

Stimulated PI3K-AKT Signaling Mediated through Ligand or Radiation-Induced EGFR Depends Indirectly, but not Directly, on Constitutive K-Ras Activity

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Abstract

Previous results showed an inducible radiation sensitivity selectively observable for K-RAS–mutated cell lines as a function of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor blockade of phosphatidylinositol 3-kinase (PI3K)-AKT signaling. Therefore, the role of K-Ras activity for a direct (i.e., through activation of PI3K by K-Ras) or an indirect stimulation of PI3K-AKT signaling (through K-Ras activity–dependent EGFR ligand production) was investigated by means of small interfering RNA and inhibitor approaches as well as ELISA measurements of EGFR ligand production. K-RAS_{mt} tumor cells presented a constitutively activated extracellular signal–regulated kinase-1/2 signaling, resulting in enhanced production and secretion of the EGFR ligand amphiregulin (AREG). Medium supernatants conditioned by K-RAS_{mt} tumor cells equally efficiently stimulated EGFR signaling into the PI3K-AKT and mitogen-activated protein kinase pathways. Knocking down K-Ras expression by specific small interfering RNA markedly affected autocrine production of AREG, but not PI3K-AKT signaling, after treatment of K-RAS–mutated or wild-type cells with EGFR ligands or exposure to ionizing radiation. These results indicate that PI3K-mediated activation of AKT in K-RAS_{mt} human tumor cells as a function of EGFR ligand or radiation stimulus is independent of a direct function of K-Ras enzyme activity but depends on a K-Ras–mediated enhanced production of EGFR ligands (i.e., most

likely AREG) through up-regulated extracellular signal–regulated kinase-1/2 signaling. The data provide new differential insight into the importance of K-RAS mutation in the context of PI3K-AKT–mediated radioresistance of EGFR-overexpressing or EGFR-mutated tumors. (*Mol Cancer Res* 2007;5(8):863–72)

Introduction

The *RAS* gene family consists of three functional genes, *H-RAS*, *K-RAS*, and *N-RAS*, which code for different proteins presenting GTPase activity (1). Ras activity is normally stimulated through the activity of membrane receptor tyrosine kinases inducing different downstream cascades primarily involved in cell signaling that controls proliferation [e.g., Raf/mitogen-activated protein kinase, phosphatidylinositol 3-kinase (PI3K), Ras GTPase-activating protein, Ral guanine nucleotide dissociation stimulator, and protein kinase C ζ ; ref. 2]. Point mutations in any of the three *RAS* genes are found in ~30% of human tumors (3) and lead to a constitutive active GTP-bound state of the Ras protein, which correlates with tumor resistance to chemotherapy and/or radiotherapy (4-9). Among different *RAS* isoforms, *K-RAS* mutations in codon 12 or codon 13 have the highest incidence and occur mainly in adenocarcinomas of pancreas (90%), colon (50%), and lung (30%). For these tumors, the importance of *K-RAS* mutation and activated status of downstream signaling cascades as prognostic factor for overall survival of patients has been described (10-13).

The Ras pathway is also an important cascade in epidermal growth factor receptor (EGFR) signaling. Ras activity is effectively stimulated by EGFR-activated adaptor proteins such as growth factor receptor binding protein 2 and SHC, which transduce the signal to Ras through the guanine nucleotide exchange factors such as Sos. Ras itself then stimulates the mitogen-activated protein kinase pathway (2). Moreover, Ras signaling through mitogen-activated protein kinase is an important regulator of the production of EGFR ligands, such as transforming growth factor α (TGF α) and amphiregulin (AREG), and contributes through this mechanism to the autocrine activation of EGFR and its downstream signaling cascades (14). For *H-RAS*–mutated tumor cells, an elevated production of the EGFR ligands TGF α and AREG has been reported (15, 16). Because EGFR signaling is a major mechanism in the control of cell proliferation and survival, a Ras-dependent autocrine loop of EGFR activation may

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contribute to cell growth, as described for tumor cells overexpressing mutated H-RAS and K-RAS (17). This mechanism may also play a role in radioresistance of RAS-mutated tumors. Moreover, experimental and clinical evidence exists that overexpression or mutation of EGFR mediates tumor resistance to both chemotherapy and especially radiotherapy (18-23), and EGFR overexpression has been correlated with accelerated repopulation of tumors undergoing radiotherapy (24).

Recent data from various groups indicate that resistance of EGFR-overexpressing tumors is a consequence of EGFR signaling into the PI3K-AKT cascade, which is known to stimulate cell survival through down-regulation of cell death pathways (25, 26). In this context, the proapoptotic Bad and caspase-9 proteins are inactivated through AKT-mediated phosphorylation (27).

The serine/threonine protein kinase AKT is found in three isoforms (i.e., AKT1/protein kinase B α , AKT2/protein kinase B β , and AKT3/protein kinase B γ). AKT kinase is efficiently induced by growth factors like EGFR ligands or by ionizing radiation through the EGFR-mediated activation of PI3K (28-31). PI3K itself is a heterodimeric complex composed of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit. Tyrosine phosphorylation of EGFR or several other growth factor receptors creates docking sites for binding of p85 through its SH2 domains on the receptor, and according to this binding, the p110 subunit is then located proximal to its membrane-bound phospholipid substrate (32). Furthermore, previous reports indicated a direct interaction of Ras protein (i.e., H-Ras) and PI3K for activation of the PI3K-AKT cascade through binding of Ras to the p85 regulatory subunit of PI3K (33, 34). Likewise, it is discussed that Ras activity is responsible for stimulation of PI3K-AKT signaling in response to ionizing radiation (35). It seems to be clear that activation of AKT via PI3K occurs through phosphorylation by phosphoinositide-dependent kinase-1 and/or phosphoinositide-dependent kinase-2 at serine and threonine residues (Ser^{472/473}, Thr³⁰⁸). Several authors reported that especially phosphorylation of AKT at Ser⁴⁷³ is associated with resistance to chemotherapy/radiotherapy (36-40), and it has been proposed that activated AKT promotes survival of cells exposed to ionizing radiation through inhibition of apoptosis (41, 42) or enhancement of DNA double-strand break repair (31).

Based on pharmacologic inhibitors as well as genetic approaches, it has been shown that targeting of Ras protein and PI3K/AKT pathway enhances radiation sensitivity of tumor cells *in vitro* and *in vivo* (7, 43, 44). In this context, Kim et al. (45) reported that Ras signaling to PI3K-AKT is an important contributor to cell survival independent of whether it results from mutation of RAS or overexpression of EGFR. Furthermore, recently, we have reported that targeting of EGFR-PI3K signaling by specific small-molecule inhibitors (i.e., BIBX1382BS and LY294002) impairs repair of radiation-induced DNA double-strand breaks (mainly through the nonhomologous end-joining mechanism), resulting in enhanced radiosensitivity selectively of those human tumor cells that present mutated *K-RAS* gene (30, 31). A similar radiosensitization was observed when K-RAS_{mt} tumor cells were transfected with specific K-RAS small interfering RNA (siRNA; ref. 46). Moreover, with the use of real-time reverse transcription-PCR,

we were able to show that K-RAS_{mt} tumor cells produce approximately twice more EGFR ligands (i.e., AREG) than K-RAS_{wt} cells (46). In that study, we also showed that conditioned medium from K-RAS_{mt} tumor cells activates EGFR-dependent AKT signaling in K-RAS_{wt} cells. As a consequence of AKT-dependent prosurvival signaling, conditioned medium from K-RAS_{mt} tumor cells significantly improves clonogenic survival of K-RAS_{wt} cells. Thus, activation of AKT signaling via an autocrine, K-RAS_{mt}-dependent elevated production of EGFR ligands and subsequent induction of EGFR signaling mediates radioresistance in a so-called K-Ras indirect manner (46).

Thus far, it is not clear whether K-Ras activity functions directly upstream of PI3K-AKT or indirectly via the autocrine EGFR ligand loop as activator of AKT through EGFR signaling. Therefore, we investigated the role of mutated as well as wild-type K-Ras for the activation process of AKT. Evidence will be provided indicating an autocrine loop of EGFR stimulation in K-RAS_{mt} tumor cells, which activates PI3K-AKT signaling independent of a direct upstream function of K-Ras activity.

Results

EGFR-Dependent Phosphorylation of AKT at Ser^{472/473} Is Dramatically Enhanced in K-RAS_{mt} Human Tumor Cells

It is known from previous report (46) that K-RAS-mutated tumor cells produce elevated amounts of EGFR ligands (i.e., TGF α and AREG), which then induce EGFR activation and further downstream signaling through PI3K-AKT as well as Ras-mitogen-activated protein kinase cascade. As shown in Fig. 1A, conditioned medium from K-RAS_{mt} tumor cells (A549) induced activation of EGFR as tested by the appearance of p-EGFR within 3 to 5 min of administration. Treatment of K-RAS_{wt} cells by BIBX1382BS for 30 min before conditioned medium administration blocked the induced phosphorylation of EGFR nearly completely (Fig. 1A). Figure 1B shows that media conditioned by two different K-RAS_{mt} tumor cells (A549 and MDA-MB-231) and tested on K-RAS_{wt} FaDu cells contained substantial amounts of p-AKT and phospho-extracellular signal-regulated kinase (p-ERK)-1/2, inducing activity. To check whether this stimulation could be blocked by specific inhibitors of EGFR, PI3K, or mitogen-activated protein kinase/ERK kinase (MEK), K-RAS_{wt} FaDu cells were treated with these inhibitors 30 min before administration of K-RAS_{mt} conditioned medium. As expected, EGFR inhibitors PD153035 and BIBX1382BS as well as PI3K-inhibitor LY294002 did block phosphorylation of AKT, but not MEK-inhibitor PD98059. Figure 1C indicates the time kinetics of the effects of conditioned media from either K-RAS_{mt} or K-RAS_{wt} cells on stimulated phosphorylation of AKT in corresponding K-RAS_{wt} or K-RAS_{mt} cell lines. p-AKT is stimulated as early as 5 min after administration of K-RAS_{mt} conditioned medium in K-RAS_{wt} cells, whereas K-RAS_{wt} conditioned medium results within 15 min in a marked reduction of the high basal activity of p-AKT in K-RAS_{mt} cells.

To explore the role of mutated K-Ras protein for the production of EGFR ligands, experiments were designed to knock down K-Ras activity by siRNA or to block the downstream effector of Ras, MEK, by the specific inhibitor PD988059. As shown in Fig. 2A, transfection of K-RAS siRNA

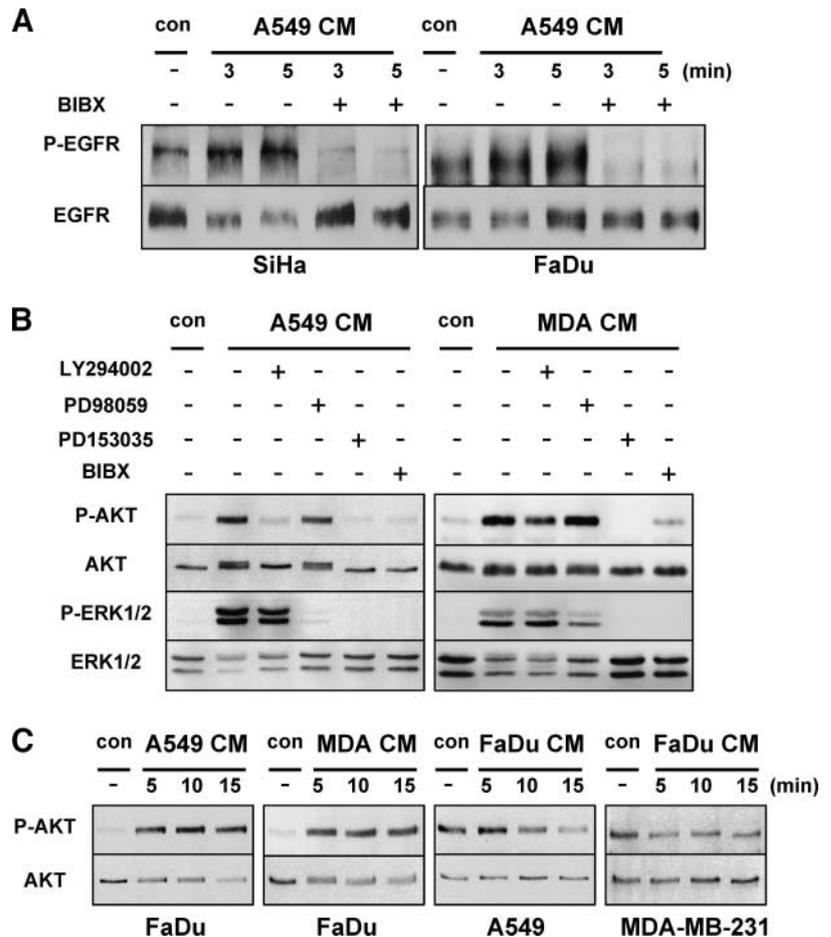


FIGURE 1. EGFR mediated phosphorylation of AKT at Ser^{472/473} in K-RAS_{wt} cells treated with conditioned medium from K-RAS_{mt} human tumor cells. **A.** K-RAS_{wt} cells (cell lines FaDu and SiHa) were serum starved for 48 h with or without pretreatment (30 min) with EGFR tyrosine kinase inhibitor BIBX1382BS (*BIBX*; 5 μ mol/L) and treated with RAS_{mt} A549 conditioned medium (CM) for 5 min. Detection of p-EGFR and total EGFR was done as described in Materials and Methods. **B.** FaDu cells were stimulated with conditioned medium from K-RAS_{mt} A549 or MDA-MB-231 for 15 min with or without pretreatment (30 min) with EGFR tyrosine kinase inhibitor BIBX1382BS (5 μ mol/L) or PD153035 (500 nmol/L), PI3K inhibitor LY294002 (10 μ mol/L), and MEK inhibitor PD98059 (20 μ mol/L). p-AKT and AKT were detected as described in Materials and Methods. **C.** FaDu cells were treated with conditioned media from K-RAS_{mt} A549 and MDA-MB-231 cells for 5, 10, or 15 min. Likewise, A549 and MDA-MB-231 cells were treated with conditioned medium from K-RAS_{wt} FaDu cells. Following detection of p-AKT with p-Ser^{472/473} antibody, blots were stripped and reprobed with antibody directed against total AKT. In each experiment, control condition (*con*) was treated with conditioned medium from autologous cells.

strongly down-regulated the expression of K-Ras protein in K-RAS_{mt} cell lines A549 and MDA-MB-231. As a consequence, basal phosphorylation of ERK1/2 was efficiently inhibited between 50% and 80% for both cell lines as determined by densitometry. Moreover, down-regulation of K-Ras also resulted in a marked reduction of basal p-AKT by \sim 40%. Treatment of these two cell lines with MEK inhibitor PD98059 also abolished ERK1/2 phosphorylation (Fig. 2B). Most interestingly, conditioned medium from the same A549 and MDA-MB-231 cells treated with the MEK inhibitor for the entire 48-h conditioning time was effectively impaired in its activity to induce AKT phosphorylation in K-RAS_{wt} cells. These results indicate the importance of K-Ras activity for the production of EGFR ligands mediating AKT phosphorylation. This was further shown by direct measurements of EGFR ligand production (e.g., AREG) in K-RAS_{mt} A549 and MDA-MB-231 cells transfected with K-RAS siRNA or treated with MEK inhibitor PD98059 with the use of ELISA technology. As shown in Fig. 2D, production of AREG by the two K-RAS_{mt} cell lines was inhibited by about 40% to 50% under both antagonistic approaches.

EGFR Ligands Differentially Enhance K-Ras-GTP in K-RAS_{wt} and K-RAS_{mt} Human Tumor Cells

EGFR ligands EGF, TGF α , and AREG stimulate K-Ras-GTP differentially in K-RAS_{wt} and K-RAS_{mt} cells. As shown

in Fig. 3, in K-RAS_{mt} A549 cells, which present already under basal conditions a pronounced amount of K-Ras-GTP, K-Ras-GTP can be stimulated about 1.2- to 1.4-fold when treated with EGFR ligands. In contrast, in K-RAS_{wt} FaDu cells, which do not present detectable or only faint amounts of K-Ras-GTP under control condition, a $>$ 10-fold, ligand-specific induction is apparent (EGF, 11-fold; TGF α , 14-fold; AREG, 19-fold).

Suppression of K-Ras Does Not Affect AKT Phosphorylation at Ser^{472/473} Induced by EGFR Ligands

Recently published results (46) and the data presented herein thus far indicate that the high basal level of p-AKT in unstimulated K-RAS_{mt} human tumor cells is due to the high activity of mutated K-Ras protein and its constitutive up-regulated stimulation of EGFR signaling through elevated EGFR ligand production. Yet, in addition to this so-called indirect effect, and based on literature (33, 34), K-Ras activity may also directly be responsible for stimulating AKT through a direct Ras-GTP-dependent activation process via PI3K. To test this, K-RAS_{mt} and K-RAS_{wt} tumor cells were transfected with K-RAS siRNA to knock down K-Ras activity. As shown in Fig. 4, in both K-RAS_{mt} (A549) and K-RAS_{wt} (FaDu and H661) cells, K-RAS siRNA effectively knocked down expression of K-Ras protein. Yet, phosphorylation of AKT

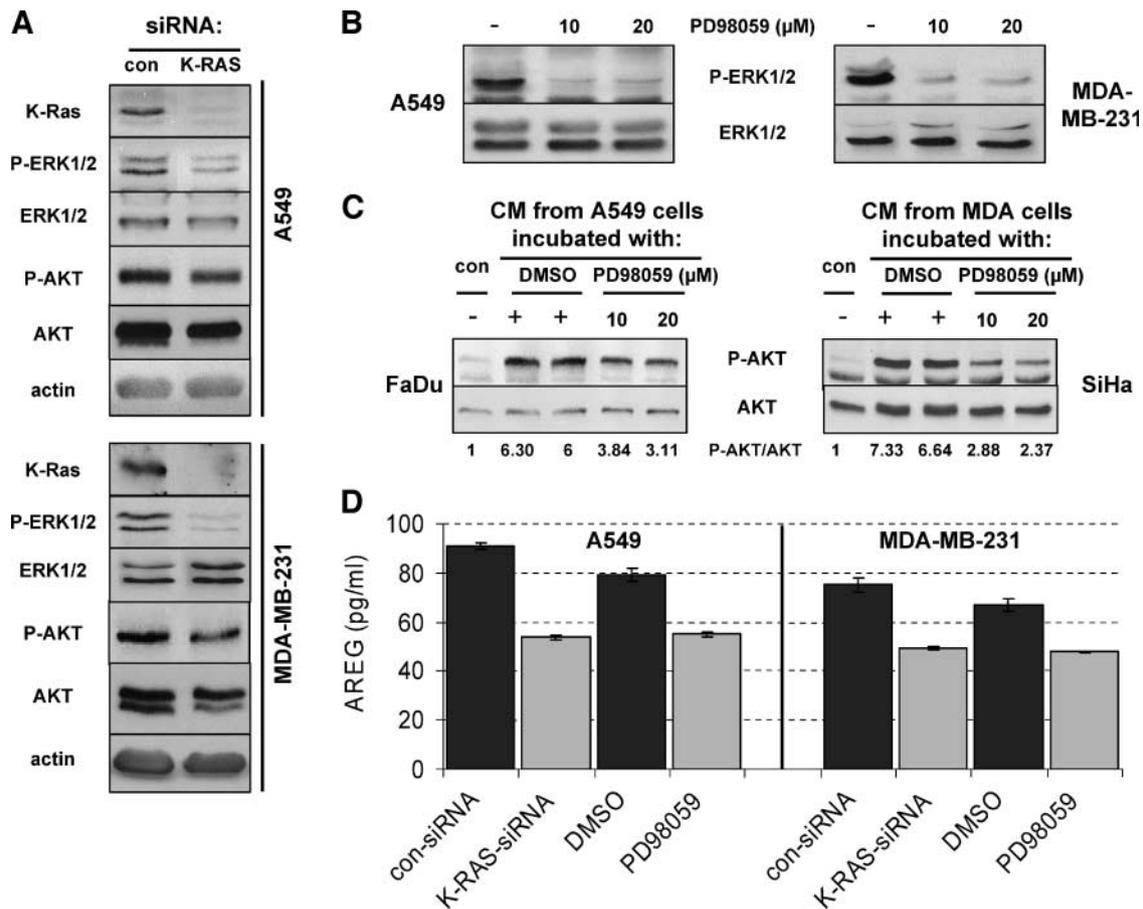


FIGURE 2. Autocrine stimulation of AKT phosphorylation in K-RAS_{mt} human tumor cells is mediated through p-ERK1/2. **A.** A549 and MDA-MB-231 cells were transfected with negative control siRNA or specific K-RAS siRNA. Down-regulation of K-Ras, p-ERK1/2, and p-AKT was shown by Western blot analysis 48 h after starvation and 4 d after transfection. To control protein loading, actin and total ERK1/2 as well as total AKT were detected. Differences in band intensities of p-ERK1/2 and p-AKT were determined by densitometry and calculation of relative intensity values based on total ERK1/2 or AKT protein. **B.** Serum-starved A549 and MDA-MB-231 cells were incubated for 48 h with vehicle or indicated concentrations of MEK inhibitor PD98059. To detect p-ERK1/2, cells were lysed and Western blot analysis was done. Blots were stripped and reprobed with pan-ERK1/2 antibody. **C.** Conditioned media from A549 and MDA-MB-231 cells used in the experiment shown in (B) were administered to K-RAS_{wt} FaDu and SiHa, respectively. By detecting p-AKT, the function of ERK1/2 activity as an effector of K-Ras in modulating secretion of EGFR ligands was analyzed. The same blots were stripped and probed with pan-AKT antibody. Control FaDu and SiHa cells were treated with autologous conditioned medium (con). In additional control experiments (data not shown), the effect of added DMSO or PD inhibitor to nonconditioned media was tested. With respect to p-AKT, no difference was apparent in comparison with autologous conditioned medium. Densitometry values shown represent the ratio of p-AKT/AKT (normalized to 1 in control). **D.** Production of AREG as measured in the supernatants of A549 and MDA-MB-231 cells is blocked by MEK inhibitor PD98059 and K-RAS siRNA. Cells (1.5×10^5) were seeded in six-well plates and, after 48 h, either medium was changed with serum-free medium containing 20 $\mu\text{mol/L}$ PD98059 or an equal volume of DMSO as vehicle or cells were transfected with control and K-RAS siRNAs. As explained in Materials and Methods, cell media were collected after and analyzed for the amount of AREG by ELISA (R&D Systems). To normalize AREG concentration to cell population, cells were trypsinized and counted. Columns, mean concentration (pg/mL) of AREG from three experiments; bars, SD.

as a consequence of treatment with EGFR ligands (EGF, TGF α , AREG) is not affected by K-RAS siRNA. However, in cells transfected with control siRNA, p-AKT is stimulated in K-RAS_{mt} as well as in K-RAS_{wt} cells. This indicates that EGFR ligand stimulation of PI3K-AKT signaling is independent of K-Ras protein directly activating PI3K.

Ionizing Radiation Enhances AKT Phosphorylation in K-RAS_{mt} and K-RAS_{wt} Cells Independent of K-Ras Activity

Activation of PI3K-AKT signaling is not only observed after EGFR stimulation by specific ligands but has also been reported to be induced in a ligand-independent manner by ionizing radiation (30, 45). Because this radiation-mediated

activation of AKT signaling is an important mechanism in resistance of EGFR-overexpressing tumor cells to irradiation, we analyzed, at clinical relevant doses of 2 Gy, the role of K-Ras protein in mediating AKT phosphorylation. As shown in Fig. 5A, K-RAS_{mt} A549 cells presented, under control condition, up-regulated K-Ras-GTP. Yet, this protein-GTP complex was only slightly (1.7-fold) enhanced after radiation exposure. K-RAS_{wt} FaDu cells, however, for which no or only faint amounts of K-Ras-GTP are detectable, showed a strong (10-fold) induction of K-Ras activity by radiation. Likewise, K-Ras-GTP expression after AREG treatment was much stronger in FaDu cells. Figure 5B indicates that phosphorylation of AKT in both K-RAS_{mt} and K-RAS_{wt} cell lines is equally inducible by ionizing radiation in control

siRNA-transfected cells. Transfection with K-RAS siRNA, which completely down-regulates K-Ras expression in both cell lines, did not affect radiation-induced AKT phosphorylation at Ser^{472/473} (Fig. 6). Again, these results suggest no direct involvement of K-Ras activity in PI3K-AKT stimulation.

Discussion

In the present study, we provide evidence that EGFR-mediated signaling into the PI3K-AKT pathway does not directly involve K-Ras-GTP but is indirectly dependent on K-Ras activity via its importance for the production of EGFR ligands, especially in human tumor cells presenting a K-RAS

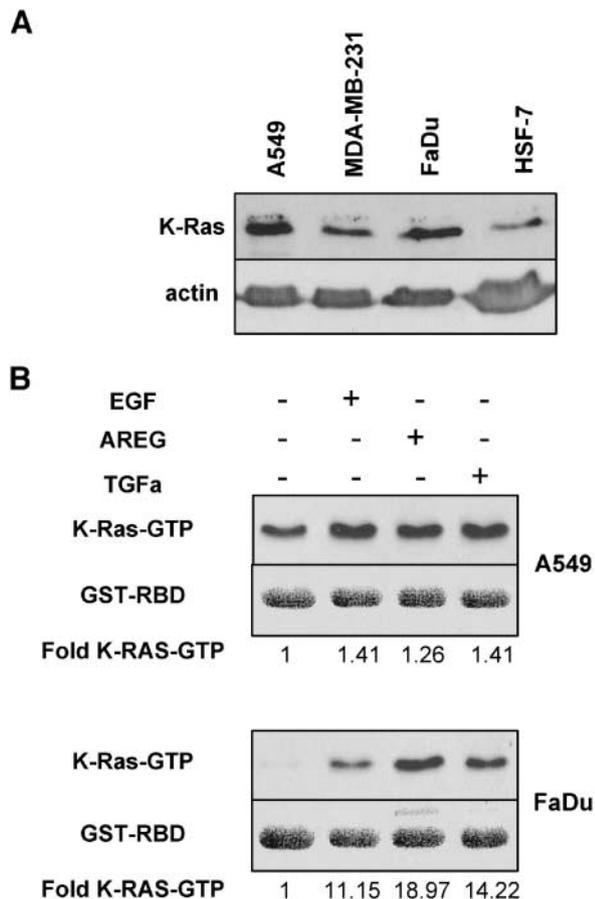


FIGURE 3. EGFR ligands enhance K-Ras activity differentially in K-RAS_{wt} and K-RAS_{mt} cells. **A.** Western blot analysis of K-Ras protein level in K-RAS_{wt} and K-RAS_{mt} cells. Cell lines of different origin were lysed and equal amounts of total protein (150 μ g) were applied to Western blot analysis with specific antibody to probe for K-Ras and actin (for protein loading). **B.** Activation of K-Ras by EGFR ligands, EGF, TGF α , and AREG in K-RAS_{mt} and K-RAS_{wt} cells was assessed 5 min after treatment with vehicle in control or after stimulation with ligands. Therefore, glutathione *S*-transferase (GST)-conjugated Raf-1 Ras binding domain (RBD) was used to precipitate activated GTP-bound K-Ras. Bound proteins were solubilized by addition of loading buffer and run on SDS-PAGE. The amount of K-Ras in the bound fraction was analyzed with specific antibody. The fusion protein (42 kDa) was detected by Coomassie staining of the polyacrylamide gel. Ligand-mediated stimulation was calculated based on densitometry (control set as 1.0).

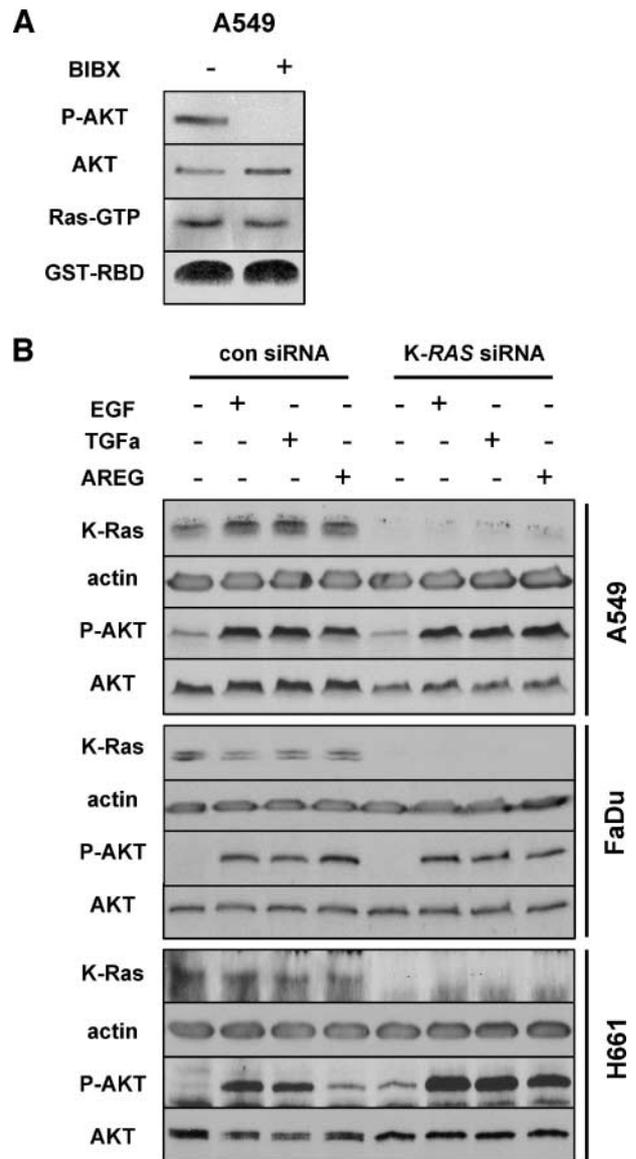


FIGURE 4. Autocrine and exogenous EGFR ligands enhance AKT phosphorylation at Ser^{472/473} in a K-Ras-independent manner. **A.** Basal level of activated Ras was determined in 48-h serum-starved A549 cells treated with EGFR tyrosine kinase inhibitor BIBX1382BS (5 μ mol/L) or vehicle as explained in the legend to Fig. 3 and in Materials and Methods. From same samples used for Ras-GTP assay, 100- μ g protein was subjected to SDS-PAGE and the level of p-AKT (Ser^{472/473}) was analyzed with specific antibody. Membrane was stripped and reincubated with total AKT antibody. **B.** Tumor cells, either K-RAS mutated (A549) or wild-type (FaDu and H661), were transfected with 50 nmol/L of control or specific K-RAS siRNA. Forty-eight hours after transfection, medium was changed with serum-free medium. After additional 48 h of starvation, cells were treated with vehicle or EGFR ligands as described in Materials and Methods. After SDS-PAGE, levels of K-Ras and p-AKT were determined. Actin and total AKT were detected as loading control.

mutation. Because it is known that both EGFR and Ras are strong modulators of radioresistance (4, 5, 19, 20), these results are of special importance for the design of targeting strategies to interfere with EGFR signaling in the context of resistance of EGFR-overexpressing or EGFR-mutated human solid tumors to radiotherapy. EGFR-dependent PI3K-AKT

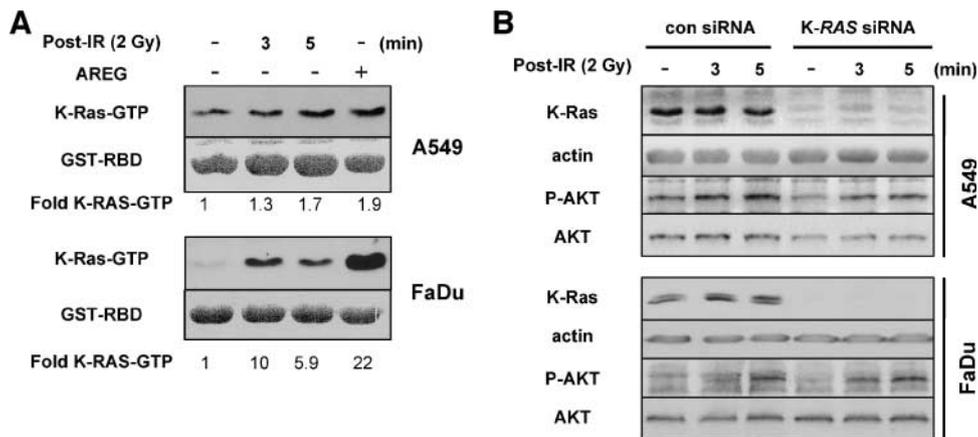


FIGURE 5. K-Ras–independent Ser^{472/473} phosphorylation of AKT by ionizing radiation. **A.** Ionizing radiation (IR)–induced K-Ras activity in 48-h serum-starved K-RAS_{mt} A549 and K-RAS_{wt} FaDu cells was analyzed 3 and 5 min after irradiation by precipitating activated GTP-bound K-Ras with glutathione *S*-transferase–conjugated Raf-1 Ras binding domain as described in Materials and Methods. As positive control, K-Ras activity of both cell lines 5 min after stimulation with AREG (100 ng/mL) is shown (+). **B.** Function of K-Ras in ionizing radiation–induced AKT phosphorylation was analyzed 4 d after transfection of cells with 50 nmol/L of control or K-RAS siRNA. Transfected and serum-starved A549 and FaDu cells were mock irradiated or irradiated with a single dose of 2 Gy. At indicated time points, cells were lysed and levels of total K-Ras and p-AKT were analyzed by Western blotting. Actin and total AKT were detected as loading control.

signaling is an important cascade to promote cell survival in response to exogenous stress factors such as exposure to ionizing radiation (38, 40, 47). It has been shown for both *in vitro* and *in vivo* systems that radiation sensitivity of various tumor xenografts or cell lines overexpressing EGFR can efficiently be enhanced by blocking EGFR with the monoclonal anti-EGFR antibody C225 (48–51). Moreover, Bonner et al. (52) and Huang and Harari (53) have shown that combining radiation with C225 results in a greater cell killing of squamous cell carcinoma cell lines and xenografts than either treatment alone. Finally, just recently, Bonner et al. (54) showed in a clinical study the clear advantage of combined C225 and radiotherapy over radiotherapy alone for head and neck cancer patients. Similar findings on enhanced radiation sensitivity have been reported for tyrosine kinase inhibitors that target the kinase activity of EGFR (55). Nevertheless, especially for tyrosine kinase inhibitors, a big heterogeneity for enhanced radiation sensitivity seems to be

obvious and even dependent on concomitant mutations as reported by us recently (30). In these studies, we showed that a highly selective experimental EGFR tyrosine kinase inhibitor induces radiation sensitivity only in K-RAS–mutated human tumor cell lines but not in K-RAS wild-type cell lines (30). This effect could be attributed to the selective blockage of EGFR–PI3K–AKT signaling and the inhibition of DNA double-strand break repair in K-RAS–mutated cell lines (31).

The detailed mechanism by which EGFR leads to altered cellular radiosensitivity is only partially understood thus far. EGFR initiates cytoplasmic signaling through autophosphorylation of the intracellular tyrosine kinase domain. As a consequence, a number of effectors like PI3K and Ras signaling are activated (2). It has been proposed by Gupta et al. (6) that Ras signaling is the important step in EGFR-mediated radiation survival. Yet, because it has also been shown that Ras activity leads to an EGFR autoocrine loop through Ras-stimulated expression of TGF α (16), this EGFR-dependent

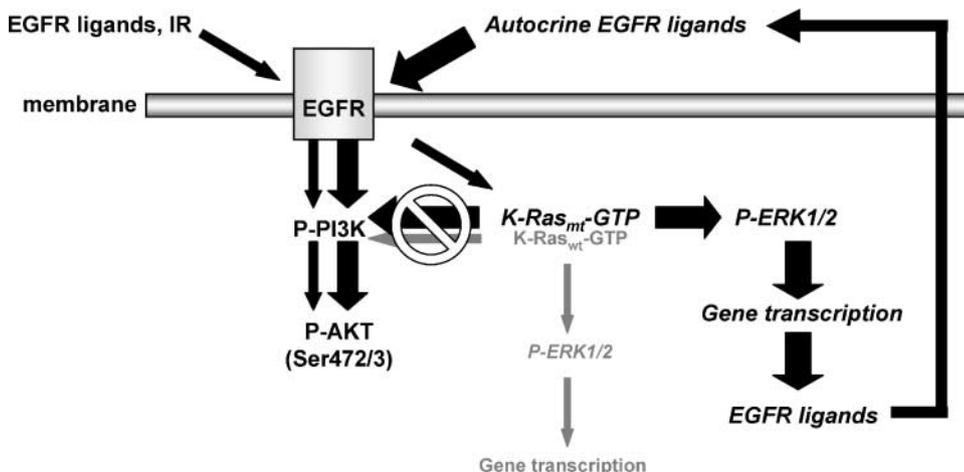


FIGURE 6. Schematic representation of pathways of AKT phosphorylation at Ser472/473 induced by K-RAS mutation or by external stimulation with EGFR ligands and ionizing radiation in K-RAS_{wt} and K-RAS_{mt} human tumor cells.

activation of PI3K-AKT survival signaling may thus be not directly but indirectly dependent on Ras activity. Especially this aspect is supported by the data presented herein. We clearly showed in the present study and earlier (46) that K-RAS-mutated tumor cells present an up-regulated production of EGFR ligands, especially AREG, which is able to efficiently stimulate EGFR-PI3K-AKT signaling. Moreover, medium conditioned by K-RAS-mutated tumor cells mediates strong induction of EGFR survival signaling and, as shown previously, resistance to ionizing radiation of K-RAS wild-type cells (46). The importance of K-Ras activity for autocrine stimulation of EGFR-PI3K-AKT signaling is documented by the results obtained from the experiments applying conditioned medium from K-RAS-mutated cells in which K-Ras activity has been knocked down by specific siRNA. Conditioned medium from these cells was not able to induce EGFR-PI3K-AKT signaling in K-RAS wild-type cells. These results clearly show the role of K-Ras activity for production of EGFR ligands and the autocrine stimulatory function of the Ras pathway for EGFR signaling as has been suggested by Grana et al. (16).

EGFR signaling into the PI3K-AKT pathway has been shown by many authors to be an important step in cell survival following exogenous cellular stress, such as exposure to ionizing radiation and chemotherapy (56-58). One important mechanism of AKT signaling to promote cell survival is the blockage of apoptosis through inactivation of the Bad protein, an important component of the mitochondrial apoptotic pathway (27). AKT-dependent phosphorylation of Bad inhibits its binding to Bcl-x1 or Bcl-2, which are antiapoptosis factors. Blockage of this process by anti-AKT or anti-EGFR strategies has been shown to increase radiation-induced apoptosis in some tumor cell systems (42, 59). Another important mechanism linked to cell survival following genotoxic stress is the repair of DNA damage. Recently, we showed that blockage of EGFR signaling and, more specifically, inhibition of the PI3K-AKT cascade lead to impaired stimulation of the catalytic subunit of DNA-protein kinase, a major enzyme in DNA double-strand break repair. As a consequence, DNA double-strand break repair is significantly reduced, resulting in enhanced radiation sensitivity of K-RAS-mutated human tumor cells (31).

For activation of PI3K-AKT signaling, previous reports suggested a direct interaction of Ras protein through binding to PI3K (33, 34), and it is discussed that Ras activity is responsible for stimulation of PI3K-AKT signaling in response to ionizing radiation (45). Evidence has been provided that physiologic activation of different Ras family members through various stimuli as well as constitutive activation of Ras via point mutations leads to activation of distinct signal transduction pathways (i.e., PI3K/AKT, c-jun NH₂-terminal kinase, p38, and focal adhesion kinase pathways; ref. 60). Especially, activated H-Ras and N-Ras seem to be markedly effective in activating the PI3K and AKT cascade (35, 58, 61). Conflicting data exist with respect to the role of K-Ras in activating PI3K-AKT signaling. Some reports indicate that K-Ras activity does directly modulate AKT phosphorylation through stimulation of PI3K (60, 62), whereas others have not found a direct stimulation of AKT activity by mutated and constitutively active K-Ras protein (35, 58, 63).

In the present study, we showed that K-Ras activity, which was stimulated strongly in K-RAS wild-type cells but only moderately in K-RAS-mutated cells by EGFR ligands or ionizing radiation, is not directly involved in PI3K-AKT stimulation. This was proved by a pronounced phosphorylation of AKT induced by EGFR ligands or ionizing radiation in spite of complete suppression of K-Ras protein by K-Ras-specific siRNA. Therefore, it can be concluded that AKT activity induced by EGFR ligands or ionizing radiation is not induced directly via interaction of K-Ras and PI3K but rather through direct binding of EGFR to PI3K. This assumption is supported by data from Mattoon et al. (32) showing that activation of AKT in mouse fibroblasts stimulated by EGF results from complex formation of EGFR and the p85 regulatory subunit of PI3K. Likewise, preliminary results from ongoing experiments in our laboratory applying K-RAS-mutated A549 cells also indicate a complex formation of EGFR and p85 on ligand stimulation. Finally, the observable reduction of basal phosphorylation of AKT in K-RAS siRNA-transfected A549 and MDA-MB-231 cells also supports the conclusion that the major proportion of PI3K-AKT signaling in these cells is activated through the K-Ras-dependent autocrine ligand loop and is independent of a direct K-Ras interaction with PI3K. Yet, as the amount of basal p-AKT reduction does not follow the efficient down-regulation of K-Ras protein by siRNA, a function of H-Ras and/or N-Ras in stimulating AKT phosphorylation directly through interaction with PI3K, as it was proposed earlier (33, 34, 45), cannot be ruled out at present. Furthermore, recently, in line with our data, it was shown that K-RAS mutation in human pancreatic and colorectal cancer cells activates EGFR and H-Ras via an autocrine production of EGFR ligands, which in turn enhances postirradiation survival (64). From the data published by Cengel et al. (64) and our data presented herein, it can be postulated that a compensatory effect may exist, which, via EGFR-dependent H-Ras activity, can enhance basal level of p-AKT independent of a direct interaction with K-Ras protein.

Taken together, this study argues against a model of direct interaction of K-Ras with the catalytic subunit of PI3K in activating AKT/protein kinase B, but supports the view of an indirect involvement of K-Ras activity in EGFR-PI3K-AKT signaling through Ras-dependent enhanced production of EGFR ligands such as AREG and TGF α . This autocrine EGFR signaling loop seems to be of special importance for induction of EGFR-mediated survival strategies following radiation exposure in the K-RAS-mutated solid human tumor cells tested in this study. Thus, the data presented are of particular interest in understanding the changes in molecular signaling pathways that occur via K-RAS mutation and may have clinical significance for molecular targeting strategies, especially in combination with radiotherapy.

Materials and Methods

Materials

Antibodies against pan-EGFR, phospho-tyrosine (PY20), phospho-AKT (Ser^{472/473}), and AKT were purchased from BD Biosciences. Pan-Ras monoclonal antibody (Ab-2) was received from Oncogene. Monoclonal K-Ras antibody, EGF,

TGF α , and AREG were obtained from Sigma-Aldrich. PI3K inhibitor LY294002, MEK inhibitor PD98059, and EGFR tyrosine kinase inhibitor PD153035 were purchased from Calbiochem (Merck). The specific EGFR tyrosine kinase inhibitor BIBX1382BS was provided by Boehringer Ingelheim Austria GmbH. Glutathione *S*-transferase-conjugated Raf-1 Ras binding domain was received from Upstate (Biozol). K-RAS and control negative siRNAs were prepared by Dharmacon. Lipofectamine 2000 and Opti-MEM medium were purchased from Invitrogen. Human AREG-ELISA kit was a product of R&D Systems.

Cell Culture

Established human tumor cell lines presenting K-RAS mutation [i.e., A549 (bronchial carcinoma; ATCC no. CCL-185) and MDA-MB-231 (breast adenocarcinoma; ATCC no. HTB-26)], K-RAS wild-type [i.e., H661 (bronchial carcinoma; ATCC no. CCL-183), FaDu (pharyngeal squamous cell carcinoma; ATCC no. HTB-43), and SiHa (cervix squamous cell carcinoma; ATCC no. HTB-35)], as well as normal human skin fibroblast (HSF-7) were used. Cells were cultured in DMEM (A549, MDA-MB-231, SiHa, and HSF-7), RPMI 1640 (H661), or MEM (FaDu), routinely supplemented with 10% FCS plus 1% penicillin-streptomycin, and incubated in a humidified atmosphere of 93% air/7% CO₂ at 37°C.

Preparation of Conditioned Medium

Exponentially growing cultures were washed twice with PBS (37°C) and incubated in serum-free medium for 48 h. Conditioned media were collected and centrifuged to remove nonadherent cells. Supernatants were fed to test cultures according to the experimental protocol. Conditioned media were designated according to the origin as K-RAS_{wt} conditioned medium or K-RAS_{mt} conditioned medium.

Inhibitor/Ligand Treatment and Irradiation

Stock solutions of the inhibitors were made at appropriate concentrations in DMSO and stored at -70°C. For treatment, inhibitor solutions were diluted to appropriate working concentrations (5 μ mol/L BIBX1382BS, 500 nmol/L PD153035, 10 μ mol/L LY294002, 20 μ mol/L PD98059) in serum-free medium. Control cultures received medium containing the solvent DMSO at appropriate concentration. Inhibitors were always supplemented to the culture media 30 min before stimulation with conditioned medium. For ligand stimulation, EGFR ligands (EGF, TGF α , and AREG; each 100 ng/mL) were added to the culture medium for 5 min. For nonstimulated controls, the same concentration of vehicle was added for 5 min. Irradiation of cells was done with the Gulmay RS225 X-ray machine (200 kVp, 15 mA, 0.5-mm Al as additional filtering) at 37°C using a dose rate of 3 Gy/min.

Western Blot Analysis and Immunoprecipitation

Total protein as well as the activated forms of EGFR, AKT, K-Ras, and ERK1/2 following stimulation with EGFR ligands, K-RAS_{wt}/K-RAS_{mt} conditioned medium, and irradiation with and without pretreatment with inhibitors or cells transfected with

control and K-RAS siRNAs were analyzed by Western blotting. Therefore, cells grown to 50% to 60% confluency were incubated in serum-free medium for 48 h. Subsequently, cells were treated with inhibitors and, at indicated time points after irradiation or ligand/conditioned medium stimulation, washed twice with ice-cold PBS and lysed with lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 50 mmol/L β -glycerophosphate, 150 mmol/L NaCl, 10% glycerol, 1% Tween 20, 1 mmol/L NaF, 1 mmol/L DTT, protease and phosphatase inhibitors]. Lysates were cleared of insoluble material and normalized for protein concentration. To analyze the activation pattern of ERK1/2 and AKT as well as total proteins, 100 to 150 μ g of protein for each sample were resolved by SDS-PAGE and blots were incubated with specific primary antibodies followed by incubation with secondary antibody conjugated to horseradish peroxidase.

To detect p-EGFR, 2 mg of protein were incubated at 4°C for 2 h using EGFR-specific antibody. Protein Sepharose beads were then added for another hour to recover the immunoprecipitates. Immunoprecipitates were washed thrice with lysis buffer, resolved by SDS-PAGE, and electrophoretically transferred to a polyvinylidene difluoride membrane. Membranes were incubated with the phospho-tyrosine-specific antibody and, after stripping, reblotted with total EGFR antibody.

Ras Activation Assay

The level of Ras-GTP was measured with a Ras activity assay kit. The assay was done following the manufacturer's protocol. Briefly, cells were stimulated with EGFR ligands or ionizing radiation and lysed with Mg²⁺-containing lysis buffer. One milligram of cell lysate was precleared with glutathione agarose. Ten micrograms of Raf-1 Ras binding domain-agarose were incubated with cell lysate at 4°C for 30 min. Agarose was collected by microcentrifugation and washed thrice with Mg²⁺-containing lysis buffer. Bound Ras proteins were solubilized in 20 μ L of protein sample loading buffer, resolved by electrophoresis, transferred to nitrocellulose, and detected with an anti-pan Ras or K-Ras antibody.

Transfection of Cells with siRNA

Interference or gene silencing for down-regulating the expression of K-RAS was done using pool siRNAs (SMART pools, Dharmacon) that contain four or more specific siRNA duplexes in a single pool. The optimal percentage of cell confluency, concentration of siRNAs, and time intervals between transfection and protein isolation were detected for each approach in pilot studies. For transfection procedure, 1.5×10^5 cells diluted in fresh medium were transferred to six-well plates 24 h before transfection. On the day of transfection, when cells were at 50% confluence, transfection of negative control and targeted siRNAs at a final concentration of 50 nmol/L was carried out using Lipofectamine 2000. Suppression of K-Ras protein was analyzed at day 4 after transfection by SDS-PAGE and immunoblotting.

ELISA

Quantitative determination of AREG was done by a specific ELISA according to the manufacturer's protocol. Briefly, cells were grown to ~60% confluence and incubated with

serum-free medium containing MEK inhibitor PD98059 or DMSO for 48 h. Conditioned media collected from each of three parallel cultures treated with inhibitor or vehicle were added in triplicates to AREG-precoated wells and incubated for 2 h at room temperature. Test samples and standards were then processed according to the manufacturer's protocol. Reactions were measured using an ELISA photometer plate reader at a wavelength of 450 nm with a wavelength correction of 570 nm. In the case of siRNA transfection, conditioned media were collected from two parallel experiments transfected with 50 nmol/L of either control siRNA or K-RAS siRNA and each sample was added in triplicates to the AREG-precoated wells.

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Molecular Cancer Research

Stimulated PI3K-AKT Signaling Mediated through Ligand or Radiation-Induced EGFR Depends Indirectly, but not Directly, on Constitutive K-Ras Activity

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