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Targeting Epidermal Growth Factor Receptor–Associated Signaling Pathways in Non–Small Cell Lung Cancer Cells: Implication in Radiation Response

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Abstract

Several studies have shown solid evidence for the potential value of targeting epidermal growth factor receptor (EGFR) signaling to enhance the antitumor activity of radiation. However, therapeutic resistance has emerged as an important clinical issue. Here, we investigated whether strategies for targeting EGFR-associated downstream signaling would radiosensitize a panel of non–small cell lung cancer cell lines. Inhibition of K-RAS using RNA interference attenuated downstream signaling and increased radiosensitivity of A549 and H460 cells, whereas inhibition of EGFR did not. A549 cells harboring a K-RAS mutation at codon V12 were radiosensitized by small interfering RNA (siRNA) targeting this codon. H460 cells having mutation at codon V61 was radiosensitized by siRNA targeting of this mutation. 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, whereas selective inhibition of extracellular signal-regulated kinase signaling did not. Inhibitors targeting the PI3K-AKT-mTOR pathway also abrogated G_2 arrest following irradiation and induced γH2AX foci formation. A dual inhibitor of class I PI3K and mammalian target of rapamycin effectively increased the radiosensitivity of A549 and H460 cells. Inhibition of PI3K-AKT signaling was associated with the downregulation of DNA-PKs. Although apoptosis was the primary mode of cell death when cells were pretreated with LY294002 or AKT inhibitor VIII, cells pretreated with rapamycin or PI-103 showed mixed modes of cell death, including apoptosis and autophagy. Our results suggest possible mechanisms for counteracting EGFR prosurvival signaling implicated in radioresistance and offer an alternative strategy for overcoming resistance to EGFR inhibitors used in combination with irradiation. *Mol Cancer Res; 8(7); 1027–36. ©2010 AACR.*

Introduction

The epidermal growth factor receptor (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 (1). Non–small cell lung cancer (NSCLC) specimens have been reported to exhibit mutations in multiple oncogenes and tumor suppressors, including EGFR, K-RAS, and tumor protein 53 (TP53). NSCLC cells express EGFR and its ligands, which together play important roles in the pathogenesis of lung cancer (2), 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 (3-5). However, therapeutic resistance resulting from several factors, including activation of downstream or alternative survival pathways, as well as molecular resistance mechanisms, has emerged as an important issue in the clinic (6, 7).

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

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identify potential therapeutic target(s) for overcoming resistance to EGFR-targeted therapy combined with radiation. 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. Specific small interfering RNA (siRNA)–induced inhibition of K-RAS, but not of EGFR, attenuated p-AKT and p-mitogen-activated protein kinase expression significantly and also increased the radiosensitivity of A549 and H460 cells. Similarly, selective inhibition of the PI3K-AKT-mammalian target of rapamycin (mTOR) pathway using pharmacologic inhibitors reduced the expression of p-AKT and p-$p70^{S6K}$ significantly, in addition to enhancing the radiosensitivity of these cells. Pretreatment with these inhibitors resulted in the abrogation of G_2 arrest following ionizing radiation and induced the prolongation of phosphorylated histone H2AX (γH2AX), indicating the impairment of DNA damage repair. In contrast, selective inhibition of the mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase-ERK pathway did not cause radiosensitization, abrogation of the G_2 checkpoint, or prolongation of γH2AX foci formation. PI-103, a dual inhibitor of class I PI3K and mTOR, effectively potentiated radiation-induced cell killing in A549 and H460 cells. Inhibition of PI3K-AKT signaling was associated with the down-regulation of DNA-PKs. 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.

Our data implicate PI3K-AKT-mTOR signaling as a potential therapeutic target to overcome resistance to EGFR-targeted therapy in combination with radiation.

Materials and Methods

Cell lines and cell culture

Three lung cancer cell lines were selected based on the known status of EGFR (8, 9), K-RAS, and TP53 (10) 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 possess 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 and were cultured in DMEM (Welgene) supplemented with 10% fetal bovine serum. Cells were maintained at 37°C in an atmosphere of 95% air/5% CO_2.

Pharmacologic inhibitors

LY294002 and rapamycin were obtained from Cell Signaling Technology, Inc. UO126, PD98059, and AKT inhibitors VIII and PI-103 (the pyridinylfuranopyrimidine inhibitor) were obtained from Calbiochem. Three-methyladenine and z-VAD were obtained from Sigma. Inhibitors were prepared as concentrated stock solutions in DMSO, stored at −20°C, and diluted in culture medium at the time of use. Control cells were treated with medium containing the same concentration of carrier DMSO.

RNA interference

Totals of 1 to 2 × 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 replaced with antibiotic-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 K-RAS 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 as control (Bioneer).

Each siRNA in reduced-serum medium (OPTIMEM, Life Technologies) was transfected into cells using Lipofectamine 2000 (Invitrogen) 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 analysis.

Clonogenic assays

Clonogenic assay was done according to a previously described protocol (11). Radiation-survival data were fitted to a linear-quadratic model using Kaleidagraph version 3.51 (Synergy Software). Sensitizer enhancement ratio (SER) was defined as the ratio of the isoeffective dose at SF 0.5 in the absence of inhibitors to that in the presence of inhibitors.

Western analysis

Cells were washed, scraped, and resuspended in lysis buffer (iNtRON Biotechnology). Proteins were solubilized by sonication, and equal amounts of protein were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Millipore Corp.). Membranes were blocked in PBS containing 0.1% Tween 20 and 5% powdered milk, and probed with primary antibody directed against p-EGFR (Tyr1068), EGFR, p-AKT (Ser473), AKT, p-ERK (Tyr202/204), ERK, p-$p70^{S6K}$ (Thr421/Ser424) and 70^{S6K}, DNA-PKs (Thr2609), Rad51, and LC3 (Cell Signaling Technology, Inc.) at 1:1,000 dilution. Monoclonal anti–K-RAS and anti–β-actin antibody (Santa Cruz Biotechnology) were used at dilutions of 1:500 and 1:5,000, respectively. Membranes were washed and incubated with secondary antibody consisting of peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch Laboratories) at a dilution of 1:2,000.

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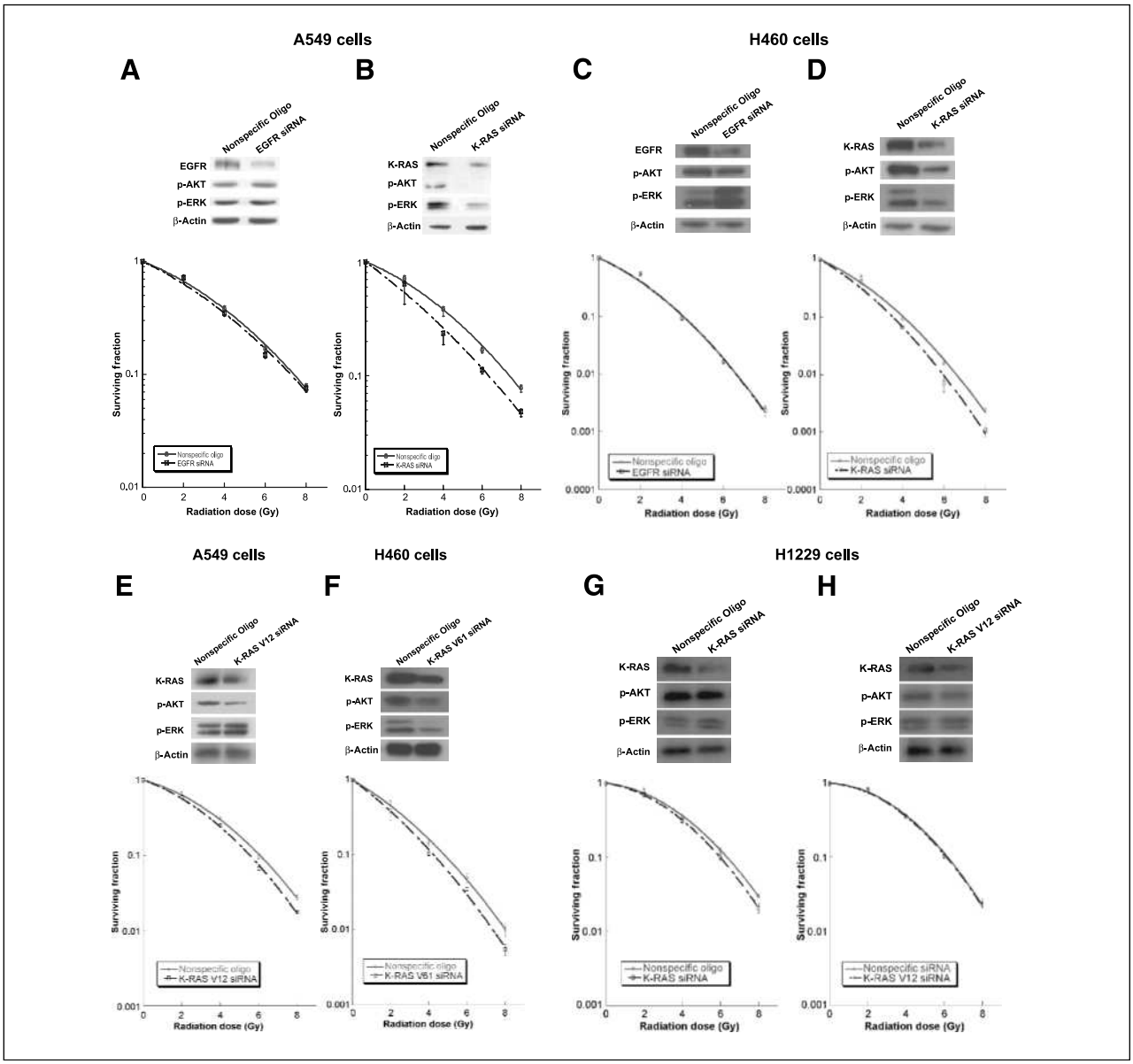

FIGURE 1. Specific inhibition of EGFR or K-RAS signaling. A and B, A549 cells were transfected with siRNAs targeting either EGFR or whole K-RAS. C and D, H460 cells were transfected with siRNAs targeting either EGFR or whole K-RAS. E, siRNAs targeting mutant K-RAS at codon V12 were transfected into A549 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 transfected with siRNA targeting either whole K-RAS or mutant K-RAS at codon V12. Western analysis was done on A549 cell lysates using antibodies specific for EGFR, K-RAS, p-ERK, p-AKT, and β-actin. Points on the survival curves, mean surviving fractions from experiments done in triplicate.

Flow cytometric analysis

Flow cytometric analysis of cell cycle status and apoptosis was done as previously described (11) using a FACScan flow cytometer (Becton Dickinson).

Immunocytochemistry

Cells were grown and treated on chamber slides. At specified times after treatment with each inhibitor, coverslips were rinsed, cells were fixed in 4% paraformaldehyde, and permeablized in methanol for 20 minutes. Cells were subsequently washed and blocked in PBS containing 2% bovine serum albumin for 1 hour. Primary antibody against γH2AX (Cell Signaling) was applied to the cells and incubated overnight. Secondary Alexa Fluor 488–conjugated donkey anti-goat antibody (Molecular Probes) was applied and incubated for 1 hour. 4′,6-Diamidino-2-phenylindole nuclear counter stain was applied at 1 μg/mL for 5 minutes. Slides were examined on an Axio Scope.A1 Imager fluorescent microscope. Images were captured and acquired using AxioCam

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MRc5 and the acquisition software AxioVision v.4.4 (Carl Zeiss).

Results

Specific inhibition of EGFR or K-RAS using RNA interference

To determine the effect of targeting EGFR-KRAS 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 ($SER_{0.5}$), 1.0; Fig. 1A]. In contrast, inhibition of K-RAS reduced p-AKT and p-ERK expression and significantly increased the radiosensitivity of A549 cells ($SER_{0.5}$, 1.5), as shown in the cell survival curve (Fig. 1B). Similar results were obtained using H460 cells expressing K-RAS mutated at codon 61 (Fig. 1C and D).

Next, we wanted to determine whether this effect was specific for the oncogenic mutant form of K-RAS. Fig. 1F 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 ($SER_{0.5}$, 1.3). The siRNAs specifically targeting mutant K-RAS at codon V61 potentiated the radiation-induced cell killing of H460 cells (Fig. 1F), whereas the siRNA targeting mutant K-RAS at codon V12 had no effect on radiosensitivity (Supplementary Figure). H1299 cells expressing mutant TP53 and wild-type K-RAS were not radiosensitized by siRNAs targeting wild-type K-RAS (Fig. 1G) or mutant K-RAS at codon V12 (Fig. 1H). Surviving fraction at 2 Gy of normal human fibroblasts was not significantly decreased by these siRNAs (Supplementary Fig. S1A).

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 Fig. 2A and C, pretreatment with 20 μmol/L′ PD98059, an inhibitor of c-RAF and mitogen-activated protein/ERK kinase kinase, did not cause radiosensitization ($SER_{0.5}$, 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

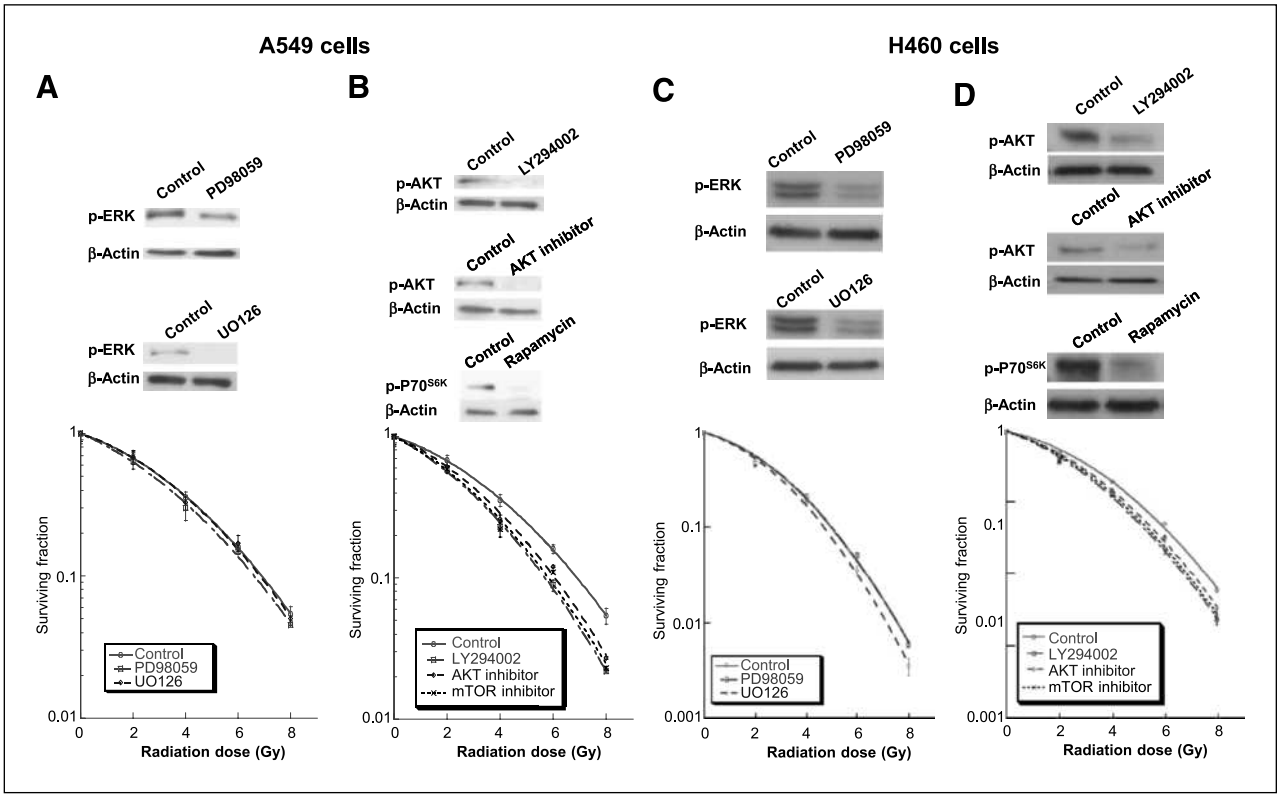

FIGURE 2. Pharmacologic inhibition of ERK or PI3K-Akt-mTOR signaling. A and C, A549 and H460 cells were pretreated with PD98059 or UO126, respectively, and subjected to Western analysis using antibodies specific for p-ERK, p-AKT, and β-actin. Points on survival curves represent mean surviving fractions 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.

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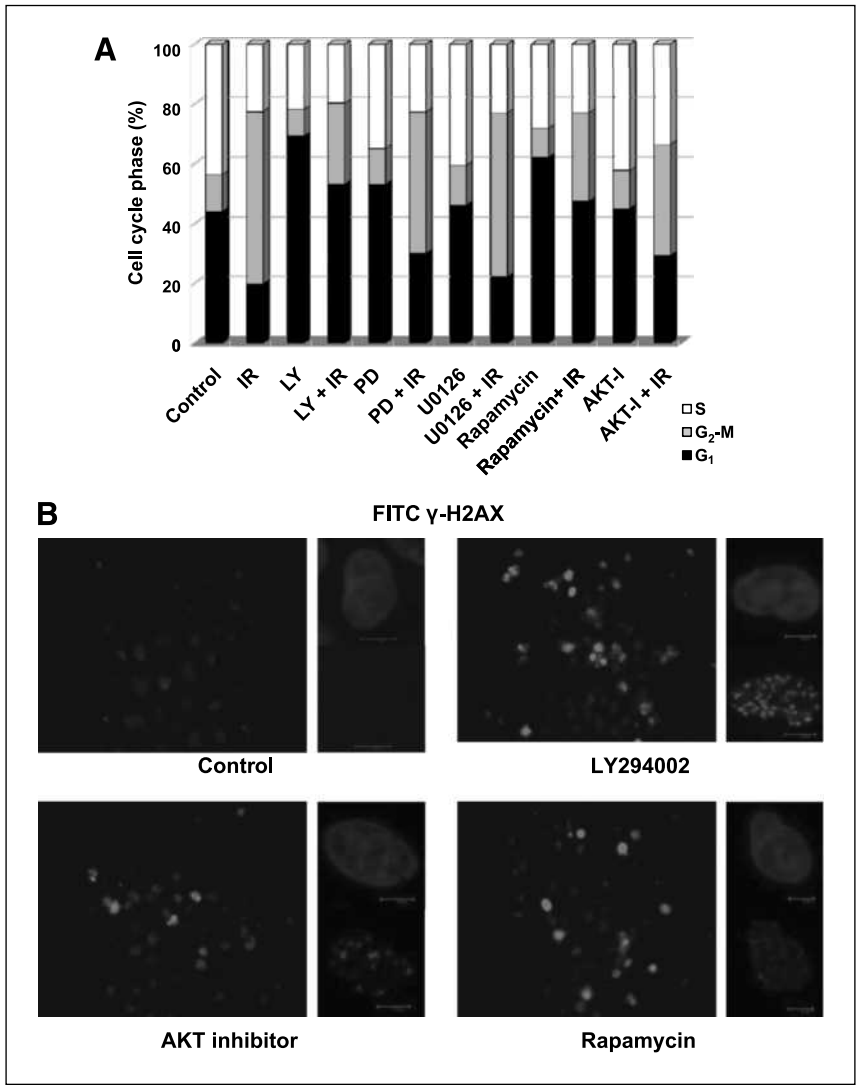

FIGURE 3. Changes in cell cycle progression and γH2AX foci formation following inhibition of PI3K-AKT-mTOR signaling. A, A549 cells were treated with the indicated inhibitors before irradiation (IR) with 6 Gy for 7 h. Subsequently, cells were stained with propidium iodide and then analyzed by flow cytometry 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.

H460 cells ($SER_{0.5}$, 1.0), although ERK phosphorylation was visibly reduced.

Pharmacologic inhibition of PI3K-AKT-mTOR signaling increased 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 ($SER_{0.5}$, 1.5; Fig. 2B and D). As expected, pretreatment with the AKT inhibitor VIII (200 nmol/L) caused significant reduction in p-AKT levels and decreased radiation survival slightly ($SER_{0.5}$, 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 $p\text{-}p70^{S6K}$ levels and significantly increased radiosensitivity in these cells ($SER_{0.5}$, 1.4). Similar results were seen with H460 cells (Fig. 2C and D).

Effects 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 G_2-M delay after irradiation alone (Fig. 3A). This effect was abrogated significantly by pretreatment with LY294002, VIII, or rapamycin. In contrast, abrogation of G_2 arrest was not observed when cells were treated with PD98059 or UO126.

Inhibition of PI3K-AKT-mTOR signaling induced prolongation of γH2AX foci

Consistent with the results discussed above, pretreatment with PI3K-AKT-mTOR pathway inhibitors caused marked prolongation of radiation-induced γH2AX foci

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formation, indicating delayed DNA damage repair compared with mock-treated control cells, 5 hours following irradiation with 6 Gy (Fig. 3B). Inhibitors targeting the ERK signaling pathway did not induce prolongation of γH2AX foci in A549 cells (data not shown).

A dual inhibitor of class I PI3K and mTOR induced effective radiosensitization

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 ($SER_{0.5}$, 1.4 and 1.25; Fig. 4A and B). This sensitizing effect was associated with prolongation of γH2AX foci 5 hours following irradiation with 6 Gy in the presence of PI-103 (Fig. 4C). Surviving fraction at 2 Gy of normal human fibroblasts was not significantly decreased by PI103 (Supplementary Fig. S2B).

Marker of 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 (Fig. 5A). Similar data were obtained for H460 cells (Fig. 5B).

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 (Fig. 6A, top). Pretreatment with 3-methyladenine, a known inhibitor of autophagy, rescued cells from radiosensitization induced by rapamycin or PI-103 (Fig. 6A, bottom). Rapamycin or PI-103 increased punctate fluorescence

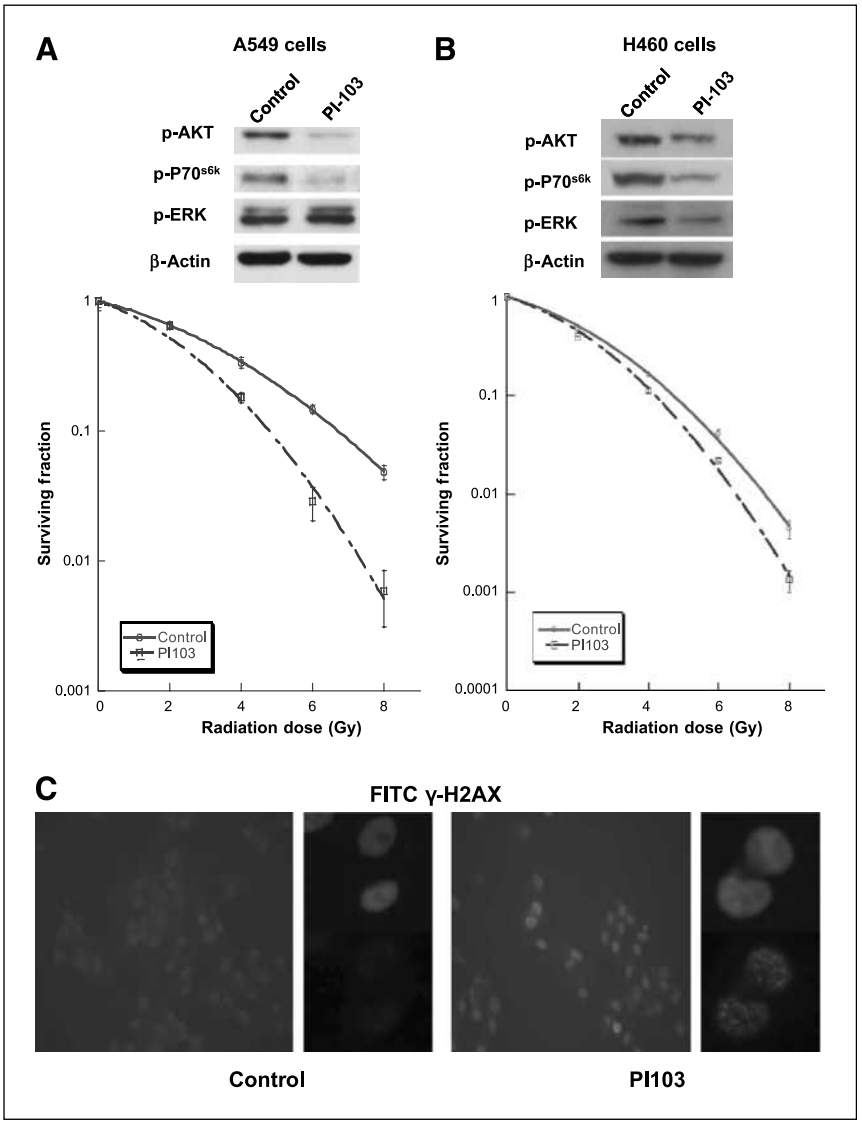

FIGURE 4. A dual inhibitor of class I PI3K and mTOR signaling induced effective radiosensitization. A and B, A549 and H460 cells were pretreated with PI-103 and subjected to Western analysis using antibodies specific for p-AKT, p-p70^{S6K}, p-ERK, and β-actin. Points on survival curves, mean surviving 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.

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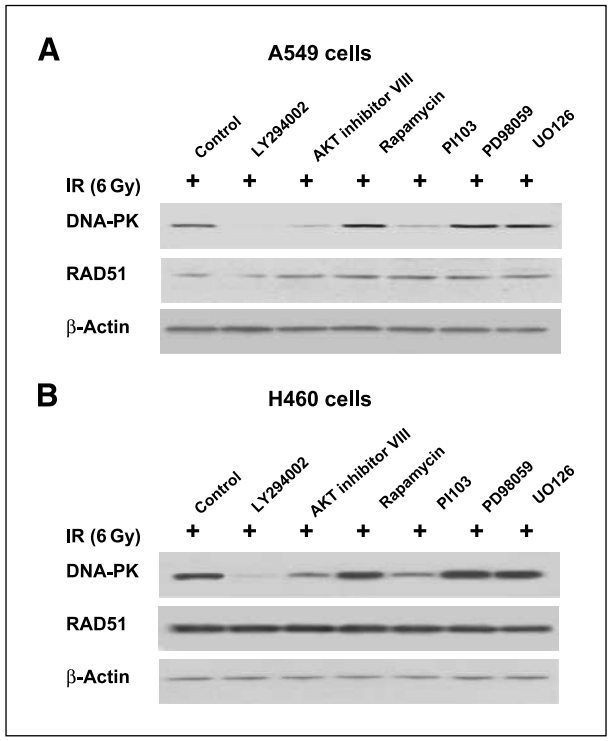

FIGURE 5. Marker of DNA damage repair. Pretreatment of A549 (A) or H460 (B) cells with PI3K inhibitor LY294002 or AKT inhibitor VIII, or a dual inhibitor, PI-103, was associated with decreased DNA-PK phosphorylation at Thr2609.

or lysosomal localization of LysoTracker within 24 hours of treatment (Fig. 6B). 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 (Fig. 6C), which is known to target the class III PI3K, although competition with its kinase domain, and inhibit the early stage of autophagic vesicle formation (12).

Discussion

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. 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 (13). 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 (14). 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 (15). Interestingly, most studies investigating cetuximab have not shown a clear association between EGFR expression and the therapeutic response (16). Moreover, whether negative predictors of responses such as those resulting from K-RAS mutations in colorectal cancer (17, 18) 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 (19, 20). 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 (reviewed in ref. 21). Das et al. (22) 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. 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 (23).

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 indicate that PI3K-mediated activation of AKT in mutant K-RAS–expressing human cancer cells in response to EGFR ligand binding or radiation is independent of a direct K-RAS function but dependent on increased production of EGFR ligands mediated by upregulation of K-RAS/ERK signaling (24). This observation provided new insight into the importance of K-RAS mutation in the context of PI3K/AKT-mediated radioresistance in EGFR-activated tumors. Another study showed that cell lines possessing activating PI3K mutations or showing loss of PTEN expression were more resistant to cetuximab than were wild-type PI3K- or PTEN-expressing cell lines. Furthermore, PI3K mutant/PTEN null and RAS/B-RAF mutant cell lines are more highly resistant to cetuximab compared with those without dual mutations or PTEN loss, indicating that constitutive and simultaneous

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activation of the RAS and PI3K pathways confers maximal resistance to this agent (25).

PI3K activity generates specific inositol lipids that have been implicated in regulating cell proliferation, differentiation, survival, and angiogenesis (26, 27). A previous report showed that inhibition of PI3K signaling increases the radiosensitivity of EGFR-activated head and neck cancer cells (28). AKT is a serine/threonine kinase

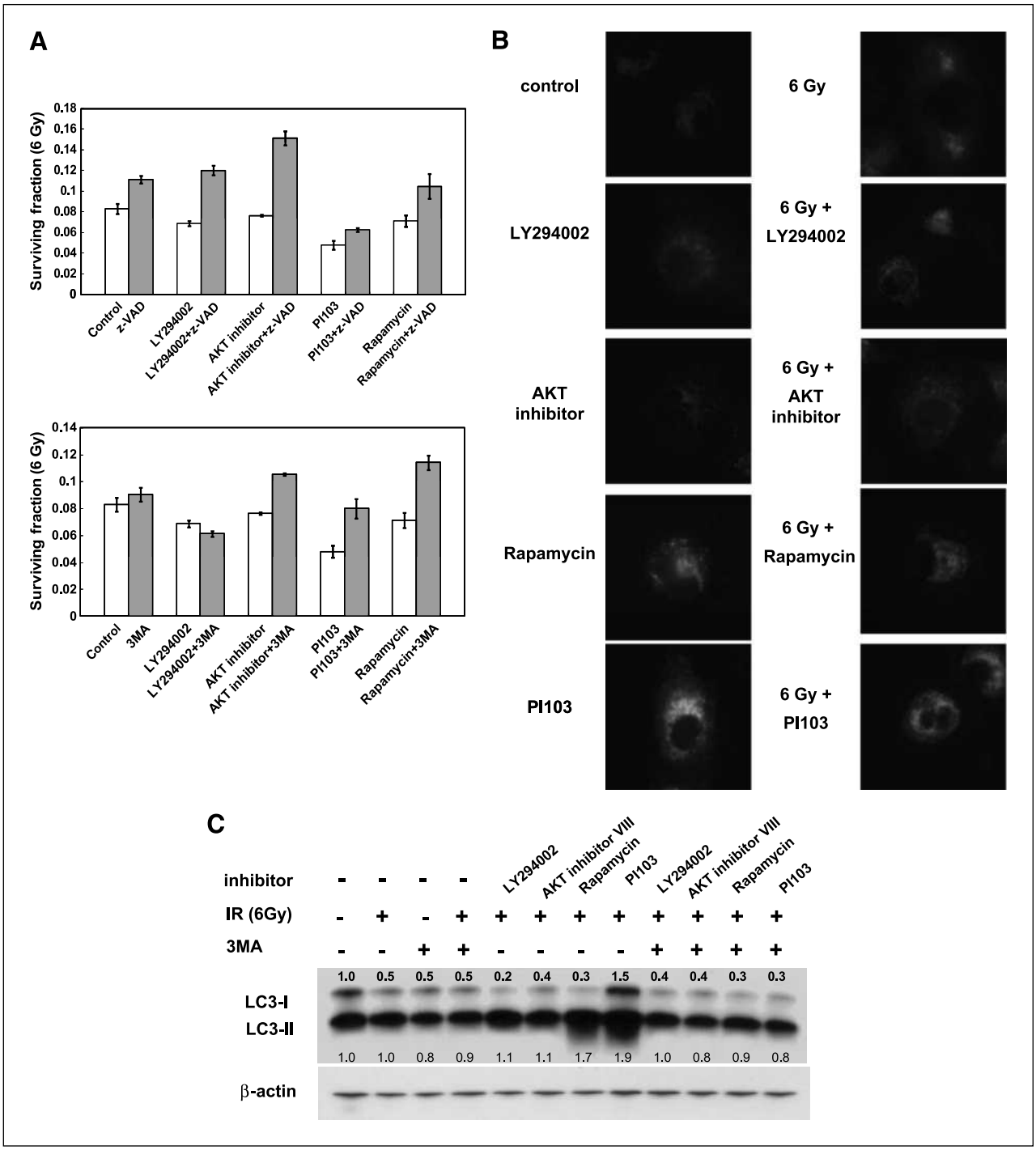

FIGURE 6. Mode of cell death. A, pretreatment with z-VAD (20 μmol/L), an inhibitor of caspase, rescued cells from the radiosensitizing effect of LY294002 or AKT inhibitor VIII, rapamycin, and PI-103 (top). Pretreatment with 3-methyladenine (250 μmol/L), a known inhibitor of autophagy, rescued cells from 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.

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that is activated in a PI3K-dependent manner by a variety of stimuli through growth factor receptors and plays an important role in cell survival. We previously reported that inhibition of Akt1using RNAi increases the radiosensitivity of EGFR- or RAS-activated cell lines (29). 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 (30). Bozulic et al. (31) recently showed that AKT activation following DNA damage requires PDK1 and DNA-PKcs, and results in increased survival of AKT1 *in vivo* following DNA damage. Pretreatment with PI3K or AKT inhibitor resulted in apoptosis as the predominant mode of cell death.

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 (32, 33). mTOR represents an attractive target because its inhibition could allow avoidance of possible side effects associated with inhibition of upstream PI3K/AKT signaling molecules with broader biological functions, including those involved in glucose signaling (34). 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 killing 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 levels of toxicity in preclinical studies (28). 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 (29). 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 (35). We observed the remarkable radiosensitizing effect of PI-103, which was associated with persistence of γH2AX foci formation and downregulation of DNA-PK.

In summary, this study revealed 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. Targeting of 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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