Blockage of Epidermal Growth Factor Receptor-Phosphatidylinositol 3-Kinase-AKT Signaling Increases Radiosensitivity of K-*RAS* Mutated Human Tumor Cells *In vitro* by Affecting DNA Repair

Mahmoud Toulany,[1] Ulla Kasten-Pisula,[2] Ingo Brammer,[2] Shaomeng Wang,[3] Jianyong Chen,[3] Klaus Dittmann,[1] Michael Baumann,[4] Ekkehard Dikomey,[2] and H. Peter Rodemann[1]

Abstract

Purpose: It is known that blockage of epidermal growth factor receptor (EGFR)/phosphatidylinositol 3-kinase (PI3K) activity enhances radiation sensitivity of human tumor cells presenting a K-*RAS* mutation. In the present study, we investigated whether impaired repair of DNA double-strand breaks (DSB) is responsible for the radiosensitizing effect of EGFR and PI3K inhibition in K-*RAS* mutated (K-RAS_{mt}) cells.

Experimental Design: The effect of the EGFR tyrosine kinase inhibitor BIBX1382BS (BIBX) on cellular radiosensitivity was determined in K-RAS_{mt} (A549) and K-RAS_{wt} (FaDu) cell lines by clonogenic survival assay. Radiation-induced phosphorylation of H2AX (Ser^{139}), ATM (Ser^{1981}), and DNA-dependent protein kinase catalytic subunit (DNA-PKcs; Thr^{2609}) was analyzed by immunoblotting. Twenty-four hours after irradiation, residual DSBs were quantified by identification of γH2AX foci and frequency of micronuclei.

Results: BIBX reduced clonogenic survival of K-RAS_{mt}-A549 cells, but not of K-RAS_{wt}-FaDu cells, after single-dose irradiation. Analysis of the radiation-induced H2AX phosphorylation revealed that BIBX, as well as the PI3K inhibitor LY294002, leads to a marked reduction of P-H2AX in K-RAS_{mt}-A549 and MDA-MB-231 cells, but not in K-RAS_{wt}-FaDu and HH4ded cells. Likewise, radiation-induced autophosphorylation of DNA-PKcs at Thr^{2609} was only blocked in A549 cells by these two inhibitors and *AKT1* small interfering RNA transfection. However, neither in K-RAS_{mt} nor in K-RAS_{wt} cells the inhibitors did affect radiation-induced ATM phosphorylation. As a consequence of inhibitor treatment, a significant enhancement of both residual DSBs and frequency of micronuclei was apparent only in A549 but not in FaDu cells following radiation.

Conclusion: Targeting of the EGFR-dependent PI3K-AKT pathway in K-*RAS*-mutated A549 cells significantly affects postradiation survival by affecting the activation of DNA-PKcs, resulting in a decreased DSB repair capacity.

The epidermal growth factor receptor (EGFR) is frequently involved in the pathogenesis of human epithelial tumors. Thus, numerous tumor entities are presenting overexpression of EGFR, which adversely affects prognosis and treatment outcome, due to EGFR-mediated therapy resistance. EGFR/erbB1 is a member of the erbB receptor family (erbB1-erbB4). Structurally, erbB receptors present an extracellular ligand binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain (1). Activation of erbB receptors by ligand binding results in downstream signaling, inducing various pathways regulating proliferation, apoptosis, angiogenesis, as well as metastasis (2). Importantly, in the case of EGFR, activation and signaling can also be mediated by ionizing radiation ligand-independently. Thus, exposure of tumor cells overexpressing EGFR to ionizing radiation does activate survival and proliferation mechanisms through signaling via the PI3K-AKT and the RAS-mitogen-activated protein kinase pathways (3). This response pattern can lead to radioresistance of

Authors' Affiliations: [1]Divison of Radiobiology and Molecular Environmental Research, Department of Radiation Oncology, University of Tuebingen, Tuebingen, Germany; [2]Department of Radiotherapy and Radiooncology, Laboratory of Experimental Radiation Oncology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [3]Division of Hematology and Oncology, Department of Internal Medicine, University of Michigan Comprehensive Cancer Center, Ann Arbor, Michigan; and [4]Department of Radiation Oncology, University of Dresden, Dresden, Germany

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Requests for reprints: H. Peter Rodemann, Division of Radiobiology and Molecular Environmental Research, Department of Radiation Oncology, Eberhard-Karls University Tuebingen, Roentgenweg 11, 72076 Tuebingen, Germany. Phone: 49-0-7071-29-85962; Fax: 49-0-7071-29-5900; E-mail: hans-peter.rodemann@uni-tuebingen.de.

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EGFR-overexpressing tumor cells, and accelerated repopulation of tumors under radiotherapy could be correlated to the expression pattern of EGFR (4–7). Consequently, antagonizing strategies to inhibit EGFR-signaling may help to overcome radioresistance of EGFR-overexpressing tumors. In this context, it has been shown that inhibition of EGFR (i.e., by monoclonal antibodies or tyrosine kinase inhibitors) can increase radiation toxicity in a variety of human tumor cells (8, 9). However, accumulating preclinical as well as clinical data indicate a very heterogeneous response pattern of different tumor cells *in vitro* and tumor entities *in vivo* especially to small-molecule tyrosine kinase inhibitors (10, 11). This may reflect cell- or tumor type–specific differences most likely based on the heterogeneity of accompanying mutations in related or separate growth regulatory pathways (12).

Previously we have shown that targeting of EGFR by the specific tyrosine kinase inhibitor BIBX1382BS (BIBX), as well as blockade of PI3K by LY294002, modulates radiation sensitivity differentially in K-*RAS*$_{mt}$ and K-*RAS*$_{wt}$ cells *in vitro* (13, 14). BIBX enhanced radiation sensitivity (i.e., clonogenic cell survival) only in tumor cells presenting a point mutation in K-*RAS* gene in codon 12 or 13 but not in wild-type *RAS* tumor cells or in a tumor xenograft model of wild-type *RAS* tumor cells (10, 15). BIBX-specific radiosensitization of K-*RAS*$_{mt}$ human tumor cells *in vitro* could be attributed to the *RAS* mutation–dependent up-regulated production and secretion of autocrine EGFR ligands (i.e., amphiregulin), which in turn selectively stimulated the EGFR-PI3K-AKT pathway (14). Consequently, inhibition of each component of the EGFR-PI3K-AKT pathway in K-*RAS*$_{mt}$ human tumor cells enhanced radiosensitivity significantly (14). Likewise, Kim et al. (16) have shown that targeting AKT activity by small interfering RNA (siRNA) approaches sensitizes human tumor cells *in vitro* to ionizing radiation.

Although different mechanisms have been proposed to be involved in the radiosensitizing effect of EGFR antagonists, such as effects on cell cycle progression, repopulation, reoxygenation, apoptosis, DNA repair, and angiogenesis (8, 17–20), the exact mechanism of radiosensitization by EGFR inhibitors is not clear thus far. Several reports discuss the possibility that blockage of the EGFR pathway predominantly leads to reduced cell survival through up-regulation of apoptosis and down-regulation of survival mechanisms, such as DNA repair after exposure to DNA-damaging agents (21, 22). DNA double-strand breaks (DSB) are the most important DNA lesions leading to cell kill following exposure to ionizing radiation (23). Two processes are primarily involved in the repair of DNA-DSBs: nonhomologous end-joining and homologous recombination (24). Nonhomologous end-joining is the major process of DNA-DSB repair responsible for survival of cells exposed to ionizing radiation. Proteins involved are the heterodimer Ku70/Ku80, the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), the ligase IV-XRCC4 complex, and, in addition, Artemis and the Mre11-NBS1-Rad50 complex (25). Any reduction in one of the nonhomologous end-joining component was shown to result in an increased number of residual DSB and/or enhanced cellular radiosensitivity (25–28).

It is known that EGFR interference by the specific antibody C225 can modulate the balance between cytoplasmic and nuclear DNA-PKcs and thereby affect DNA repair (8, 17). Furthermore, a recent report by Friedmann et al. (22) describes the potential of the EGFR tyrosine kinase inhibitor gefitinib (ZD1839), as well as the PI3K inhibitor LY294002, to block repair of DNA damage induced by chemotherapeutic agents, such as cisplatin and melphalan, and proposes that the EGFR-PI3K pathway may be involved in regulating repair of DNA damage through control of DNA-PK activity.

Thus, in the context of these reports (8, 22) and our own previous results (13, 14), we addressed the question whether small-molecule inhibitors of EGFR tyrosine kinase, PI3K activity, or AKT (e.g., BIBX, LY294002, or API-59CJ-OH, a derivative of API-59CJ-OMe; refs. 29, 30), as well as *AKT1* siRNA, are able to modulate radiation-induced activation of proteins involved in repair of DNA damage. As a consequence, radiation sensitivity of K-*RAS*$_{mt}$ human tumor cells is significantly enhanced. Evidence is provided indicating a prominent role of EGFR-PI3K-AKT-signaling in triggering DSB repair as one functional end point of radiation response of K-*RAS*$_{mt}$ human tumor cells.

Materials and Methods

Antibodies and inhibitors. P-H2AX (Ser^{139}) and P-ATM (Ser^{1981}) antibodies were purchased from Upstate/Biomol (Hamburg, Germany). Antiactin antibody and cytochalasin B were obtained from Sigma (Taufkirchen, Germany). DNA-PKcs antibody was a product of BD PharMingen (Heidelberg, Germany). PI3K inhibitor LY294002 is a product of Calbiochem (Merck, Schwalbach-Germany). The specific EGFR tyrosine kinase inhibitor BIBX was provided by Boehringer Ingelheim Austria GmbH (Vienna, Austria). The AKT inhibitor API-59CJ-OH is a derivative of API-59-OMe, recently described by Jin et al. (29) and Tang et al. (30). As tested in pilot experiments, API-59CJ-OH at a concentration of 10 μmol/L completely blocked EGF-induced AKT-dependent GSK3 phosphorylation at Ser^{21} when cells were pretreated for 72 hours with this inhibitor. P-Thr^{2609} antibody was kindly provided by Prof. Dr. David Chen (Division of Molecular Radiation Biology, University of Texas Southwestern Medical Center, Dallas, TX).

Cell lines. Established human tumor cell lines presenting K-*RAS* mutation [i.e., A549 (bronchial carcinoma, ATCC no. CCL-185, mutated in codon 12) and MDA-MB-231 (breast adenocarcinoma, ATCC no. HTB-26, mutated in codon 13)], as well as normal *RAS* cell lines [i.e., FaDu (pharyngeal squamous cell carcinoma, ATCC no HTB-43) and transformed human skin fibroblast HH4ded], were used. Cells were cultured in DMEM (A549, MDA-MB-231, and HH4ded) or MEM (FaDu) routinely supplemented with 10% FCS and 1% penicillin-streptomycin and incubated in a humidified atmosphere of 93% air/7% CO_2 at 37°C.

Inhibitor treatment. Stock solutions of the EGFR inhibitor BIBX, the PI3K inhibitor LY294002, and the AKT pathway inhibitor API-59CJ-OH were made at appropriate concentration in DMSO and stored at −70°C. For treatment, inhibitor solutions were diluted 1:1,000 to appropriate working concentrations (5 μmol/L BIBX, 10 μmol/L LY294002, and 10 μmol/L API-59CJ-OH) in serum-free medium. Control cultures received medium containing the solvent DMSO at a concentration of 0.1%. BIBX and LY294002 were supplemented to the culture media 30 minutes before irradiation whereas, based on pilot studies, preirradiation treatment with API-59CJ-OH was 72 hours.

Clonogenic assay. Clonogenic cell survival following radiation exposure was analyzed by means of colony formation assay. Subconfluent cell cultures were irradiated with X-rays (200 kVp, 18 mA, 0.5 mm Cu additional filtering) at room temperature using a dose rate of 4 Gy/min. After irradiation, cultures were incubated for 24 hours at 37°C. Thereafter, cells were trypsinized, plated at a constant cell density (500 per 50-cm^2 Petri dish), and incubated for 14 days to allow for

colony growth. Colonies of >50 cells were scored as survivors. Clonogenic fraction of irradiated cells was normalized to the plating efficiency of unirradiated controls.

AKT1 siRNA transfection. Transfection of A549 and FaDu cells with *AKT1* siRNA (50 nmol/L; Dharmacon, Chicago, IL), a predominant isoform of AKT in tumor cells, was done using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) according to the protocol of the manufacturer. Time-course experiments revealed a maximum suppression of AKT1 protein at day 4 after transfection.

Western blot analysis. Total protein contents as well as activation patterns (i.e., phosphorylation of H2AX, ATM, and DNA-PKcs) following ionizing radiation with and without inhibitor pretreatment were analyzed by Western blotting. Therefore, cells grown to 50% to 60% confluency were incubated in serum-free medium for 48 hours. Subsequently, irradiated cells treated with inhibitors or vehicle before ionizing radiation were washed twice with ice-cold PBS and lysed with lysis-buffer [50 mmol/L Tris-base (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] at the indicated time points after irradiation. Lysates were cleared of insoluble material and normalized for protein concentration. To analyze the activation pattern of H2AX, DNA-PKcs, and ATM, as well as total proteins, 100 to 200 μg 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.

Determination of γH2AX-foci. γH2AX focus assay (31) was applied for determination of residual DNA-DSB. Therefore, cells grown on glass slides were irradiated with X-rays (2 and 6 Gy) and incubated at 37°C for 24 hours to allow repair of ionizing radiation–induced DNA-DSB (32). Thereafter, cells were fixed by 2% formaldehyde in PBS for 15 minutes at room temperature. Then cells were washed thrice in PBS for 10 minutes and were permeabilized by 0.2% Triton X-100 in PBS/1% bovine serum albumin (BSA) on ice for 5 minutes. After washing (PBS/1% BSA), samples were blocked at room temperature (PBS/3% BSA) for 1 hour and then incubated with anti–phospho-histone H2AX antibody at room temperature (1:100 in PBS/1% BSA/0.5% Tween 20) for 1 hour. After washing with PBS/1% BSA/0.5% Tween 20 (3×, 10 minutes each), incubation with second antibody was done at room temperature for 1 hour as described elsewhere (31). After washing (4×), cells were incubated with 4′,6-diamidino-2-phenylindole/antifade (Appligene Oncor, Eschwege, Germany). The number of foci was counted using a fluorescence microscope (Axioplan 2, Zeiss). Fifty to 70 cells were counted per coded slide.

Micronucleus assay. Micronucleus assay was done according to standard procedure (33). In brief, exponentially growing cells were pretreated with inhibitor or vehicle for 30 minutes before irradiation (single dose of 2 Gy); 2 μg/mL of cytochalasin-B was added to the culture media for 24 hours. Cells were collected by centrifugation and incubated in a hypotonic solution (0.075 mol/L KCl) for 5 minutes. After centrifugation (1,000 rpm), cells were fixed with methanol/glacial acetic acid (3:1) and spread onto cooled glass slides. After air-drying and staining (4% Giemsa), micronuclei were scored microscopically in 400 binucleated cells.

Statistics and densitometry. Student's t test was used to compare data between two groups. Values are expressed as mean ± SE. $P < 0.05$ was considered statistically significant. Densitometric quantification analyses of immunoblots were done with Scion Image computer software.

Results

Differential effect of BIBX on radiosensitivity. As previously shown (13), the EGFR inhibitor BIBX enhances toxicity of fractionated dose irradiation in K-*RAS*$_{mt}$ and K-*RAS*$_{wt}$ cells differentially. Radiation sensitivity was significantly affected in K-*RAS*$_{mt}$ cells whereas cells presenting normal *RAS* were not sensitized by this compound. This differential radiosensitizing effect of BIBX is now confirmed here by single-dose irradiation with doses up to 6 Gy in K-*RAS*$_{mt}$ A549 and K-*RAS*$_{wt}$ FaDu cells (Fig. 1). These data were used to calculate the dose D_{37}, which is required to reduce survival to 37%. For A549 the respective values without and with BIBX amounted to 5.1 and 3.6 Gy, and for FaDu, 5.0 and 4.9 Gy, respectively. The resulting dose modifying factors are 1.4 for A549 and 1 for FaDu cells.

Differential modulation of H2AX phosphorylation by inhibitors of EGFR and PI3K. To analyze how BIBX as well as LY294002 affects radiation-induced H2AX phosphorylation as indicator of DNA damage signaling, A549, MDA-MB-231, FaDu, and HH4ded cells were irradiated with single doses of ionizing radiation (1, 2, and 4 Gy). H2AX phosphorylation at Ser^{139} reached a maximum at 30 minutes post ionizing radiation (Fig. 2). The densitometric analysis of P-H2AX after ionizing radiation indicates a dose-dependent increase in H2AX phosphorylation, especially at the 30-minute time point.

Figure 3 shows the effect of BIBX and LY294002 on the level of H2AX phosphorylation in K-*RAS*$_{mt}$ A549 and MDA-MB-231 as well as in K-*RAS*$_{wt}$ FaDu and HH4ded cells. Cells were irradiated with a single dose of 8 Gy in the presence and absence of both inhibitors. For all cell lines H2AX phosphorylation was enhanced immediately (i.e., 1 minute) after irradiation, whereby the level of H2AX phosphorylation is markedly more pronounced in A549 and MDA-MB-231 cells than in FaDu or HH4ded cells. Most notably, however, pretreatment with either inhibitor markedly reduced/abolished radiation-induced H2AX phosphorylation in A549 and MDA-MB-231 cells whereas it was affected only slightly in FaDu and HH4ded cells (Fig. 3). In contrast to H2AX, ionizing radiation–induced phosphorylation of ATM (Ser^{1981}) remained unaffected by the inhibitors in either cell line (Fig. 3).

Effect of EGFR, PI3K, and AKT inhibitors on ionizing radiation–induced activation of DNA-PKcs. It is widely accepted that ATM and DNA-PKcs are the major determinants of phosphorylation of H2AX in response to DNA-DSBs (34–36). As shown in Fig. 3, although H2AX phosphorylation in K-*RAS*$_{mt}$ cells is markedly inhibited by the EGFR and PI3K inhibitors, ATM phosphorylation was not affected. Therefore,

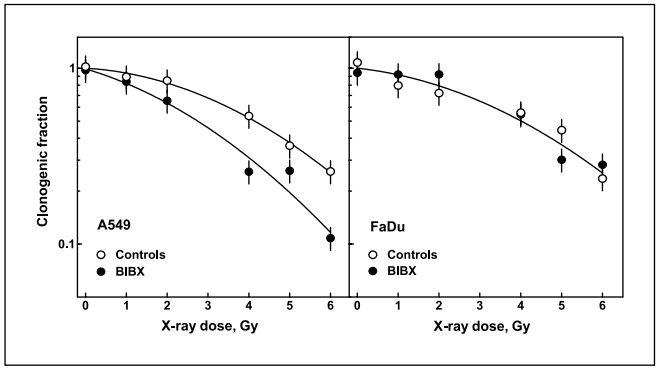

Fig. 1. Differential radiosensitization of K-*RAS*$_{mt}$ A549 and K-*RAS*$_{wt}$ FaDu cells by BIBX. Log-phase A549 and FaDu cells were pretreated for 30 minutes with BIBX (5 μmol/L) and irradiated with X-ray doses up to 6 Gy. Cells were trypsinized and plated for colony formation 24 hours after ionizing radiation. Fourteen days later, colonies formed were counted and survival fractions were calculated as described in Materials and Methods. Data points represent the mean surviving fraction ± SEM of three parallel experiments.

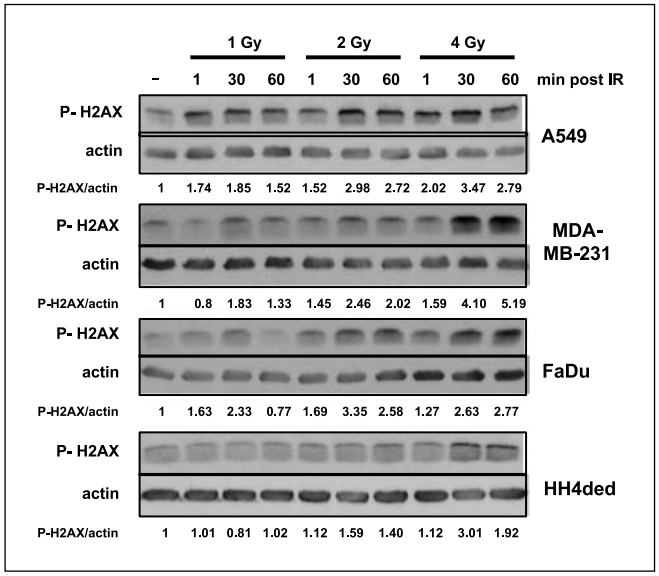

Fig. 2. Time kinetics of radiation-induced H2AX phosphorylation. Forty-eight-hour serum-starved K-*RAS*$_{mt}$ (A549 and MDA-MB-231) and K-*RAS*$_{wt}$ (FaDu and HH4ded) cells were exposed to single doses of ionizing radiation of 1, 2 or 4 Gy. Cells were lysed at the times indicated, subjected to SDS-PAGE, and incubated with antibodies against P-H2AX (Ser^{139}) and actin. Densitometry values shown represent the ratios of P-H2AX/actin (normalized to 1.0 in control).

we asked the question whether radiation-induced DNA-PK activity is modulated by either of these inhibitors. DNA-PKcs phosphorylation at Thr^{2609} has previously been shown to be relevant for both DSB repair and cellular radiosensitivity (18, 37). In both A549 and FaDu cells irradiated with a single dose of 2 Gy, Thr^{2609} phosphorylation of DNA-PKcs was clearly enhanced (Fig. 4A). Pretreatment with either BIBX or LY294002 significantly reduced phosphorylation only in K-*RAS*$_{mt}$ A549 cells but not in K-*RAS*$_{wt}$-FaDu cells. Yet, in either cell line the expression level of total DNA-PKcs was not affected by inhibitor treatment.

Furthermore, a specific inhibition of AKT signaling, i.e., API-59CJ-OH (29, 30), markedly inhibited phosphorylation of DNA-PKcs in A549 but not in FaDu cells, indicating the dependence of the activity status of DNA-PKcs on AKT signaling in the K-*RAS*$_{mt}$ A549 cells (Fig. 4B). To further test this hypothesis, both A549 and FaDu cells were transfected with *AKT1* siRNA, which abolished expression of AKT1 protein in both cell lines very efficiently (Fig. 5). Yet, only in A549 cells, and not in FaDu cells, was phosphorylation of DNA-PKcs at Thr^{2609} markedly blocked (Fig. 5).

Differential effects on DNA-DSB repair by inhibitors of EGFR and PI3K. Pretreatment with BIBX resulted in a significant differential effect on the number of residual foci in the analyzed K-*RAS*$_{mt}$ A549 and K-*RAS*$_{wt}$ FaDu cell lines. For A549, but not for FaDu cells, the number of residual γH2AX foci was slightly enhanced when BIBX was combined with 2 Gy but was significantly elevated when given before 6 Gy. Furthermore, the PI3K inhibitor LY294002 led to an even more pronounced effect on residual γH2AX foci in A549 cells (Fig. 6A). Neither BIBX nor LY294002 treatment significantly affected the structure or the intensity of γH2AX foci remaining in A549 and FaDu cells 24 hours after irradiation (Fig. 6A). Likewise, pretreatment with BIBX significantly enhanced the frequency of micronuclei in K-*RAS*$_{mt}$ A549 cells, but not in K-*RAS*$_{wt}$ FaDu cells (Fig. 6B). This result corresponds to the difference in DSB repair capacity seen in Fig. 6A.

Discussion

Previously we have shown that inhibition of EGFR activity with the specific EGFR tyrosine kinase inhibitor BIBX enhances radiation toxicity only of K-*RAS*$_{mt}$ cells, but not of K-*RAS*$_{wt}$ human tumor cells, via blockage of EGFR-PI3K-AKT cascade (13, 14). The present study now shows for the first time that EGFR-PI3K-AKT signaling in K-*RAS*$_{mt}$ A549, but not in K-*RAS*$_{wt}$ FaDu tumor cells, is directly involved in the activation of proteins regulating DNA-DSB repair (i.e., H2AX and DNA-PKcs).

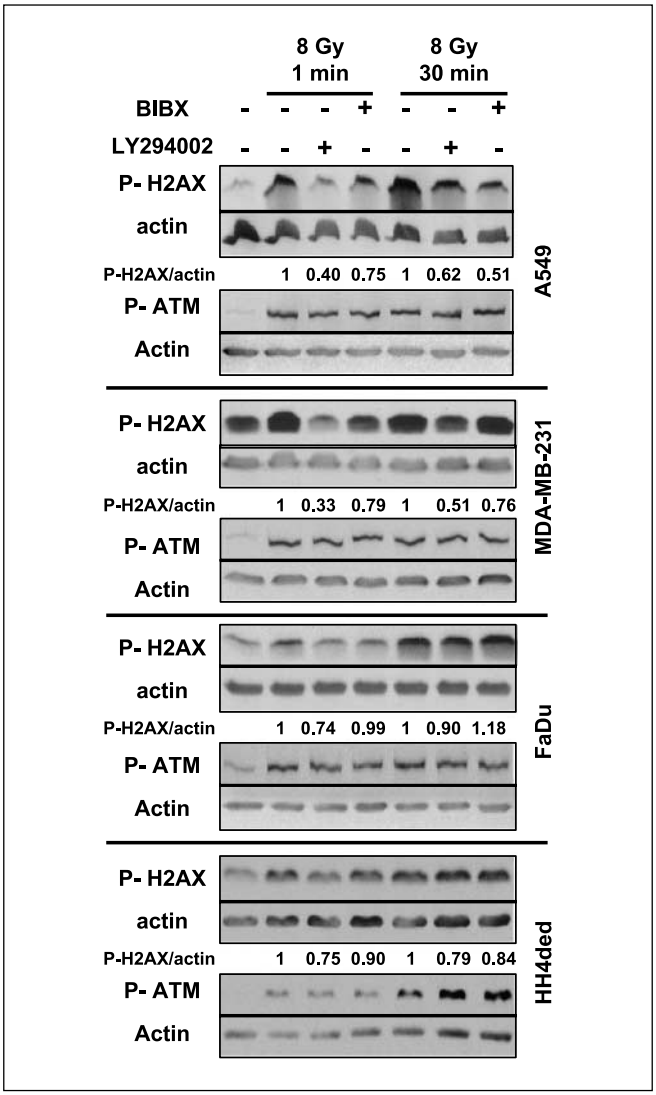

Fig. 3. Radiation-induced H2AX and ATM phosphorylatiom in K-*RAS*$_{mt}$ and K-*RAS*$_{wt}$ cells after inhibitor pretreatment. Serum-starved K-*RAS*$_{mt}$ (A549 and MDA-MB-231) and K-*RAS*$_{wt}$ (FaDu and HH4ded) cells were pretreated for 30 minutes with BIBX, LY294002, or DMSO (vehicle) and irradiated (8 Gy). Cells were lysed at the times indicated, subjected to SDS-PAGE, and incubated with antibodies against P-H2AX (Ser^{139}), P-ATM (Ser^{1981}), and actin. Densitometry values shown represent the ratios of P-H2AX/actin (normalized to 1.0 in control).

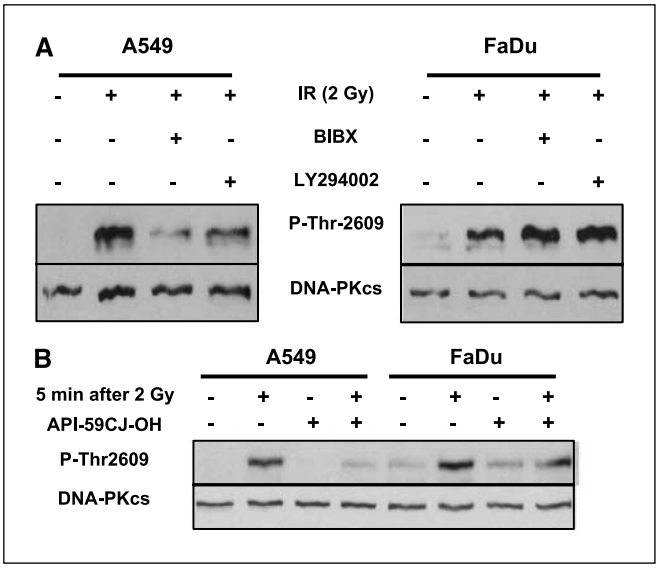

Fig. 4. Phosphorylation of DNA-PKcs at Thr^{2609} as a function of inhibitor treatment. Forty-eight-hour serum-starved A549 and FaDu cells were treated with BIBX (5 μmol/L; *A*), LY294002 (10 μmol/L; *A*), API-59CJ-OH (10 μmol/L; *B*), or vehicle, as described in Materials and Methods, and exposed to single-dose ionizing radiation of 2 Gy. Five and/or 10 minutes after ionizing radiation, cells were lysed and lysates were subjected to SDS-PAGE and P-Thr^{2609} immunoblotting. Subsequently, blots were stripped and reprobed with total DNA-PKcs antibody.

Various groups reporting on chemosensitizing or radiosensitizing effects of EGFR antagonists (tyrosine kinase inhibitors as well as EGFR-specific antibodies; i.e., C225) proposed that inhibition of EGFR signaling may, in addition to proliferation and apoptosis, also affect DNA damage repair (8, 17, 18, 22). Therefore, in the present study, DNA repair was investigated after selective inhibition of EGFR-PI3K-AKT pathway. As we could clearly show on the basis of residual DSB or chromosomal damage detected either by the H2AX- or the micronucleus assay, preincubation with BIBX led to a significant impairment of DNA repair only in K-RAS_{mt} A549 but not in K-RAS_{wt} FaDu cells. In mammalian cells, efficient and successful repair of radiation-induced DNA-DSB is mainly done by nonhomologous end-joining in which DNA-PKcs is one of the critical proteins. Targeting of this protein either by siRNA or antisense (26, 28) results in an elevation of both residual DSBs and cellular radiosensitivity. In this context, reports (17, 18, 38) on the physical interaction of EGFR with DNA-PKcs are of special interest. As recently shown by us, internalization and translocation of activated EGFR into the nucleus seem to be an important step in the activation of DNA-PK. Inhibition of EGFR by the monoclonal antibody C225 not only radiosensitized cultured human K-RAS_{mt} tumor cell lines but also prevented nuclear translocation of radiation-induced EGFR, resulting in a significant inhibition of DNA-PK activity as well as DNA-DSB repair (17, 18). These results indicate that nuclear translocation and direct interaction of EGFR and DNA-PKcs are necessary for effective DNA-DSB repair.

The present study additionally revealed that EGFR signaling through the PI3K-AKT pathway is also involved in the regulation of DNA-PKcs and DNA repair of K-RAS_{mt} tumor cells. As shown, the EGFR tyrosine kinase inhibitor BIBX abrogated radiation-induced DNA-PKcs phosphorylation at Thr^{2609} only in K-RAS_{mt} A549, but not in K-RAS_{wt} FaDu cells. Thr^{2609} phosphorylation is indicative for activated DNA-PKcs (37). Recently, Shintani et al. (21) reported that inhibition of EGFR by the tyrosine kinase inhibitor gefitinib/Iressa (ZD1839) results in a reduced nuclear fraction of DNA-PKcs protein, which correlates with enhanced radiation sensitivity of human oral cancer cell mouse xenografts. Without giving any information to functional aspects of DNA-PKcs (i.e., Thr^{2609} phosphorylation, kinase activity, or DNA repair measurements), these authors propose that gefitinib treatment impairs repair of radiation-induced DNA-DSB. Likewise, reduction of nuclear protein fraction of DNA-PKcs due to blockage of EGFR function by the specific antibody C225 has been correlated to C225-mediated radiosensitization (8). Yet, in our study, the differential effect of BIBX on DNA-PKcs phosphorylation was not due to a BIBX-mediated decrease in total DNA-PK protein because the inhibitor did not alter the content of DNA-PKcs. Thus, it is currently not clear how the results by Shintani et al. (21) and Huang and Harari (8) obtained for gefitinib and C225 compare to our results shown for BIBX at the functional level of DNA-PKcs. Nevertheless, our data of reduced DNA-PKcs phosphorylation at Thr^{2609} clearly correlate with an impaired DNA repair activity as determined via residual DSBs and the number of micronuclei 24 hours post ionizing radiation. Furthermore, the blockage of ionizing radiation–induced DNA-PKcs activation by BIBX, LY294002, and API-59CJ-OH, as well as *AKT1* siRNA, clearly indicates the functional requirement of the EGFR-PI3K-AKT pathway in regulating DNA-DSB repair after exposure to ionizing radiation.

Differential effects in K-RAS_{mt} and K-RAS_{wt} cells were also apparent at the level of H2AX phosphorylation. Only in K-RAS_{mt} A549 and MDA-MB-231 cells inhibition of EGFR tyrosine kinase by BIBX or of PI3K by LY294002 led to a pronounced reduction of ionizing radiation–induced H2AX phosphorylation. Phosphorylation of H2AX is considered to be a signal for the accumulation of repair proteins in the vicinity of damaged chromatin (39–41). Consequently, inhibition of phosphorylation will retard this accumulation and thereby impede DNA repair. The observed inhibitory effect of BIBX, LY294002, and API-59CJ-OH on H2AX phosphorylation is in

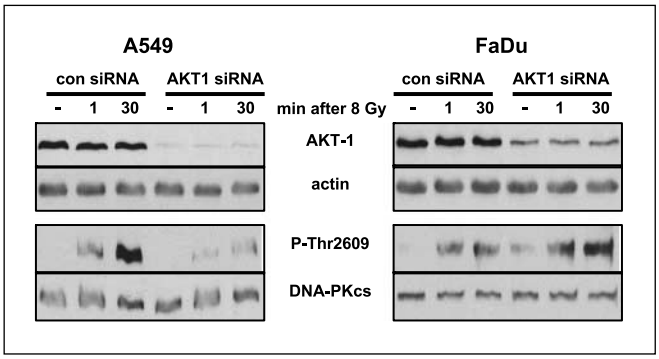

Fig. 5. Phosphorylation of DNA-PKcs at Thr^{2609} as a function of *AKT1* siRNA. A549 and FaDu cells were transfected with control siRNA or *AKT1* siRNA as described in Materials and Methods. Four days after transfection, cells were irradiated with single dose of 8 Gy. At time points indicated, cells were lysed and suppression of AKT1 was checked by immunoblotting. On the same gels, phosphorylation of DNA-PKcs at Thr^{2609} was determined. For loading control, total DNA-PKcs protein and actin were detected.

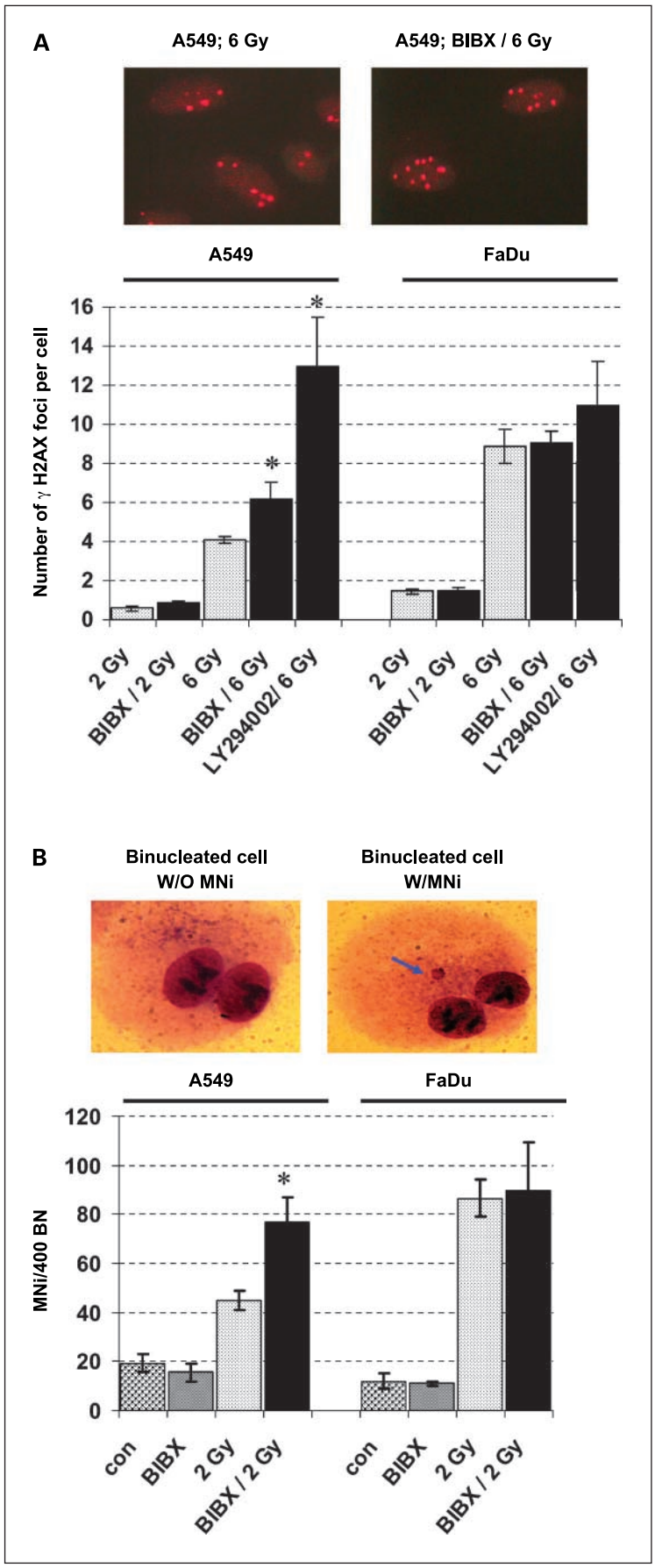

Fig. 6. Differential effect of BIBX on residual DSBs and frequency of micronuclei. *A,* cells grown on glass slides were pretreated with BIBX (5 μmol/L), LY294002 (10 μmol/L), or DMSO for 30 minutes before irradiation with a single-dose ionizing radiation of 2 and/or 6 Gy. After irradiation, cells were incubated at 37°C for 24 hours before DSBs were detected via detection of γH2AX foci formation. *, $P < 0.05$, statistically significant enhancement in frequency of radiation-induced γH2AX foci in A549 cells pretreated with BIBX or LY294002 (Student's *t* test). *B,* exponentially growing cells were pretreated with BIBX (5 μmol/L) or DMSO for 30 minutes and irradiated at 2 Gy. Immediately after irradiation, cells were processed for determination of micronuclei (*MNi*) as described in Materials and Methods. *, $P < 0.05$, statistically significant enhancement in frequency of radiation-induced micronuclei in A549 cells pretreated with BIBX (Student's *t* test). Columns, mean of four independent experiments; bars, SE.

line with the data found for wortmannin, which also blocks H2AX phosphorylation (34, 36). Furthermore, our data also reveal that inhibition of EGFR-PI3K-AKT signaling might affect DSB repair by two different ways. First, blocking of Thr^{2609} phosphorylation of DNA PKcs will render the enzyme inactive and thereby directly impair nonhomologous end-joining. Second, inhibition of H2AX phosphorylation might indirectly interfere with DNA repair via a retarded assembly of nonhomologous end-joining proteins at the DSB.

In response to ionizing radiation–induced DNA-DSBs, phosphorylation of the histone H2AX is considered to be done by two kinases (i.e., ATM and DNA PKcs; refs. 34–36). Data suggest a complementary contribution of these two enzymes because an inhibition of only one kinase results only in a moderate reduction whereas simultaneous blockage of both kinases leads to a pronounced reduction of H2AX phosphorylation (35, 36). It is now shown here for K-RAS_{mt} A549 cells that BIBX-, LY294002-, or API-59CJ-OH-dependent reduction of H2AX phosphorylation correlates with a blocked activation of DNA-PKcs but not of ATM. With respect to phosphorylation of H2AX, these results indicate that in K-RAS_{mt} cells the balance between DNA PKcs and ATM is shifted towards DNA-PKcs. Our data also show that DNA-PKcs, but not ATM, is activated via ionizing radiation–induced EGFR-PI3K-AKT signaling, probably via a direct interaction of AKT1 and DNA PKcs as it has already been suggested (42, 43). However, further investigations are necessary to elucidate the role of other kinases (i.e., ATR) in mediating phosphorylation of H2AX downstream of EGFR.

Earlier reports (8, 22) and our data presented here and published recently (13, 14, 17, 18) further support the hypothesis that EGFR-signaling regulates cellular radiation sensitivity through modulating DNA repair processes. As shown here and previously (13, 14), K-RAS_{mt} human tumor cells present a selective sensitivity to inhibitors of both EGFR tyrosine kinase as well as PI3K. Although various studies (8, 22, 44, 45) addressed the importance of receptor-mediated PI3K-AKT signaling in regulation of DNA repair, the present report provides the first direct evidence that erbB receptor–mediated stimulation of the PI3K-AKT pathway, as shown here for erbB1/EGFR, can directly regulate the activation profile of DNA-PKcs as well as the repair of DNA-DSBs in irradiated K-RAS_{mt} human tumor cells.

In summary, the data presented clearly indicate a differential response of K-RAS_{mt} versus K-RAS_{wt} human tumor cells to EGFR- or PI3K-targeting approaches in terms of radiosensitization mediated primarily at the level of DNA repair. Although the detailed regulatory steps linking EGFR-PI3K-AKT signaling to DNA repair have not been identified yet, the data presented add important information to the understanding of EGFR-mediated regulation of pathways involved in the response profile of human tumor cells to ionizing radiation.

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