretreated with PP2, TMZ, or both, for 24 h. U251 cells, which have a high proportion of methylated MGMT, showed a high degree of radiosensitization by PP2 plus TMZ. Conversely, T98G cells, with a high proportion of unmethylated MGMT, showed less radiosensitization by PP2 plus TMZ (Fig. 12A).

In previous studies[70], we demonstrated an important role of PI3K-Akt-mTOR signaling in the radiation response; we now wanted to determine whether inhibition of these targets would further increase the radiosensitizing effect of TMZ. Since inhibition of mTOR is a way to avoid possible side effects associated with inhibition of PI3K-Akt, we tested whether rapamycin would cause radiosensitivity in glioma cells. Pretreatment with rapamycin (0.1μM) caused a dramatic reduction in the level of p-p70S6K, but did not discernibly potentiate the radiosensitizing effect of TMZ in either cell line (Fig. 12B). PI103, a dual inhibitor of class I PI3K and mTOR, markedly reduced p-Akt and p-p70S6K protein levels, and effectively potentiated the radiosensitizing effect of TMZ in both cell lines (Fig. 12C). A STAT3 inhibitor, Cpd188, preferentially potentiated the radiosensitizing effect of TMZ in U251 cells showing high levels of p-STAT3 expression (Fig. 12D).

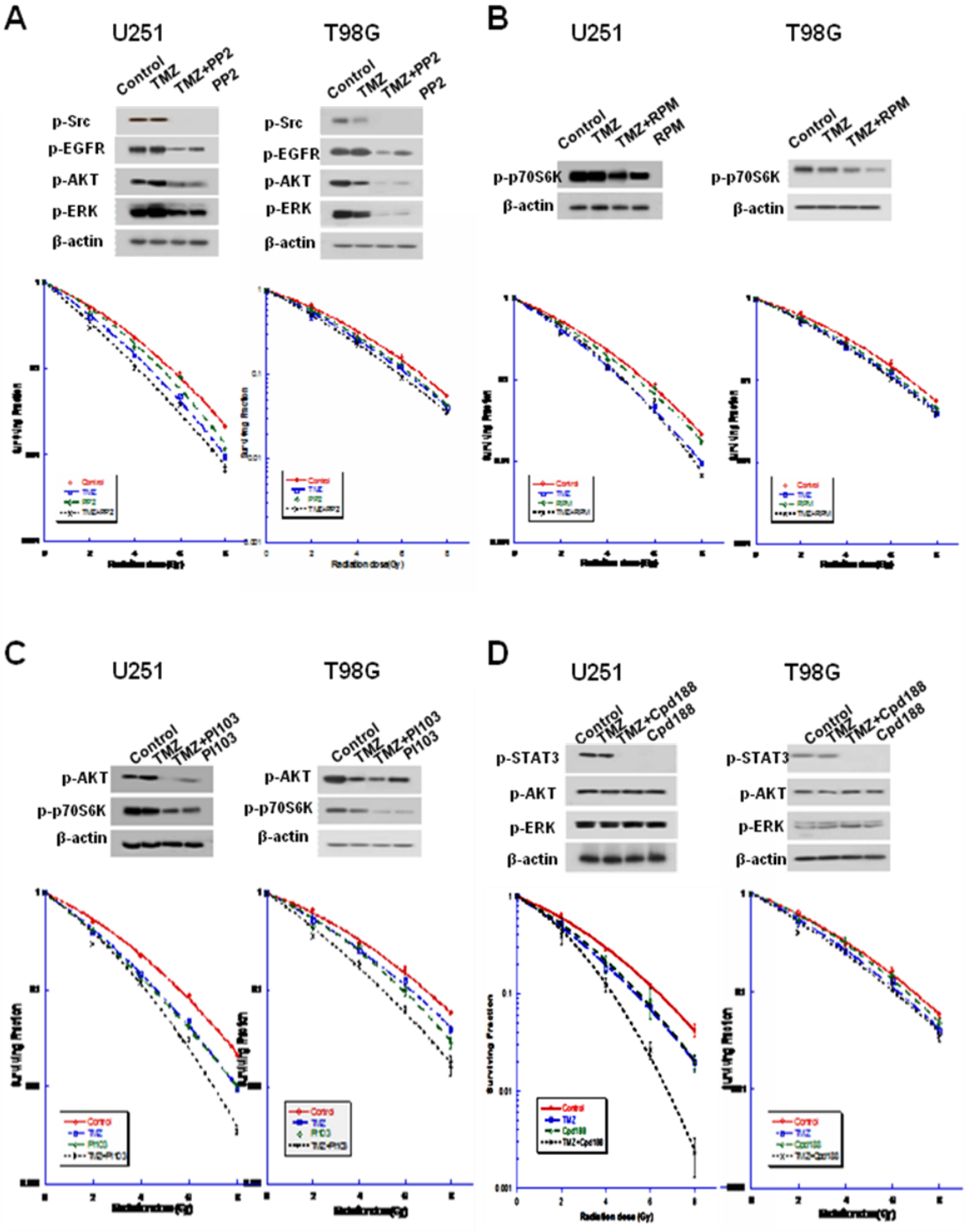


Figure 12. Targeting downstream signaling of EGFR.

A. U251 and T98G cells were pretreated with the Src inhibitor PP2 (10μM) plus TMZ (25μM) for 24 h and subjected to western blot analysis using the indicated antibodies. Whereas U251 cells showed a high degree of radiosensitization, T98G cells showed less radiosensitization after combined treatment with PP2 and TMZ.

B. U251 and T98G cells were pretreated with rapamycin (100nM) and TMZ (25μM) for 24 h. Pretreatment with rapamycin plus TMZ did not have a synergistic radiosensitizing effect on U251 and T98G cells compared with treatment with TMZ alone.

C. U251 and T98G cells were pretreated with a dual inhibitor of class I PI3K and mTOR signaling, PI103 (0.4μM), and TMZ (25μM) for 24 h. PI103 effectively enhanced the radiosensitizing effect of TMZ in both U251 and T98G cells.

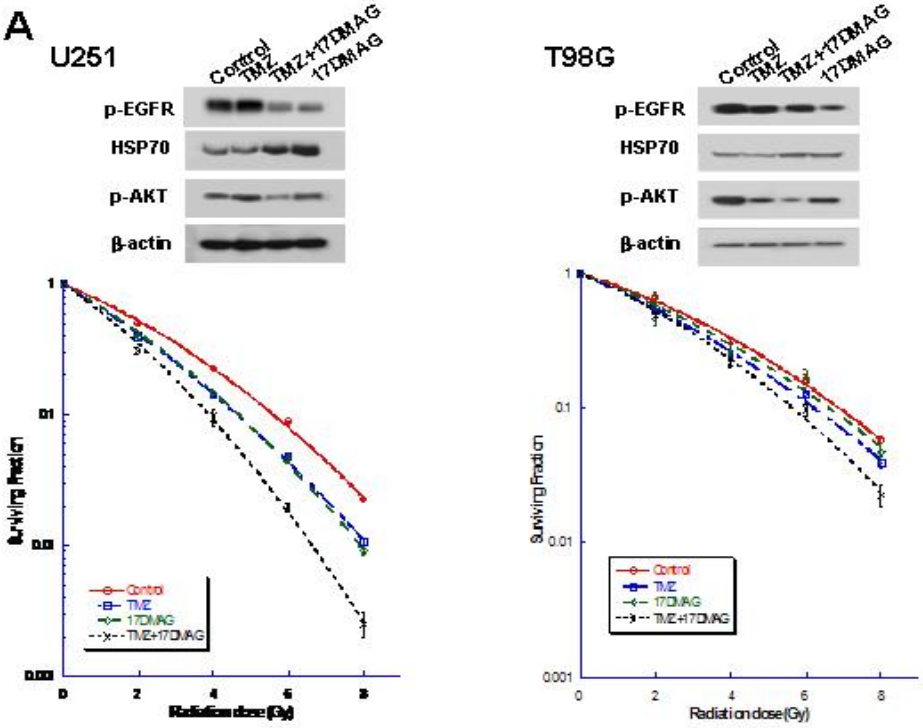
D. U251 and T98G cells were pretreated with the STAT3 inhibitor Cpd188 (10μM) and TMZ (25μM) for 24 h. Cpd188-induced radiosensitization was greater in U251 cells with high levels of p-STAT3 than in T98G cells. Points on survival curves represent mean surviving fractions from experiments performed in triplicate.

3-3. Ligand-independent modulation using HSP90 inhibitor

The molecular chaperone HSP90 stabilizes Akt and oncogenic forms of mutant EGFR, both of which contribute to the growth of a variety of cancers including gliomas [72]. Given that HSP90 is over-expressed in human glioblastoma tissues compared to normal human brain tissue[73], we hypothesized that targeting HSP90 enhances the cytotoxic effect of radiation therapy plus TMZ. As shown in Figure 13A, pretreatment with a HSP90 inhibitor, 17-DMAG (25nM), increased expression of HSP70 and attenuated levels of its client proteins, p-EGFR and p-Akt. 17-DMAG effectively potentiated the radiosensitizing effect of TMZ. This effect was more pronounced in U251 cells showing high levels of HSP90 expression than in T98G cells at the higher radiation doses (Tables 1 and 2).

3-4. Epigenetic modulation using HDAC inhibitor

We previously reported that HDIs potentiate radiation-induced cell killing in a panel of human cancer cells with activated EGFR signaling through diverse mechanisms[54]. A recent study also showed that the HDAC inhibitor LBH589 induced acetylation of HSP90, resulting in reduced association of HSP90 with key chaperone proteins in NSCLC with EGFR mutations[74]. As shown in Figure 13B, pretreatment with a HDAC inhibitor, LBH589 (20nM), induced acetylation of histone H3, leading to acetylation of HSP90 and down-regulation of its client proteins p-EGFR and p-Akt. LBH589 effectively potentiated the radiosensitizing effect of TMZ. This effect was more pronounced in U251 cells than in T98G cells and occurred at higher radiation doses (Tables 1 and 2).


A
U251
Control
TMZ
TMZ+17DMAG
17DMAG
p-EGFR
HSP70
p-AKT
β-actin
Surviving fraction
Radiation dose(Gy)
Control
TMZ
17DMAG
TMZ+17DMAG
T98G
Control
TMZ
TMZ+17DMAG
17DMAG
p-EGFR
HSP70
p-AKT
β-actin
Surviving Fraction
Radiation dose(Gy)
Control
TMZ
17DMAG
TMZ+17DMAG

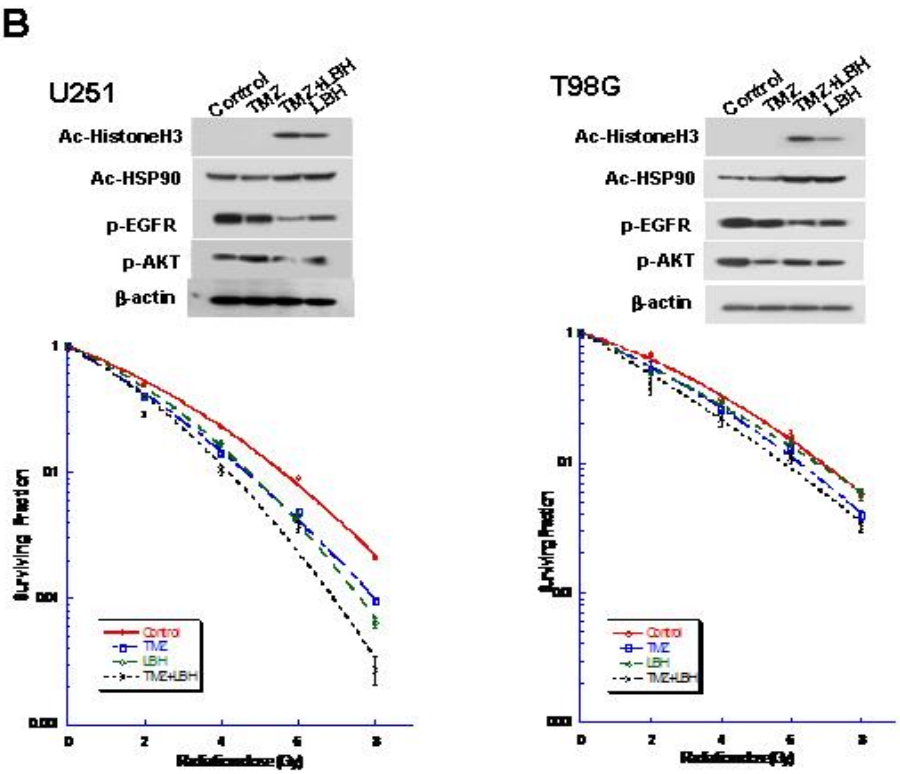

B
U251
Control
TMZ
TMZ+LBH
LBH
Ac-HistoneH3
Ac-HSP90
p-EGFR
p-AKT
β-actin
Surviving fraction
Control
TMZ
LBH
TMZ+LBH
T98G
Control
TMZ
TMZ+LBH
LBH
Ac-HistoneH3
Ac-HSP90
p-EGFR
p-AKT
β-actin
Surviving fraction
Control
TMZ
LBH
TMZ+LBH

Figure 13. Ligand-independent modulation and epigenetic modulation.

A. U251 and T98G cells were pretreated with the HSP90 inhibitor 17-DMAG (25nM) and TMZ (25μM) for 24 h. 17-DMAG enhanced the radiosensitizing effect of TMZ in U251 and T98G cells.

B. U251 and T98G cells were pretreated with TMZ (25μM) and LBH589 (20nM) for 24 h. LBH589 effectively potentiated the radiosensitizing effect of TMZ. Points on survival curves represent mean surviving fractions from experiments done in triplicate.

3-5. Impairment of DNA damage repair following irradiation

DSB repair is an established determinant of radiosensitivity; inhibition of critical components such as DNA-PK, genetically or pharmacologically, consistently induces radiosensitization[23]. Immunostaining was done to study γH2AX foci present at DNA double-strand breaks (DSB). U251 cells were pretreated with the indicated inhibitors plus TMZ before assessment of γH2AX foci formation. Mock-treated control cells were analyzed 6 h after irradiation with 6 Gy. Pretreatment of U251 cells with the dual inhibitor PI103, the HSP90 inhibitor 17-DMAG, or the HDAC inhibitor LBH589 combined with TMZ caused marked prolongation of radiation-induced γH2AX foci formation 6 h after irradiation with 6 Gy (Fig. 14A), indicating delayed DNA damage repair. Pretreatment of U251 with PI103, 17-DMAG, or LBH589 combined with TMZ attenuated expression of p-DNA-PK (Fig. 14B).

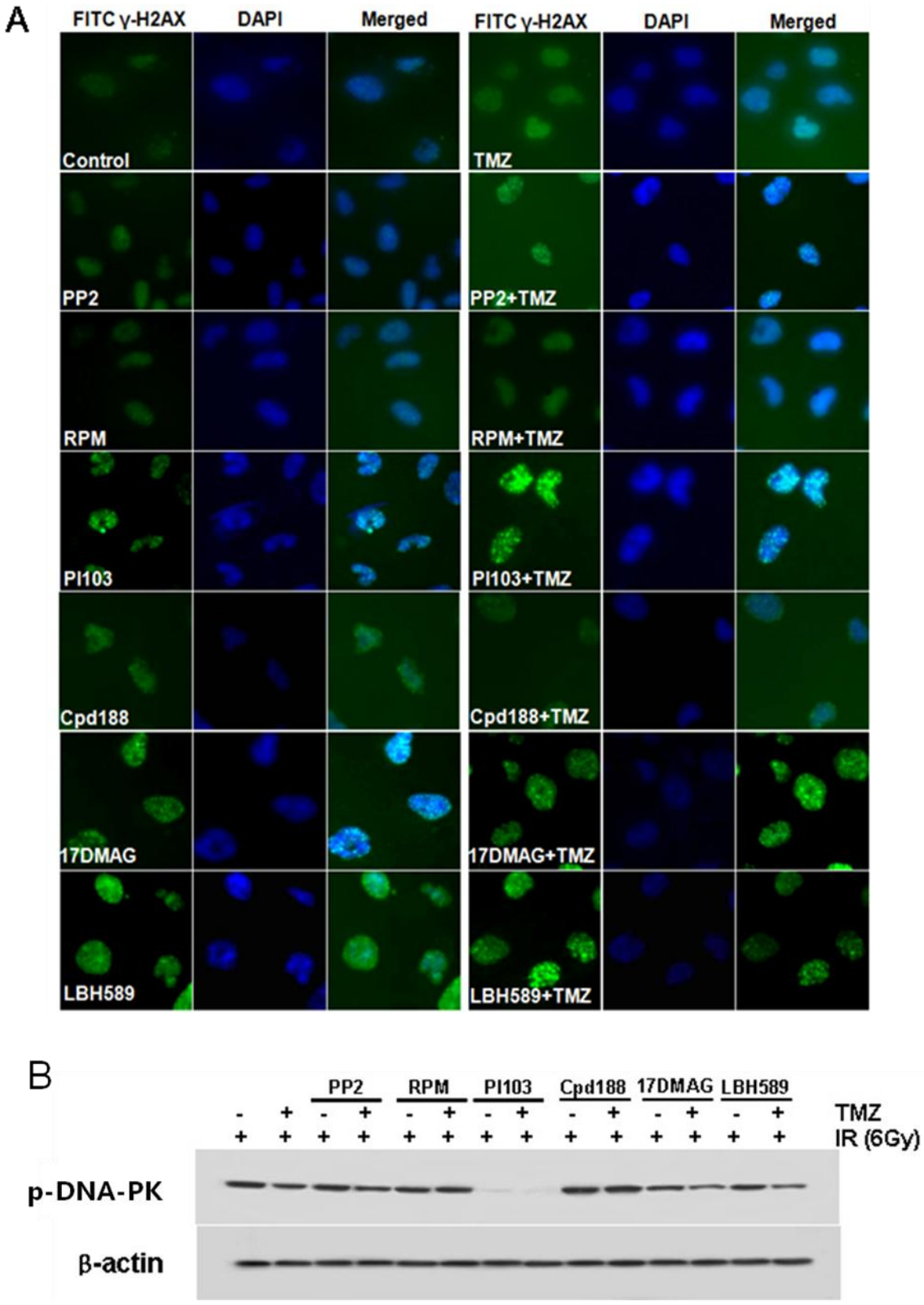

A
FITC γ-H2AX
DAPI
Merged
FITC γ-H2AX
DAPI
Merged
Control
TMZ
PP2
PP2+TMZ
RPM
RPM+TMZ
PI103
PI103+TMZ
Cpd188
Cpd188+TMZ
17DMAG
17DMAG+TMZ
LBH589
LBH589+TMZ
B
PP2
RPM
PI103
Cpd188
17DMAG
LBH589
TMZ
IR (6Gy)
p-DNA-PK
β-actin

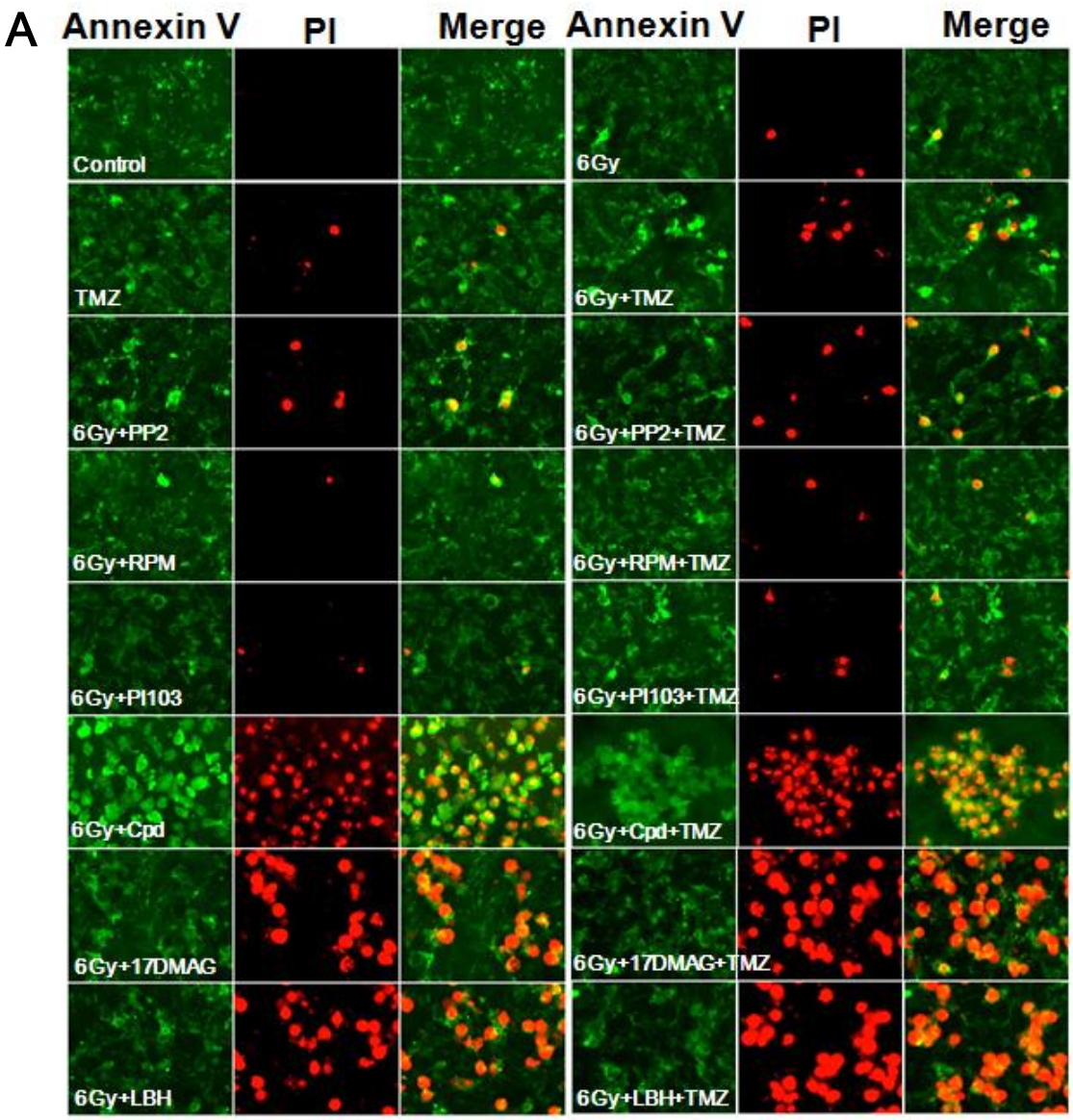
Figure 14. Impairment of DNA damage repair following irradiation.

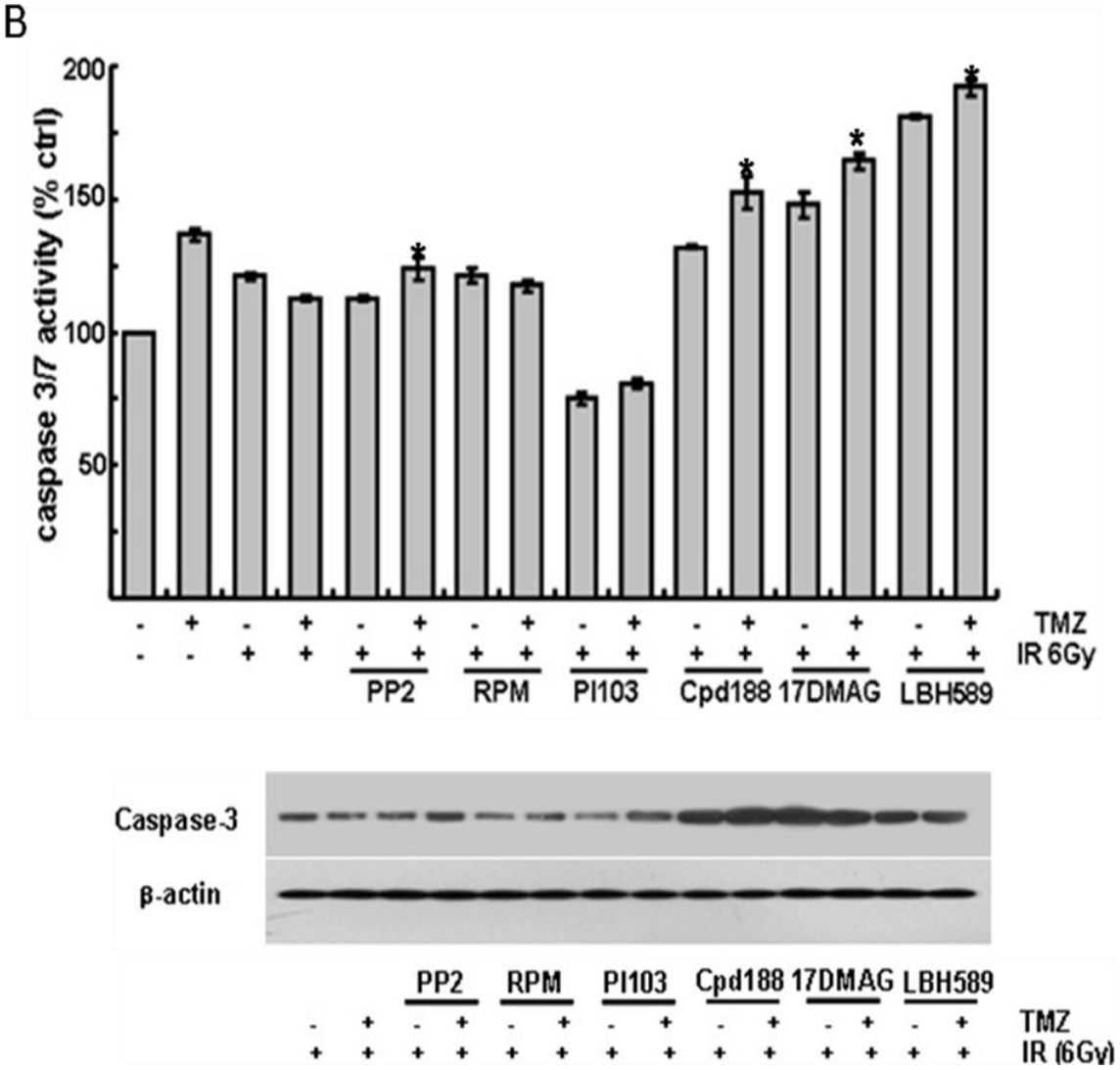
A. U251 cells were pretreated with the indicated inhibitors plus TMZ before assessment of γH2AX foci formation. Mock-treated control cells were analyzed 6 h after irradiation with 6 Gy. Pretreatment of U251 cells with the dual inhibitor PI103, the HDAC inhibitor LBH589, or a HSP90 inhibitor 17-DMAG plus TMZ caused marked prolongation of radiation-induced γH2AX foci formation 6 h after irradiation with 6Gy.

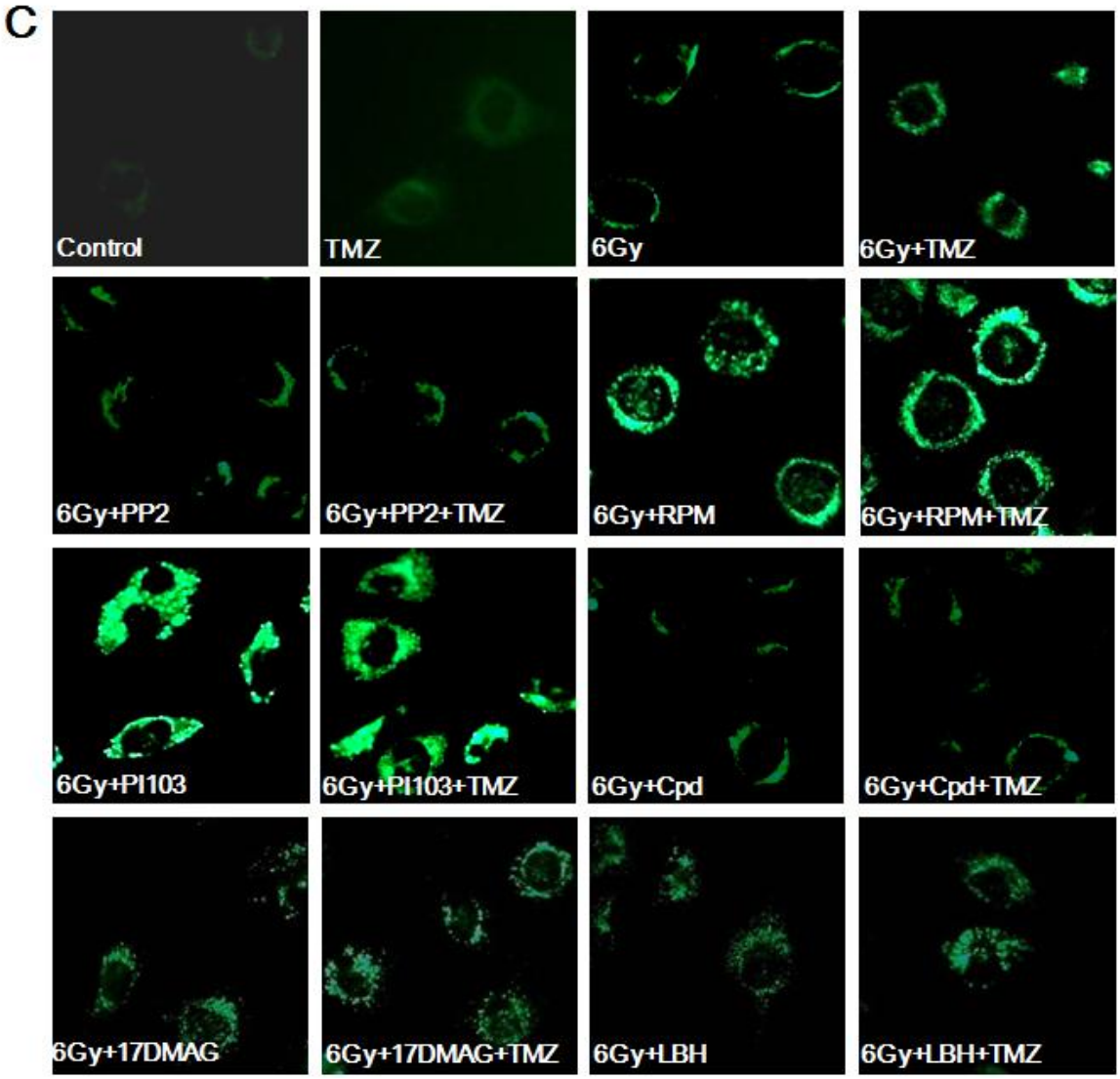
B. Pretreatment of U251 cells with TMZ combined with PI103, LBH589, or 17-DMAG attenuated p-DNA-PK expression.

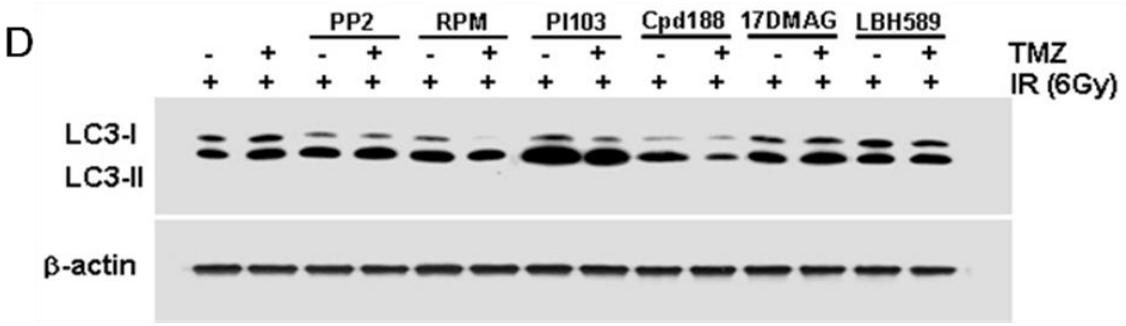
3-6. Mode of cell death

Annexin V-FITC/PI double staining and Caspase 3/7 assay method were employed to examine apoptotic cell death. Annexin-V-FITC staining targets the membranes of apoptotic cells, showing green fluorescence, while PI staining targets the nuclei of apoptotic cells, showing red fluorescence. As shown in Figure 15A, the combined treatment of TMZ with PP2 or Cpd188, or 17-DMAG or LBH589 showed fluorescent green cell membranes and fluorescent red nuclei. Additionally, treatment of TMZ with PP2 or Cpd188, or 17-DMAG or LBH589 increased cleaved caspase3 expression and caspase-3/7 activity within 24 h after combination treatment on U251 cells (Figure 15B). Pretreatment with TMZ combined with rapamycin or PI103 increased punctate fluorescence or lysosomal localization of LysoTracker in U251 cells at 24 h (Figure 15C). To further elucidate the mechanism underlying autophagy in U251 cells, we examined the effect of the combination treatment of each inhibitor with or without TMZ on the conversion of microtubule-associated protein light chain (LC3). Treatment with rapamycin or PI103 in the presence or absence of TMZ increased LC3–II (16kDa) expression in U251 cells at 24 h after each combined treatment (Figure 15D). Senescence was examined by detecting the activity of β-galactosidase and no discernable change was detected in U251 cultures within 7 days after the treatment (Figure 15E).


A
Annexin V
PI
Merge
Annexin V
PI
Merge
Control
6Gy
TMZ
6Gy+TMZ
6Gy+PP2
6Gy+PP2+TMZ
6Gy+RPM
6Gy+RPM+TMZ
6Gy+PI103
6Gy+PI103+TMZ
6Gy+Cpd
6Gy+Cpd+TMZ
6Gy+17DMAG
6Gy+17DMAG+TMZ
6Gy+LBH
6Gy+LBH+TMZ


B
caspase 3/7 activity (% ctrl)
200
150
100
50
TMZ
IR 6Gy
PP2
RPM
PI103
Cpd188
17DMAG
LBH589
Caspase-3
β-actin
PP2
RPM
PI103
Cpd188
17DMAG
LBH589
TMZ
IR (6Gy)


C
Control
TMZ
6Gy
6Gy+TMZ
6Gy+PP2
6Gy+PP2+TMZ
6Gy+RPM
6Gy+RPM+TMZ
6Gy+PI103
6Gy+PI103+TMZ
6Gy+Cpd
6Gy+Cpd+TMZ
6Gy+17DMAG
6Gy+17DMAG+TMZ
6Gy+LBH
6Gy+LBH+TMZ


D
PP2
RPM
PI103
Cpd188
17DMAG
LBH589
TMZ
IR (6Gy)
LC3-I
LC3-II
β-actin

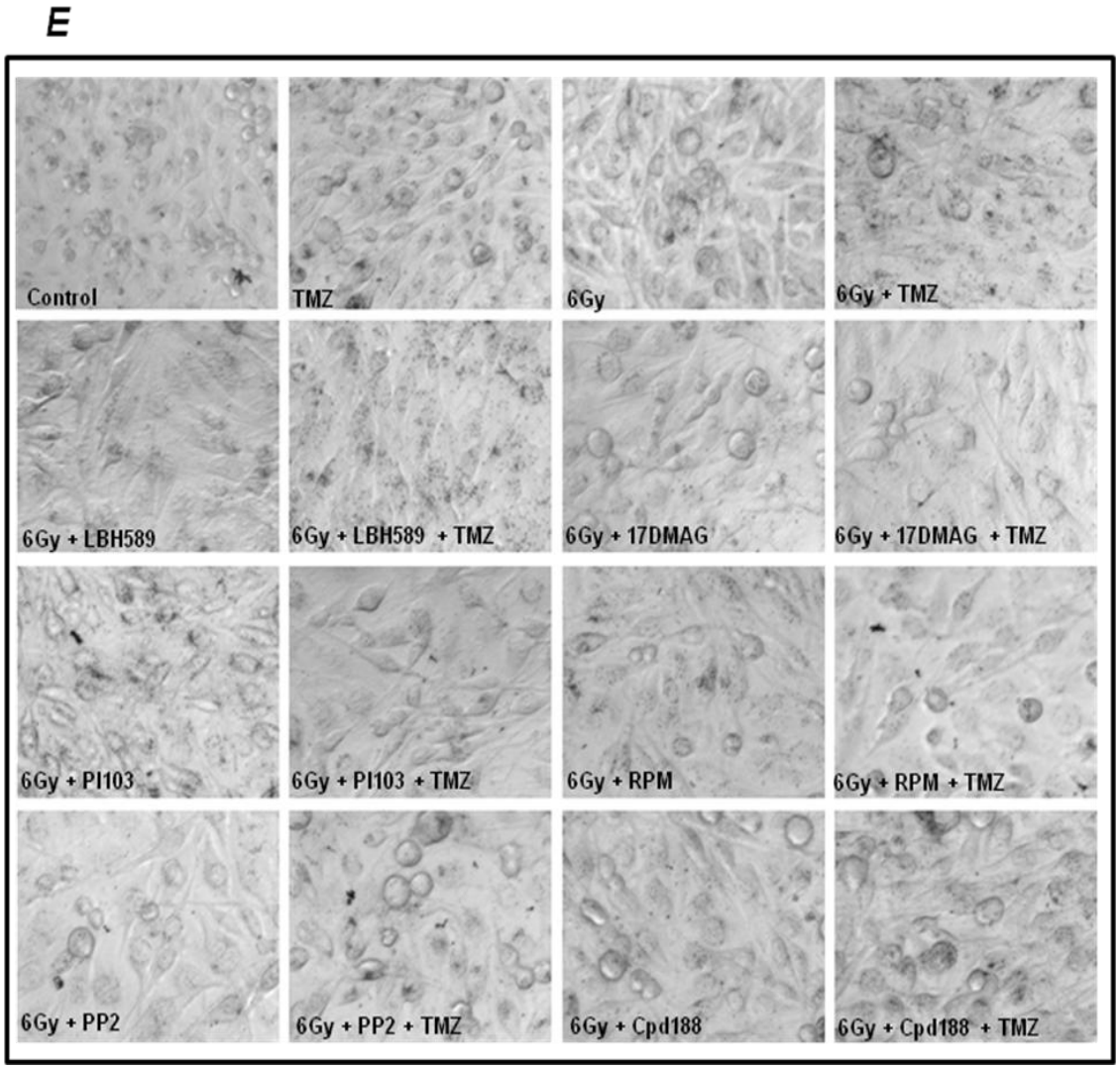


Figure 15. Mode of cell death.

A. Annexin V-FITC/PI double-staining method was employed to examine cell apoptosis with 6Gy irradiation. U251 cells are analyzed for green fluorescence (FITC) and red fluorescence (PI). The combination treatment of TMZ with PP2, Cpd188, 17-DMAG, or LBH589 increased green and red fluorescence on U251cells.

B. The combination treatment of TMZ with PP2 or Cpd188 or 17-DMAG or LBH589 increased caspase-3/7 activity within 24 h after combination treatment on U251 cells. Data

are presented as the mean ± SD of three experiments. *$P<0.05$ *versus* IR and TMZ

C. Pretreatment of U251 glioblastoma cells with TMZ combined with rapamycin or PI103 increased punctate fluorescence or lysosomal localization of LysoTracker at 24 h.

D. Treatment with rapamycin or PI103 in the presence or absence of TMZ increased LC3–II (16kDa) expression in U251 glioblastoma cells at 24 h after each combined treatment but had no detectable effect on T98G cells.

E. Senescence was examined by detecting the activity of β-galactosidase and no discernable change was detected in U251 cultures within 7 days after the treatment.

3-7. Toxicity towards normal human astrocytes

As shown in Figure 16, the surviving fraction at 2 Gy (SF2) of normal human astrocytes was not significantly decreased by pretreatment with the indicated inhibitors, alone or combined with TMZ.

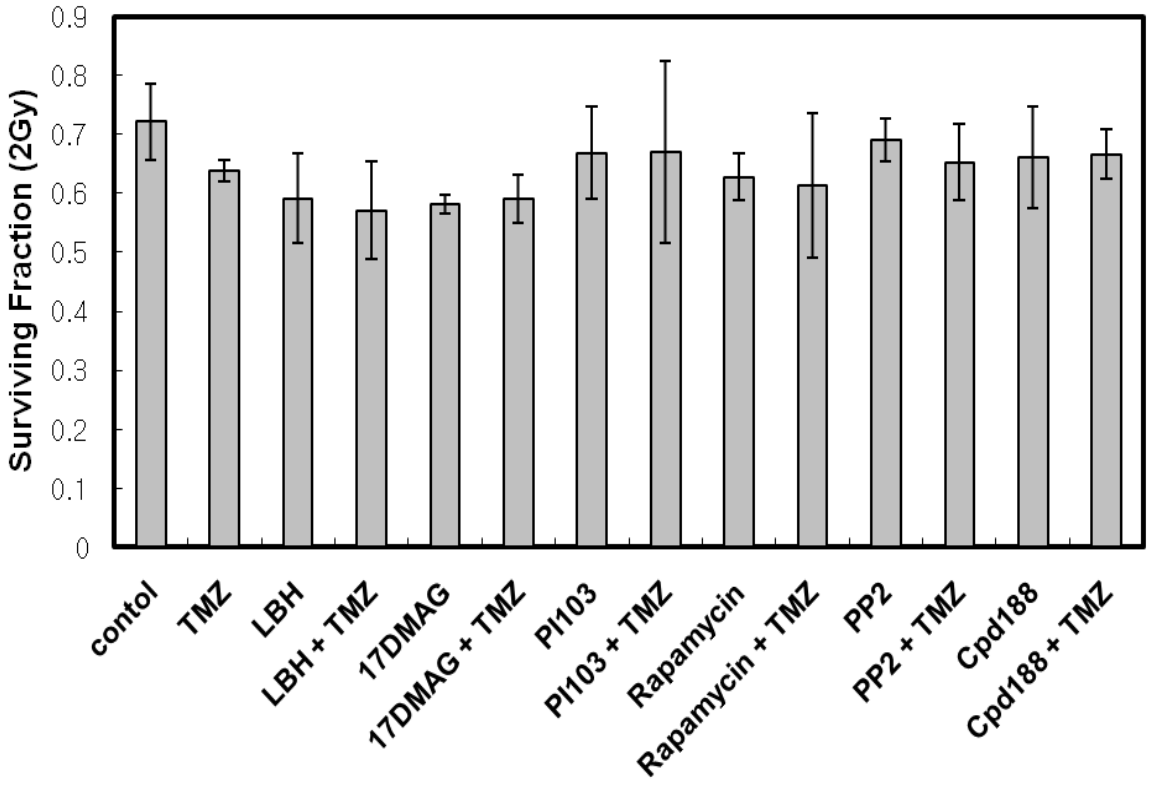


Figure 16. Toxicity towards normal human astrocytes.

Effect of combined treatment with TMZ and the indicated inhibitors on the growth of normal astrocytes. Surviving fraction at 2 Gy (SF2) is shown for normal human astrocytes treated with the indicated inhibitors in the presence and absence of TMZ compared with control. Each value represents the mean surviving fraction calculated from cells treated in triplicate.

Supplementary Table 1. Sensitizer enhancement ratio of U251 cells

Inhibitor	$SER_{0.5}$	$SER_{0.05}$
PP2	1.15	1.08
TMZ	1.41	1.17
TMZ+PP2	**1.54**	**1.27**
RPM	1.06	1.04
TMZ	1.41	1.17
TMZ+RPM	**1.21**	**1.18**
PI103	1.41	1.20
TMZ	1.41	1.17
TMZ+PI103	**1.55**	**1.34**
Cpd188	1.25	1.17
TMZ	1.32	1.17
TMZ+Cpd188	**1.43**	**1.51**
17DMAG	1.31	1.18
TMZ	1.41	1.17
TMZ+17DMAG	**1.55**	**1.39**
LBH589	1.27	1.10
TMZ	1.41	1.17
TMZ+LBH589	**1.50**	**1.35**

Supplementary Table 2. Sensitizer enhancement ratio of T98G cells

Inhibitor	$SER_{0.5}$	$SER_{0.05}$
PP2	1.16	1.05
TMZ	1.26	1.07
TMZ+PP2	**1.38**	**1.11**
RPM	1.13	1.04
TMZ	1.26	1.07
TMZ+RPM	**1.23**	**1.10**
PI103	1.26	1.14
TMZ	1.26	1.07
TMZ+PI103	**1.65**	**1.30**
Cpd188	1.05	1.05
TMZ	1.26	1.07
TMZ+Cpd188	**1.31**	**1.10**
17DMAG	1.16	1.03
TMZ	1.26	1.07
TMZ+17DMAG	**1.29**	**1.20**
LBH589	1.24	1.01
TMZ	1.26	1.07
TMZ+LBH589	**1.40**	**1.14**

4. Discussion

The current standard of care for malignant glioma is initial treatment with radiation therapy combined with TMZ; however, malignant gliomas usually recur with a median time to progression of approximately 7 months[63]. Recently, several studies have focused on aberrant signal transduction in GBM, resistance mechanisms of GBM to TMZ and radiotherapy. Inhibition of signal transduction pathways may provide the basis for a new paradigm of GBM therapy, based on the fact that most human gliomas exhibit aberrant activation of a pro-survival/pro-growth signaling network.

EGFR is one of the most attractive therapeutic targets in GBM since the gene is amplified and over-expressed in approximately 40% of primary GBMs, especially those of the classical subtype. Nearly half of tumors with EGFR amplification also express a constitutively active EGFR mutant, EGF variant VIII (EGFRvIII), which has an in-frame deletion of exons 2-7 within the EGFR extracellular domain[75, 76]. Clinical trials with EGFR kinase inhibitors such as gefitinib and erlotinib did not show a significant benefit on overall survival or progression-free survival in patients with malignant glioma[66]. Given the role of this growth factor receptor in gliomagenesis[77], the failure of EGFR inhibitors in GBM patients was particularly disappointing. Understanding the molecular mechanism of resistance may provide insight into the development of alternative strategies to tackle this issue.

Some studies found that tumors with EGFRvIII[78] and intact PTEN and tumors with low p-Akt levels are more likely to respond to EGFR inhibitors[79]. Several investigators have identified loss of the PTEN tumor suppressor as a resistance factor for EGFR kinase

inhibitor therapy[10, 11, 80]. Vivanco et al. also showed a critical role of PTEN in downregulation of activated EGFR. The PI3K/Akt/mTOR pathway is a critical regulator of tumor cell metabolism, growth, proliferation, and survival. In malignant gliomas, activity of this signaling network is frequently increased because of receptor tyrosine kinase over-activity, loss of PTEN tumor suppressor, and/or mutated oncogenic PI3K subunits[67]. Attempts to inhibit the PI3K pathway with pan-PI3K inhibitors such as LY294002 have not progressed to clinical use due to concerns over organ toxicity and poor bioavailability[81, 82]. Inhibition of the pathway using rapamycin resulted in paradoxical activation of Akt through loss of negative feedback in a subset of patients, which in turn was related to shorter time-to-progression during postsurgical maintenance rapamycin therapy[83]. The limited single-agent activity of rapamycin analogs in several GBM trials[84, 85] provides a rationale for ongoing clinical trials with dual PI3K/mTOR inhibitors in GBM. A clinical trial of a dual PI3K/m-TOR inhibitor, XL765, in combination with TMZ is currently underway for GBM[86]. Our results are in line with previous reports since combined treatment with TMZ and a dual PI3K/m-TOR inhibitor, XL765, has been successfully tested in glioma cell lines[82, 86]. Based on our results, we propose dual targeting PI3K/m-TOR with PI103 as a therapeutic strategy with potential to inhibit DNA-PK, and induce increased autophagy in GBM.

The Src family of tyrosine kinases consists of nine non-receptor membrane associated proteins. Although the mechanisms by which Src acts in tumorigenesis and progression are not completely defined, it is likely that Src is a central regulator at the interface between extracellular signals and intracellular pathway activation. Src can be activated by integrin engagement or by activation of cell surface receptors, including EGFR and platelet-derived

growth factor receptor, and can mediate phosphorylation of multiple intracellular substrates including EGFR, STAT3, focal adhesion kinase, and cyclin D[87]. Furthermore, there is biological synergy between intracellular Src and EGFR[68]. The cancer promoting potential of Src also can be explained, in part, by downstream activation of PI3K and ERK pathways. In addition to promoting tumor growth, Src plays a role in cell adhesion, invasion[88], angiogenesis[89], and inhibition of apoptosis[90].

STAT3 is a member of the STAT family of transcription factors, which are located in the cytoplasm in their inactive forms. Following stimulation by an extracellular signal such as a growth factor, Janus kinases induce phosphorylation of STAT3, leading to its dimerization via Src-homology 2 (SH2) domains and translocation to the nucleus where it regulates the expression of critical genes involved in cell proliferation, survival, migration, and invasion[91]. Previous studies showed that aberrant activation of STAT3 was identified not only in glioblastoma but also in many other cancers[92]. Further, it has recently been demonstrated that STAT3 is essential for the aggressiveness of gliomas [93]. Interestingly, STAT3 does not seem to be essential for the survival of untransformed cells, but it is indispensable for many different tumor cells[94]. Additionally, down-regulation of STAT3 induces apoptosis of human glioma cells[95]. In our study, a STAT3 inhibitor, Cpd188 especially potentiated the radiosensitizing effect of TMZ in U251 cells showing high levels of p-STAT3 expression via the induction of apoptosis.

Data on glioblastoma in The Cancer Genome Atlas indicate that tumorigenesis and progression involve multiple molecular abnormalities[96]. HSP90, a molecular chaperon, is essential for the stability and function of many oncogenic client proteins that are frequently dysregulated in malignant glioma, such as mutant EGFR, Akt, and p53. Since HSP90 is

essential for the function of normal cells as well as tumor cells, one might be concerned that inhibition of its functions might not be selective for malignancy. Both preclinical and clinical observations, however, have shown that HSP90 inhibitors can be given in vivo at doses and schedules that exert antitumor activity without causing host toxicity[97]. In addition to counteracting pro-survival signaling, HSP90 can play a role in DSB repair and the activation of cell cycle check point[98]. Inhibition of multiple signaling circuitries through the abrogation of HSP90 may be an effective treatment strategy for highly recalcitrant tumors such as glioblastoma[99-101].

HDAC inhibitors (HDIs) target epigenetic modifications that interfere with transcriptional regulation and can induce growth arrest and cell death[102-104]. We previously reported that HDIs potentiate radiation-induced cell killing in a panel of human cancer cells through diverse mechanisms[105]. LBH589 preferentially radiosensitized human glioma cells that exhibited activated EGFR signaling due to the EGFRVIII mutation. Treatment with LBH589 or specific inhibition of HDAC6 led to acetylation of HSP90, which induced down-regulation of the client oncoproteins EGFR and decreased levels of p-Akt[105]. LBH589 has also been reported to induce apoptosis, and delay of DNA damage repair in lung cancer cells[106].

Our results suggest mechanisms that may be potential targets for counteracting the pro-survival signaling implicated in radioresistance of malignant glioma cells and offer insight into potential strategies to improve the therapeutic outcome of radiotherapy and TMZ in the management of GBM.

General discussion

EGFR is an important determinant of tumor response to ionizing radiation[5]. Elevated levels of EGFR activity and expression are frequently correlated with the radioresistance of tumors, including NSCLC[3] and malignant gliomas[4]. Therefore, the molecular inhibition of EGFR is a promising therapeutic strategy for enhancing the cytotoxic effects of radiotherapy. However, therapeutic resistance for anti-EGFR agents resulting from several factors, including activation of downstream pathways or alternative survival pathways, as well as molecular resistance mechanisms, has emerged as an important issue in the clinic[33-35]. In this study, we showed that targeting signaling pathways downstream of the EGFR could enhance radiosensitivity of a panel of NSCLC and glioma cell lines expressing activated EGFR.

In the first part, we demonstrated that siRNA–induced inhibition of K-RAS, but not of EGFR, increased the radiosensitivity of A549 and H460 lung cancer cells. These results suggest that targeting the EGFR alone is not an effective strategy for modulating the radiation response in NSCLC cells possessing constitutive activation of downstream signaling due to mutations in K-RAS. Similarly, selective inhibition of the PI3K-AKT-mammalian target of rapamycin (mTOR) pathway using pharmacologic inhibitors, not of ERK signaling, enhanced the radiosensitivity of these cells. Treatment of these inhibitors resulted in the abrogation of G2 arrest following ionizing radiation and induced the prolongation of γH2AX, indicating the impairment of DNA damage repair. Inhibition of PI3K-AKT signaling was associated with the downregulation of p-DNA-PK. Although

apoptosis was the predominant mode of cell death when cells were pretreated with LY294002 or AKT inhibitor VIII, mixed modes of cell death, including autophagy, were observed when the cells were pretreated with rapamycin or PI-103. These results showed that targeting PI3K-AKT-mTOR signaling causes significant radiosensitization of lung cancer cells expressing activated EGFR and K-RAS by inhibiting prosurvival signaling and DNA damage repair. PI3K-AKT-mTOR signaling seems to be the common downstream pathway implicated in radioresistance in NSCLC cells. Further, agents to inhibit RAS protein synthesis or RAS membrane localization have not demonstrated efficacy against NSCLC containing activated K-RAS[27]. Accordingly, inhibition of effector molecules downstream of K-RAS may hold promise and further efforts to develop therapies for patients with K-RAS mutant NSCLC are urgently needed. Therefore, targeting of the PI3K-AKT-mTOR signaling pathway using these inhibitors could be a viable approach to simultaneously counteracting EGFR and K-RAS prosurvival signaling, and an alternative strategy in NSCLC showing activated EGFR and K-RAS to overcome therapeutic resistance of currently available EGFR inhibitors combined with irradiation.

In the second part, we evaluated whether targeting EGFR-associated signaling would radiosensitize glioma cells having activated EGFR and mutated PTEN and further increase the radiosensitizing effect of TMZ. Although the activation of EGFR-associated signaling was frequent and poor prognosis, the outcome of EGFR targeted therapy for high grade glioma has been disappointing due to diverse mechanism of therapeutic resistance[75]. Therefore, we first evaluated the efficacy of targeting downstream molecules in EGFR-associated signaling pathway such as Src, PI3K-AKT-mTOR and STAT3 in two glioma cell lines, U251 and T98G. We also tested the effect of an HSP90 inhibitor and a histone

deacetylase (HDAC) inhibitor, focusing on targeting pro-survival signaling from EGFR. U251 cells that have a high proportion of methylated-MGMT compared to unmethylated-MGMT were highly susceptible to the radiosensitizing effect of TMZ. The cytotoxic effects of radiotherapy plus TMZ in this cell line were further increased by treatment with a Src inhibitor, PP2; a dual inhibitor of Class I PI3K and mTOR, PI103; a STAT3 inhibitor, Cpd188; a HSP90 inhibitor, 17-DMAG; or a histone deacetylase (HDAC) inhibitor, LBH589. Conversely, T98G cells that have a high proportion of unmethylated-MGMT were less susceptible to the radiosensitizing effect of TMZ. Treatment with PI103, 17-DMAG, and LBH-589 enhanced the cytotoxic effect of radiotherapy plus TMZ in this cell line. However, treatment with a mTOR inhibitor, rapamycin, did not discernibly potentiate the radiosensitizing effect of TMZ in either cell line. The mechanism of enhanced radiosensitizing effects of TMZ was multifactorial, involving impaired DNA damage repair, induction of autophagy or apoptosis. Overall, these results suggest that targeting downstream molecules in an EGFR-associated signaling network, ligand-independent modulation using an inhibitor of HSP90, or epigenetic modulation through inhibition of HDAC could be potential strategies to improve the therapeutic outcome of GBM. Current single agent receptor targeting does not achieve a maximal therapeutic effect in GBM, and activation of downstream pathways confer resistance to current available agents[75]. Therefore, the inhibition of molecules more downstream of the EGFR might be more effective to circumvent resistance of GBM to TMZ and radiotherapy. To our best knowledge, no study has been reported about the antitumor effects in vitro of PP2, Cpd188, or LBH589 on malignant glioma in combination with TMZ. Further, agents such as PI103 or Cpd188 that selectively sensitize glioma cells to the cytotoxic effects of RT plus TMZ

while not increasing side effects to normal astrocytes, might further improve GBM therapy. Although future work will be required to elucidate whether these signaling occur in vivo, these results suggest possible targets for overcoming radioresistance in GBM. Since the biology of malignant glioma involves a complex network of interconnected signaling pathways resulting in cell growth, survival and the aggressive phenotype, careful preclinical interrogation is necessary to determine optimal treatment combinations.

Taken together, these results suggest possible mechanisms for counteracting EGFR prosurvival signaling implicated in radioresistance of NSCLC and malignant glioma cells, and provide possible targets for preventing radioresistance to anti-EGFR agents combined with radiation therapy.

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국 문 초 록

비소세포성 폐암과 악성 교종 세포종에서 EGFR 신호전달계 조절을 통한 방사선 감수성 증강작용에 관한 연구

EGFR 신호전달계는 폐암과 악성 교종의 발병과 관련하여 중요한 역할을 하며, 따라서 EGFR 신호전달계의 억제가 방사선 항암 치료 효과를 증대시키기 위한 좋은 표적이 되고 있으나, EGFR 저해제에 대한 치료 저항성이 주요 임상적 문제로 떠오르고 있다. 본 논문에서는 EGFR 신호전달 경로와 관련된 다양한 하위경로의 조절을 통해 EGFR이 활성화 되어 있는 비소세포성 폐암과 악성 교종 세포종에서 방사선 감수성 증강작용 및 그 기전에 관하여 연구하였다.

첫 번째 파트에서는, 활성화된 EGFR 또는 K-RAS 변이 또는 두 가지 모두를 갖고 있는 비소세포성 폐암주에서 EGFR 하위 신호전달경로 조절이 방사선 감수성에 어떠한 영향을 미치는지 연구하였다. RNA 간섭효과를 이용한 K-RAS의 억제는 A549, H460 세포의 방사선 감수성을 증가시켰지만, EGFR의 억제는 영향이 없는 것으로 나타났다. K-RAS 코돈 V12 변이를 보이는 A549 세포주와, K-RAS 코돈 V61 변이를 보이는 H460 세포주는 각각의 코돈을 타겟팅하는 K-RAS siRNA에 의해 방사선 감수성이 증대되었다. 반면에, wild type K-RAS를 보이는 H1299 세포주는 K-RAS siRNA에 의해 방사선 감수성이 증가하지 않은 것으로 나타났다. PI3K-AKT-mTOR 신호전달경로의 차

단은 두 가지 세포주에서 모두 방사선 감수성을 증가시켰으나, ERK 신호전달 경로의 차단은 방사선 감수성에 영향이 없었다. PI3K-AKT-mTOR 신호전달 경로의 차단은 방사선과 병합 후 G2 arrest 억제와 γH2AX foci 지연을 유도하였다. Class I PI3K/mTOR 이중 억제제는 A549와 H460 세포주에서 모두 효과적으로 방사선 감수성을 증가시켰다. PI3K/AKT 신호전달경로의 차단은 DNA-PK의 인산화 억제와 관련이 있었다. 주요 세포사멸기전으로 LY294002와 AKT inhibitor VIII의 경우는 apoptosis를, rapamycin이나 PI-103의 경우는 apoptosis와 autophagy를 모두 보이는 것으로 나타났다.

두 번째 파트에서는 EGFR이 활성화 되어 있는 악성 교종 세포종에서 EGFR 신호전달계 억제 시 방사선 감수성에 미치는 효과를 보고, 이와 더불어 temozolomide (TMZ)와의 병합 처리 시 방사선 감수성에 미치는 영향에 관하여 연구하였다. 악성 교종 세포종의 방사선 항암 치료 효과는 TMZ에 의해 증진되었으나, 여전히 예후가 좋치 않다. 따라서 Src, PI3K-AKT-mTOR, STAT3과 같은 EGFR 하위 신호전달경로의 조절과 HSP90 억제제를 이용한 ligand-independent modulation, histone deacetylase (HDAC) 억제제를 이용한 epigenetic modulation 등을 통해 방사선 감수성에 미치는 영향에 관하여 연구하였다. TMZ에 의한 방사선 감수성 증강작용은 MGMT가 과메틸화된 U251 세포주에서 더 크게 나타났다. U251 세포주에서는 Src 억제제인 PP2와 Class I PI3K /mTOR 억제제인 PI103, STAT3 억제제인 Cpd188, HSP90 억제제인 17-DMAG, HDAC 억제제인 LBH589가 TMZ와 병합처리 하였을 때

방사선 감수성을 효과적으로 증가시켰다. 반면에, 저메틸화된 MGMT를 보이는 T98G 세포주에서는 TMZ와 PI103, 17-DMAG, LBH-589와의 병합처리가 TMZ 단독처리 군에 비하여 방사선 감수성을 증가시키는 것으로 나타났으며, 이러한 효과는 U251 세포주보다 낮았다. 이와 같은 방사선 감수성 증강작용은 손상된 DNA damage repair 기전과 autophagy 또는 apoptosis의 증대 등을 수반하는 다양한 요인과 관련이 있는 것으로 나타났다.

이상의 결과는 EGFR pro-survival 신호전달계와 관련된 다양한 하위신호전달경로의 조절을 통해, 비소세포성 폐암과 악성 교종 세포종에서 기존 EGFR 항암제의 방사선 치료저항성을 극복할 수 있는 가능성 있는 기전과 대안을 제시한다.

주요어: 비소세포성 폐암, 악성 교종 세포종, Epidermal growth factor receptor (EGFR), K-RAS, Temozolomide (TMZ), 방사선감수성증대

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