

# Non–Small Cell Lung Cancers with Kinase Domain Mutations in the Epidermal Growth Factor Receptor Are Sensitive to Ionizing Radiation

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## Abstract

**Non–small cell lung cancers (NSCLCs) bearing mutations in the tyrosine kinase domain (TKD) of the epidermal growth factor receptor (EGFR) often exhibit dramatic sensitivity to the EGFR tyrosine kinase inhibitors gefitinib and erlotinib. Ionizing radiation (IR) is frequently used in the treatment of NSCLC, but little is known how lung tumor–acquired EGFR mutations affect responses to IR. Because this is of great clinical importance, we investigated and found that clonogenic survival of mutant EGFR NSCLCs in response to IR was reduced 500- to 1,000-fold compared with wild-type (WT) EGFR NSCLCs. Exogenous expression of either the L858R point mutant or the  $\Delta$ E746-E750 deletion mutant form of EGFR in immortalized human bronchial epithelial cells, p53 WT NSCLC (A549), or p53-null NSCLC (NCI-H1299) resulted in dramatically increased sensitivity to IR. We show that the majority of mutant EGFR NSCLCs, including those that contain the secondary gefitinib resistance T790M mutation, exhibit characteristics consistent with a radiosensitive phenotype, which include delayed DNA repair kinetics, defective IR-induced arrest in DNA synthesis or mitosis, and pronounced increases in apoptosis or micronuclei. Thus, understanding how activating mutations in the TKD domain of EGFR contribute to radiosensitivity should provide new insight into effective treatment of NSCLC with radiotherapy and perhaps avoid emergence of single agent drug resistance.** (Cancer Res 2006; 66(19): 9601-8)

## Introduction

Recent discoveries have linked somatic mutations in the tyrosine kinase domain (TKD) of the *epidermal growth factor receptor* (*EGFR*) gene in a subset of non–small cell lung carcinoma (NSCLC) to tumor responsiveness to the EGFR tyrosine kinase inhibitor (TKI) gefitinib (1–3). The mutations seem to favor an adenocarcinoma histology, East Asian ethnicity, female gender, and never-smoker status. These mutations were mapped in exons 18 to 21 of the *EGFR* that encodes the ATP-binding region of the TKD (4). Three classes of EGFR mutations have been identified: an L858R

missense mutation, deletions in exon 19, and insertions in exon 20 (5, 6). Studies show that the mutant *EGFR* is often amplified, constitutively active in most tumors, and exhibits dramatically high sensitivity to gefitinib (2, 7–12). The prevailing hypothesis is that gefitinib-sensitive NSCLCs are “addicted” to activated EGFR signaling (13), and thus, chemotherapeutic agents targeting the TKD of EGFR are initially highly effective. Recent studies have reported that nine patients harboring mutant EGFR NSCLCs that initially responded to gefitinib later developed progressive NSCLC. Six of these patients had a secondary, somatically acquired, threonine to methionine mutation at position 790 (T790M) that seems to confer resistance to gefitinib (14–16). This secondary mutation has also been observed in a NSCLC cell line, H1975 (16).

Radiotherapy, either alone or in combination with chemotherapy, is routinely used in the treatment of lung cancer with curative intent in primary lesions as well as palliative therapy of metastatic disease. In addition to the well-known differences in radioresponse between SCLC and NSCLC, great differences in response exist between NSCLCs with the same histology. Expression and activity of EGFR are important determinants of radioresponse in several neoplasias, including NSCLCs, and loss of function mutations in the EGFR or anti-EGFR therapy has been shown to sensitize tumor cells to radiation (17–19). The discovery of activating mutations in the EGFR raises important questions about the response of mutant EGFR NSCLC to ionizing radiation (IR). (a) For example, do NSCLCs “addicted” to activated EGFR signaling exhibit differences in radioresponse compared with those that do not have activated EGFR? (b) In addition, would anti-EGFR therapy influence the response to radiation? To address these issues, we have compared the *in vitro* radiation responses of NSCLCs with and without activating mutations in the TKD of EGFR. Of great basic and translational interest, we have found that the presence of such mutations significantly sensitizes NSCLCs to IR.

## Materials and Methods

### Cell Culture

All NSCLC cell lines in the study were obtained from the Hamon Cancer Center for Therapeutic Oncology Research (University of Texas Southwestern Medical Center, Dallas, TX) and tested negative for *Mycoplasma* contamination in periodic evaluation. In addition, their identity was verified by DNA fingerprinting using the PowerPlex 1.2 System (Promega, Madison, WI). NSCLC cell lines were cultured at 37°C and 5% CO<sub>2</sub> in RPMI 1640 with glutamine (Hyclone, Logan, UT) containing 5% fetal bovine serum (FBS; Gemini Biosciences, Woodland, CA). Immortalized human bronchial epithelial cell (HBEC) lines (13) were stably transfected with either an empty vector, a cytomegalovirus promoter-driven LacZ construct, the wild-type

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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(WT) *EGFR*, a mutant *EGFR* construct with an L858R replacement in the exon 21, or an *EGFR* construct with a deletion in the exon 19 as described previously (20). HBECs were grown at 37°C and 5% CO<sub>2</sub> in keratinocyte serum-free medium (Invitrogen/Life Technologies, Carlsbad, CA) supplemented with 0.2 ng/mL recombinant human EGF and 30 µg/mL bovine pituitary extract.

### Western Blot Analysis

Lysates from HBEC or NSCLC cell lines transfected with LacZ, WT *EGFR*, ΔE746-E750, or the L858R mutant *EGFR* were electrophoresed and transferred to polyvinylidene difluoride membranes. Membranes were probed with anti-total *EGFR* antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

### Cell Viability Assay

NSCLC cell lines were seeded in replicates of six in 96-well plates at a density of 5,000 per well. Cells were either left untreated or irradiated in an X-Rad 300 irradiator (Precision X-Ray, East Haven, CT) and allowed to grow for 7 days. Cells were lysed in 50 µL of CellTiter-Glo reagent (Promega), and luminescence was measured. Percentage cell viability of irradiated samples was calculated as the fraction of cell viability relative to 100% viability of untreated samples at 7th day and plotted as a function of radiation dose.

### Clonogenic Cell Survival Assay

NSCLC cells were seeded in triplicate 60-mm dishes containing RPMI 1640 plus 10% FBS at various densities commensurate with the dose of radiation. Cells were irradiated at various doses using the <sup>137</sup>Cs irradiator (Model Mark I-68, JL Shepherd Associates, San Fernando, CA). Colonies were stained with crystal violet and manually counted using a microscope. Surviving fraction values were plotted as a function of radiation dose.

### Epifluorescence Scanning Microscopy

**DNA synthesis.** DNA synthesis was measured using the Cell Proliferation Assay kit (GE Healthcare, Piscataway, NJ) according to the manufacturer's instruction. Briefly, NSCLC cell lines were seeded in replicates of six in 96-well plates at a density of 10,000 to 20,000 per well and either left untreated or irradiated at various doses of radiation. After 18 hours, cells were maintained in bromodeoxyuridine (BrdUrd) containing medium for 3 hours. They were then fixed and stained with a monoclonal antibody against BrdUrd and detected by anti-mouse Cy5-labeled secondary antibody (GE Healthcare). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) to obtain total nuclei count. Images were acquired at ×4 magnification with the IN Cell Analyzer 1000 (GE Healthcare).

**Mitotic nuclei.** NSCLC cells were fixed 9 hours following radiation treatment and probed with an antibody against phosphorylated Ser<sup>10</sup> histone H3 (Upstate Cell Signaling, Lake Placid, NY) and detected by a Cy5-labeled secondary antibody. Phosphorylated histone H3-positive nuclei were quantified and normalized to total number of DAPI-stained nuclei by imaging using the IN Cell Analyzer 1000.

**Apoptosis assays.** Forty-eight hours following radiation, cells were fixed and stained with DAPI. Apoptotic cells were scored for the presence of intensely DAPI-stained apoptotic bodies and normalized to total number of nuclei. Apoptotic events were quantified using the image analysis software.

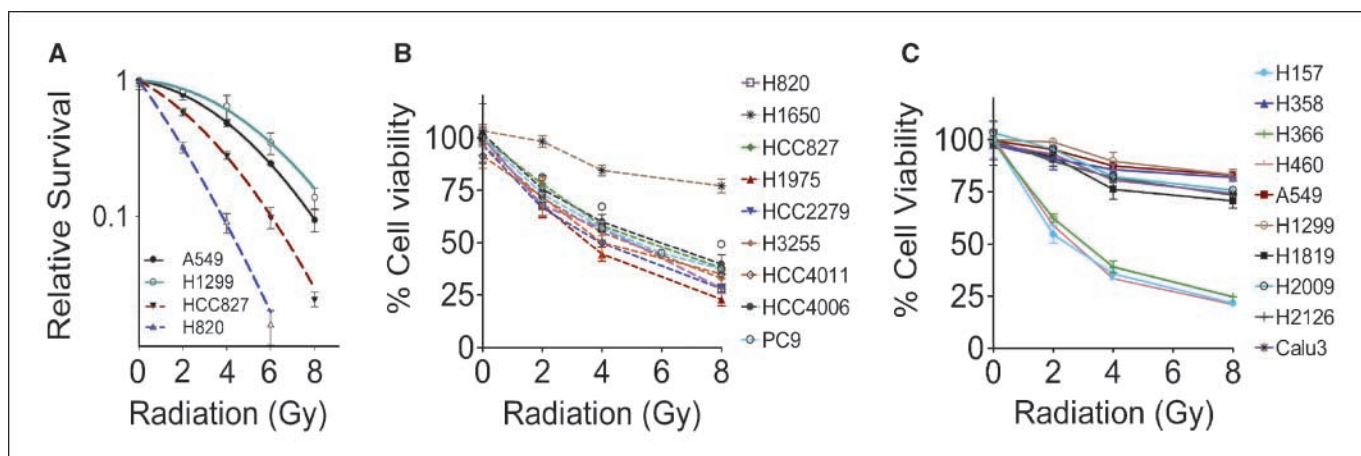
**H2AX foci determination.** Cells were left untreated or irradiated at 1 Gy and fixed in 1% paraformaldehyde and 0.1% Triton X-100 in PBS at various time points following IR. H2AX foci were identified by standard immunocytochemistry with anti-phosphorylated Ser<sup>139</sup> histone H2AX antibody (Upstate Cell Signaling) and Cy5-labeled anti-mouse secondary antibody. Images were acquired on the IN Cell Analyzer 1000. Cy5-positive H2AX foci per nucleus were then measured using Developer Toolbox software.

## Results

In this study, we investigated the effects of IR on multiple physiologic end points in 19 NSCLC cell lines. Of these, 10 NSCLC cell lines expressed the WT receptors (NCI-H157, H358, H366, H460, H1299, H1819, H2009, H2126, A549, and Calu3), a second set of 6 cell lines contained an in-frame deletion (ΔE746-E750) in the exon 19 of the *EGFR* (H820, HCC827, H1650, HCC2279, HCC4006, and PC-9), whereas a third set of 3 cell lines had the L858R replacement in the exon 21 of the *EGFR* (NCI-H1975, HCC3255, and HCC4011). All mutant *EGFR* cell lines, except NCI-H3255, were initiated in the laboratory of Dr. A.F. Gazdar (University of Texas Southwestern Medical Center), and mutational analysis in these cell lines has been previously reported (9).

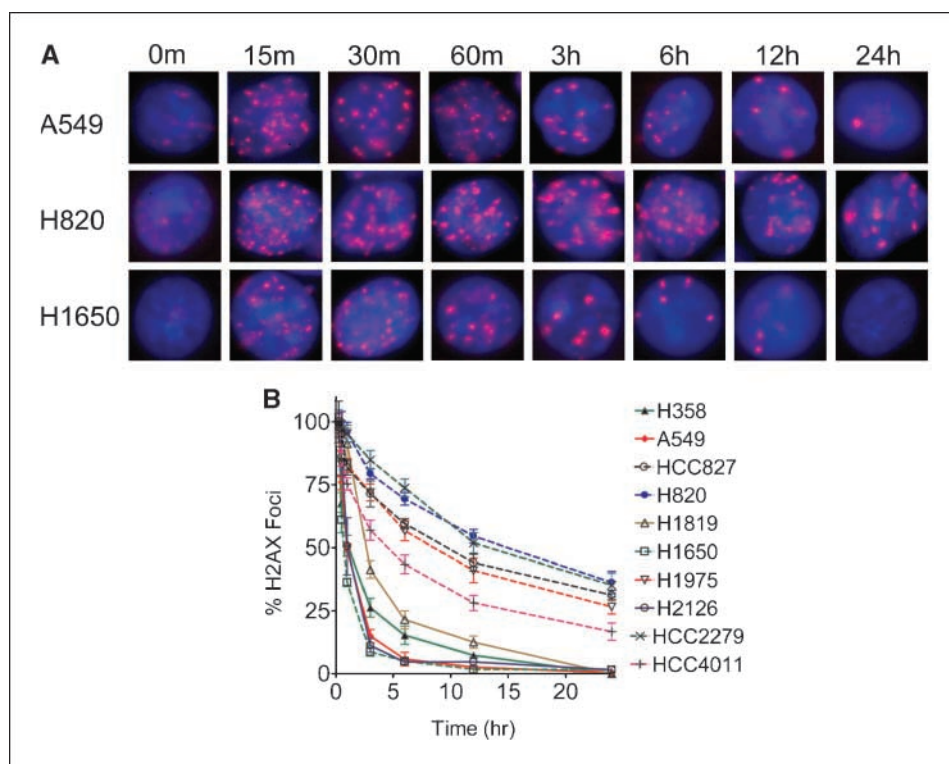
### Effect of IR on clonogenic survival in mutant *EGFR* NSCLCs.

We first compared the effect of radiation on clonogenic survival in two WT *EGFR* NSCLCs, H1299 and A549, and in two mutant *EGFR* NSCLCs, HCC827 and H820. Two cell lines, NCI-H820 and HCC827, formed clear colonies in clonogenic survival assays. Results of a typical experiment are shown in Fig. 1A. The WT *EGFR* cell lines A549 and H1299 showed a significant tolerance to radiation and modest loss of colony-forming ability at 8 Gy. In contrast, the mutant *EGFR*-expressing NSCLC cell line H820 exhibited high



**Figure 1.** Mutant *EGFR*-expressing cell lines show enhanced sensitivity to radiation. A, clonogenic cell survival assay. Colonies were counted on the 10th day following radiation, and surviving fractions were plotted as a function of dose. Error bars, SD from mean of triplicate measurements from a single experiment. Shown here is a representative of three independent experiments with similar result. Cell viability assay in mutant *EGFR*-expressing (B) and WT *EGFR*-expressing (C) cell lines. Cells were irradiated, and cell viability relative to untreated samples on the 7th day following IR was measured. Representative of three independent experiments is shown. Points, mean of six replicates each from two independent experiments; bars, SD.

**Figure 2.** Mutant EGFR-expressing cell lines show dissimilar kinetics of DNA repair at 1 Gy as WT-expressing NSCLC cell lines. Cell lines were irradiated at 1 Gy, and samples were fixed at various time points and probed with anti-phosphorylated histone  $\gamma$ H2AX antibody that was detected with a Cy5-labeled secondary antibody by fluorescence microscopy. *A*, images of H2AX foci in nuclei at  $\times 40$  magnification from three representative NSCLC cell lines. *B*, graphical representation of results from four WT (solid lines) and six mutant (broken lines) EGFR NSCLCs. Points, mean values of  $>6,000$  nuclear events taken from 30 fields; bars, SD.



radiosensitivity and retained a surviving fraction  $\sim 2$  orders of magnitude lower at 6 Gy compared with unirradiated controls. In comparison, HCC827, which also harbors the  $\Delta E746-E750$  mutant EGFR, seemed only moderately sensitive to radiation.

**Effect of IR on cell viability in mutant EGFR NSCLCs.** The effect of IR on other mutant EGFR NSCLCs was examined using ATP as a surrogate for cell viability. Cell viability assessments at the 7th day time point following IR reflect combined effects of cell death and cell proliferation in the entire population and differ from clonogenic assays that measure surviving fraction of individual clonogens. Our objective was to examine whether WT and mutant EGFR NSCLCs show differences in long-term cell viability in response to IR. The results in Fig. 1B and C indicate that, relative to cell viability in samples that received no radiation (0 Gy) set at 100%, viability in eight of nine cell lines that expressed the mutant EGFR (Fig. 1B) decreased with radiation in a dose-dependent manner to as low as 30% at 8 Gy radiation. In contrast, 8 of 11 NSCLCs that expressed the WT EGFR (Fig. 1C) showed less dramatic decreases in viability with radiation and retained  $\sim 70\%$  of their viability at 8 Gy. Three WT EGFR-expressing cell lines, H157, H366, and H460, however, showed the same dramatic decreases in cell viability as the mutant-expressing lines, whereas one mutant EGFR NSCLC, H1650, seemed relatively radioresistant. Data from the cell viability assay in Fig. 1B and C are consistent with those from clonogenic survival assay in Fig. 1A.

**NSCLCs with mutant forms of EGFR exhibit delayed kinetics of DNA repair.** Several studies have linked defects in DNA repair mechanisms to enhanced sensitivity to radiation (21). An early step in double-strand DNA break (DSB) repair involves the rapid phosphorylation of the histone  $\gamma$ H2AX, which then forms distinct foci at sites of DSB in the nucleus (22, 23). There is a linear relationship between the number of phosphorylated  $\gamma$ H2AX foci and DSBs (24). Using  $\gamma$ H2AX foci as a surrogate for DSBs, we

compared rates of DSB repair between four representative radioresistant WT and six mutant EGFR NSCLCs. NSCLC cells were exposed to 1 Gy IR and stained with antibodies against phosphorylated  $\gamma$ H2AX at various time points over a 24-hour period. Figure 2A shows images of  $\gamma$ H2AX foci from three representative NSCLCs, A549, H820, and H1650, captured at various time points during a 24-hour period following radiation at 1 Gy. The data in Fig. 2B indicate that five of six mutant EGFR-expressing cell lines (broken lines) show a significantly lower rate of DSB resolution and retain nearly 60% to 90% of  $\gamma$ H2AX foci at 3 hours and 30% to 50% foci at 24 hours following IR. The only exception to this trend was the radioresistant mutant EGFR cell line H1650, which exhibited a strikingly high rate of  $\gamma$ H2AX foci resolution (Fig. 2A and B). By contrast, WT EGFR NSCLC cell lines (solid lines) have higher rates of  $\gamma$ H2AX resolution and eliminate  $\sim 90\%$  of their foci by 3 hours. The data indicate that mutant EGFR expression is often associated with delayed DSB repair kinetics.

**NSCLCs with mutant forms of EGFR circumvent radiation-induced G<sub>1</sub> cell cycle checkpoint and fail to halt DNA synthesis.** Numerous studies have shown that IR-induced DNA damage results in arrest at the G<sub>1</sub>, intra-S, and/or the G<sub>2</sub>-M transition point of the cell cycle. The prevailing view is that cell cycle arrest prevents replication of damaged DNA and promotes cell survival (25–29). Cells were transiently exposed to the nucleotide analogue BrdUrd, and BrdUrd incorporation between 18 and 21 hours following IR was used as an end point for active DNA synthesis. Results from a representative experiment involving eight mutant EGFR and one WT EGFR NSCLCs are shown in Fig. 3. In response to radiation, the WT EGFR-expressing NSCLC line A549 showed an 80% to 90% dose-dependent decrease in BrdUrd-positive nuclei compared with untreated samples at 18 hours of radiation. A similar trend was observed in four other WT EGFR-expressing NSCLC lines, H358, H2009, H2126, and Calu3

(Supplementary Fig. S1A). Such decreases in BrdUrd incorporation were not significant at 6 and 9 hours of radiation (data not shown), suggesting that the IR-induced block in the DNA synthesis in WT EGFR NSCLCs occurred later than 9 hours and persisted at least until 18 hours following IR. In striking contrast to WT EGFR NSCLCs, six of eight mutant EGFR NSCLCs showed no significant IR-induced decreases in BrdUrd incorporation even after high dose radiation. One exception was the radioresistant, mutant EGFR-expressing NSCLC H1650, which showed dose-dependent decreases in BrdUrd-positive nuclei similar to those observed with A549. The data indicate that IR-sensitive mutant EGFR NSCLC cell lines lack the ability to block DNA synthesis in response to IR.

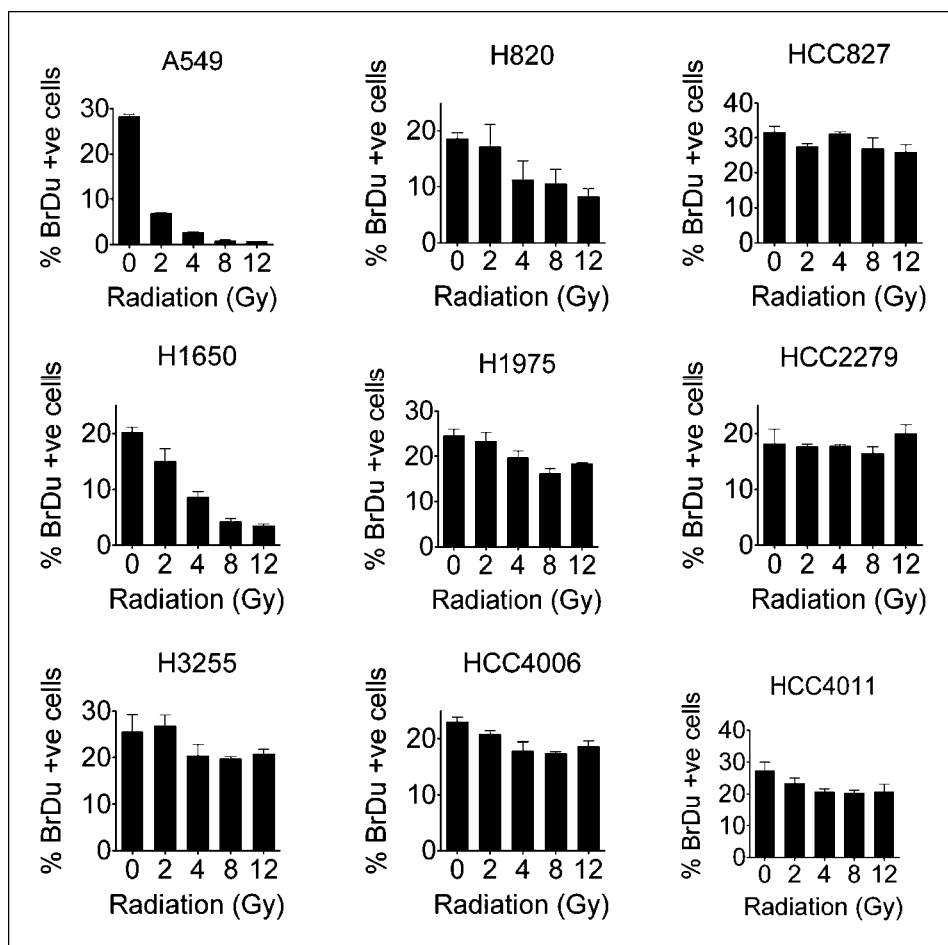
#### WT and mutant EGFR-expressing cell lines exhibit similar patterns of radiation-induced arrest at the G<sub>2</sub>-M checkpoint.

In most cell lines, arrest in the G<sub>2</sub> phase of the cell cycle is a characteristic response to IR-induced DNA damage, which is manifested as a decrease in the mitotic phase (M phase) fraction (29). We used the M-phase-specific phosphorylation of histone H3 at 9 hours following IR as an end point for mitosis (Fig. 4). In six of eight mutant EGFR NSCLC cell lines, mitotic fractions decreased in a dose-dependent manner relative to untreated samples. Similar IR-dependent decreases in the mitotic fraction were observed in A549 (Fig. 4) as well as in five other WT EGFR cell lines, H358, H1299, H1819, H2009, and H2126 (Supplementary Fig. S1B). The data indicate that, except for two mutant EGFR NSCLCs, NCI-H820 and HCC4011, IR-induced decrease in mitosis is

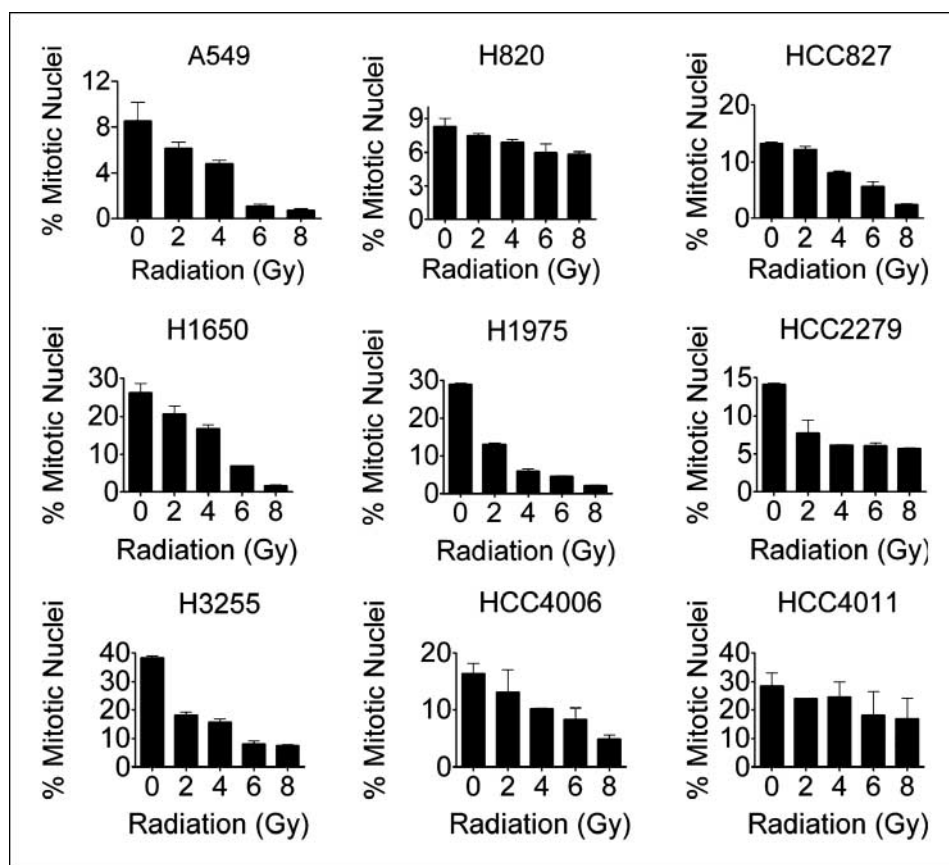
a common response to radiation in most NSCLC cell lines regardless of EGFR status.

**Radiosensitive mutant EGFR NSCLCs exhibit apoptosis or micronuclei in response to IR.** An early outcome of IR is the interphase death by apoptosis observed in some cell types (30, 31). We evaluated the effect of IR on apoptosis in eight mutant EGFR-expressing cell lines at 48 hours after radiation by counting DAPI-stained apoptotic bodies and nuclei (Fig. 5). The WT EGFR-expressing cell line A549 showed relatively low basal and radiation-induced apoptosis. Similar lack of IR-induced apoptosis was observed in five other radioresistant WT EGFR NSCLCs (Supplementary Fig. S1C). By contrast, five mutant NSCLCs, H1975, HCC2279, HCC3255, HCC4006, and HCC4011, exhibited relatively high basal levels of apoptotic bodies (5-18%), which increased to 20% to 35% at 8 Gy in a radiation dose-dependent manner. A second subset of mutant EGFR-expressing NSCLC cell lines that included the radiosensitive cell lines NCI-H820 and HCC827 and the radioresistant H1650 showed relatively very low basal apoptotic fractions (0.5-2%), which increased up to only 5% to 8% at 8 Gy.

One outcome of IR-induced DNA damage is the generation of acentric chromosomes that develop into circular chromatin-bound micronuclei after cells complete one or more cycles of mitotic division (32-34). Three mutant EGFR NSCLCs, H820, H1975, and H2279, showed an IR dose-dependent increase in micronuclei, which were absent in WT EGFR-expressing cell lines



**Figure 3.** Mutant EGFR-expressing NSCLC cell lines do not halt DNA synthesis in response to IR. BrdUrd incorporation at 18 hours following radiation was detected by fluorescence microscopy. BrdUrd-positive nuclei were imaged at  $\times 4$  magnification and calculated as a percentage of a total number of DAPI-stained nuclei, and mean values were plotted as a function of radiation dose. Columns, mean values of  $>8,000$  nuclear events; bars, SD. Representative of two independent experiments that showed similar results.



**Figure 4.** Most NSCLC cell lines regardless of EGFR status exhibit dose-dependent decreases in the mitotic fractions. Mitotic phase-specific histone H3 phosphorylation was detected by fluorescence microscopy, and images were acquired at  $\times 10$  magnification. Phosphorylated histone H3-positive nuclei are represented as percentage of total nuclei stained with DAPI. Columns, mean values of  $>5,000$  nuclear events from 24 fields; bars, SD.

(Supplementary Fig. S2). Of note, H820 did not show marked IR-dependent apoptosis but did exhibit a 40-fold increase in micronuclei after IR.

**Ectopic expression of mutant EGFR subtypes sensitizes HBEC and NSCLC cells to radiation.** We tested the hypothesis that ectopic expression of either the WT or the mutant form of EGFR in an isogenic background would confirm the differences in radiation responses observed in NSCLC lines. The effect of radiation on clonogenic cell survival was examined in three different cell lines, HBEC, A549, and H1299, which were stably transfected with either the WT EGFR construct, the L858R mutant EGFR, or the  $\Delta E746-E750$  deletion mutant. (Fig. 6A-C). Ectopic expression of either L858R or the  $\Delta E746-E750$  deletion mutant significantly reduced clonogenic survival after IR in both HBEC and the originally radioresistant WT EGFR NSCLCs A549 and H1299. In contrast, the expression of WT EGFR had a radioprotective effect in these NSCLC lines. The data indicate that the WT and mutant forms of the EGFR are distinctly different in effecting response to radiation in an isogenic background.

It was of interest to examine whether ectopic expression of the mutant EGFR forms similarly sensitized H1299 cells to gefitinib. Results in Supplementary Fig. S3A and B show that ectopic expression of either the L858R or the deletion mutant EGFR in these cells did not reduce the  $EC_{50}$  for gefitinib and had no effect on cell viability compared with WT EGFR or LacZ vector-transfected controls. The absence of a gefitinib-sensitizing effect of the mutant EGFR forms is in striking contrast to their radiosensitizing effect. The data suggest that gefitinib and radiation may affect cell viability through different mechanisms.

## Discussion

In this study, we provide evidence that eight of nine NSCLC cell lines that harbor mutations in the TKD of EGFR exhibit a predominantly radiosensitive phenotype, which is associated with incomplete DSB repair, failure to halt DNA synthesis or mitosis, and either induction of apoptosis or development of micronuclei in response to IR. By contrast, NSCLC cell lines with WT EGFR were nearly always radioresistant and exhibited efficient DSB repair and IR-dependent inhibition of DNA synthesis and mitosis and did not show apoptosis or micronuclei.

How do activating mutations in EGFR contribute to IR-induced cell death? There are two possible mechanisms by which TKD mutations in the EGFR may contribute to radiosensitivity: (a) amplification of IR-induced DNA damage through abrogation of IR-induced cell cycle checkpoints and (b) interference in the repair of IR-induced DSB.

Our study shows that most mutant EGFR NSCLCs do not halt DNA synthesis or progression into mitosis in response to IR, suggesting that IR-induced checkpoints are defective in these cell lines. It is possible that elevated or aberrant signaling from the mutant EGFR may override IR-induced checkpoint controls in these cell lines. We<sup>8</sup> and others have observed that mutations in the TKD of EGFR are associated with elevated ligand-independent EGFR tyrosine kinase activity (2), aberrant signaling (9, 12), or different substrate specificity compared with WT EGFR (35).

<sup>8</sup> Unpublished data.

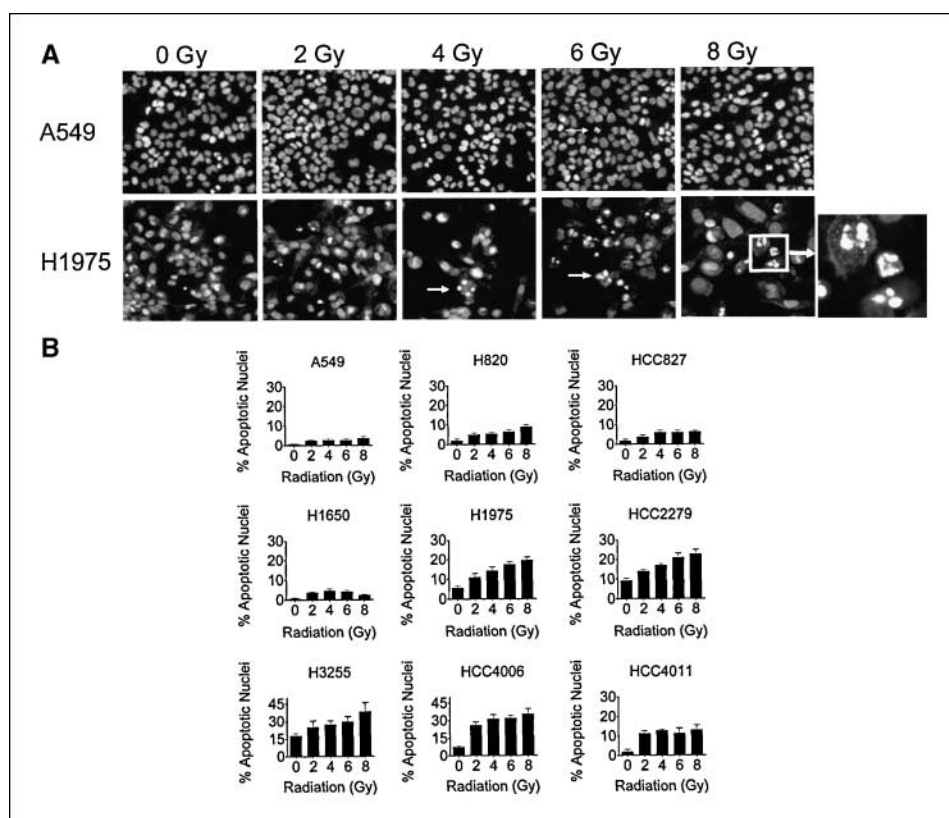
Moreover, in a recent study, Sato et al. (20) have shown that ectopic expression of the L858R or the  $\Delta$ E746-E750 mutant forms of EGFR in HBECs significantly increases phosphorylation at tyrosine residues 854, 992, and 1,068 of the EGFR as well as the activity of protein kinase B/AKT and the signal transducers and activators of transcription STAT3. This pattern of EGFR signaling is known to promote cell cycle progression, cell survival, and cell proliferation. In mutant EGFR-expressing cell lines, constitutive activity and signaling from the EGFR likely promotes progression through IR-induced cell cycle checkpoints and leads to unchecked DNA synthesis, interference in DNA repair, and amplification of DNA damage and results in catastrophic chromosomal aberrations and/or apoptosis. Thus, consequences of EGFR constitutive activity and elevated expression may contribute to decreased clonogenic survival or long-term viability in mutant EGFR-expressing cell lines when exposed to IR.

A second possibility is that mutations in the TKD of the EGFR or aberrant mutant EGFR signaling may alter the function of cellular DNA repair mechanisms. Our study shows that most mutant EGFR NSCLC cell lines exhibit delayed DSB repair kinetics. The relationship between EGFR and DNA repair is not well understood. Recently, Dittmann et al. (36) showed that, at least for WT EGFR, the receptor translocates to the nucleus in response to IR and binds to DNA-dependent protein kinase (DNA-PK), a key enzyme in nonhomologous end-joining repair. The physiologic significance of these interactions is yet to be elucidated. However, membrane sequestration of the receptor through anti-EGFR monoclonal antibody, cetuximab, prevents EGFR-DNA-PK interactions and decreases clonogenic survival (37). Preliminary evidence from our laboratory suggests that EGFR-DNA-PKcs or EGFR-Ku80 interactions are detectable in the radioresistant, WT EGFR-expressing

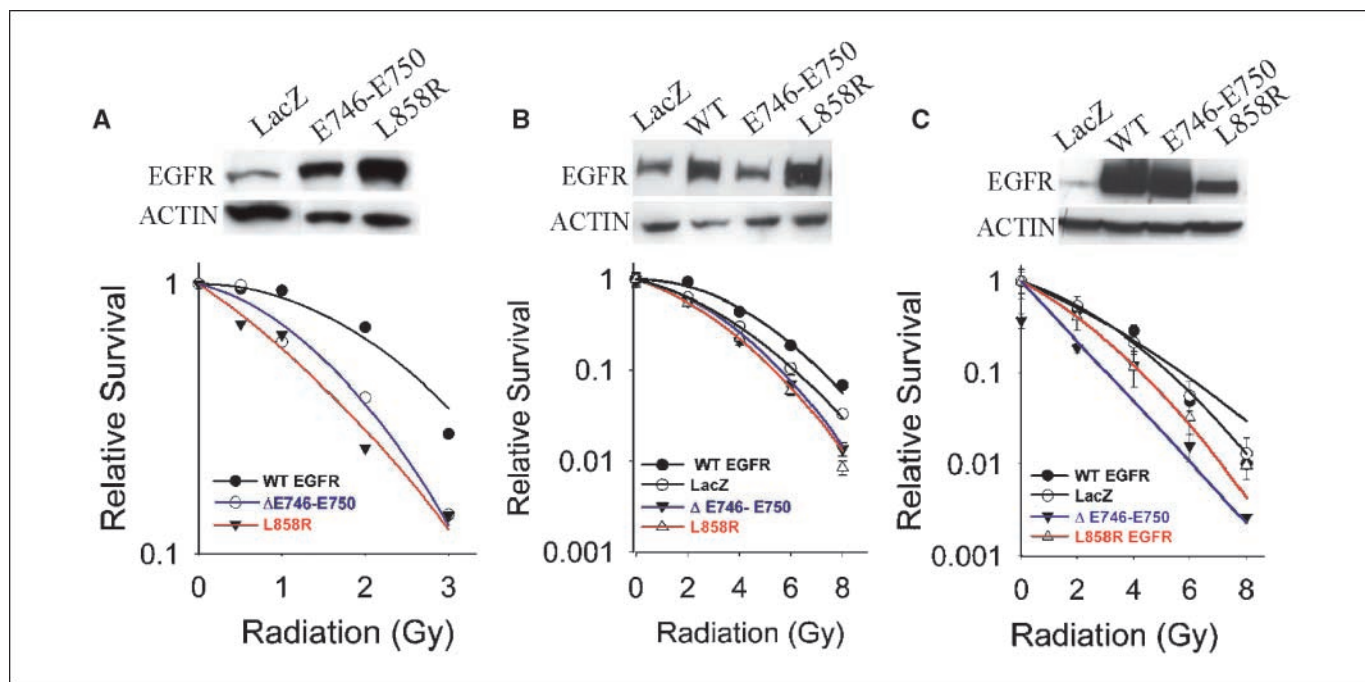
cell line A549 but are absent in at least two mutant EGFR NSCLCs, NCI-H820 and NCI-HCC827. Thus, disruption of EGFR interactions with the DNA repair machinery may account for the delayed kinetics of DSBs in mutant EGFR-expressing NSCLC cell lines.

The radioresistant phenotype of the mutant EGFR-expressing cell line H1650 is intriguing. This cell line, which harbors the  $\Delta$ E746-E750 deletion in the EGFR, was radioresistant and exhibited IR dose-dependent inhibition of DNA synthesis and mitosis and efficient DSB repair and showed no apoptotic or micronuclear fractions. One possible reason could be that expression or activity of the mutant EGFR in this cell line relative to the WT allele may be insufficient to exert a radiosensitizing effect. Alternatively, the radioresistant phenotype of this cell line could be due to factors other than EGFR or lie downstream to the EGFR.

Our studies involving ectopic expression in HBEC, A549, and H1299 cell lines clearly illustrate the radiosensitizing effect of both the L858R as well as the  $\Delta$ E746-E750 mutant forms of EGFR, which contrasts with the radioprotective effect of the WT EGFR. This radiosensitizing potential of the mutant EGFR forms was evident in both the p53 WT (A549) as well as the p53-null (H1299) backgrounds, suggesting that the mutant EGFR-mediated radiosensitization may involve a p53-independent process. Interestingly, ectopic expression of the mutant EGFR did not significantly sensitize H1299 cells to the TKIs gefitinib or erlotinib. Moreover, H1975, which contains the T790M mutant and is resistant to gefitinib, still exhibited enhanced sensitivity to radiation. Thus, despite the differences in sensitivity to gefitinib, mutant EGFR NSCLCs show similar responses to IR. It would be of considerable clinical interest to investigate whether gefitinib alters the radioresponse of mutant EGFR cell lines. Findings from such studies would be relevant for treatment decisions in the clinic and



**Figure 5.** Radiation induces apoptosis in majority of radiosensitive mutant EGFR-expressing NSCLC cell lines. Cell lines were irradiated and stained 48 hours later with DAPI. Images were acquired at  $\times 20$  magnification. *A*, images of nuclei from two representative cell lines, A549 and H1975. *Inset*, arrows, apoptotic nuclei were detected by the presence of high-intensity apoptotic bodies. *B*, percentage apoptotic nuclei were plotted as a function of radiation dose. *Columns*, mean values of  $>5,000$  nuclear events from 60 fields; *bars*, SD.



**Figure 6.** Ectopic expression of L858R or deletion mutant EGFR constructs sensitizes cells to radiation. Effect of stable expression of a LacZ vector (green), WT (black), L858R mutant EGFR (red), or  $\Delta$ E746-E750 deletion mutant EGFR on clonogenic survival was examined in HBECs (A), A549 NSCLC cells (B), or H1299 NSCLC cells (C). Points, mean of two independent experiments each done in triplicate; bars, SD. Inset, levels of stable EGFR expression in various transfectants.

establish the molecular basis of a combined modality treatment involving TKI-based chemotherapy and radiotherapy.

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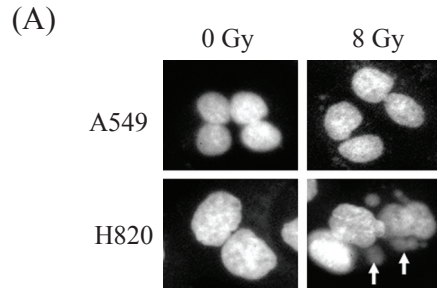
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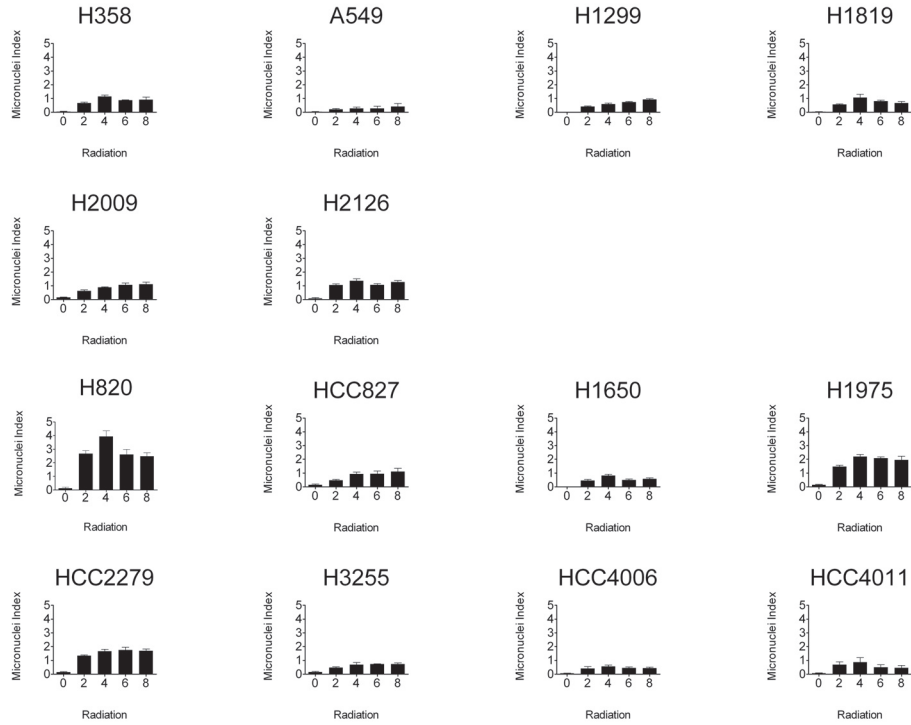
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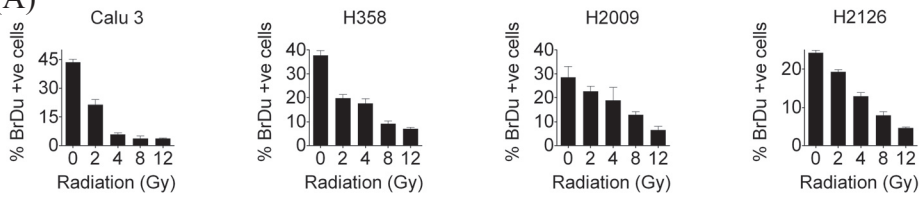
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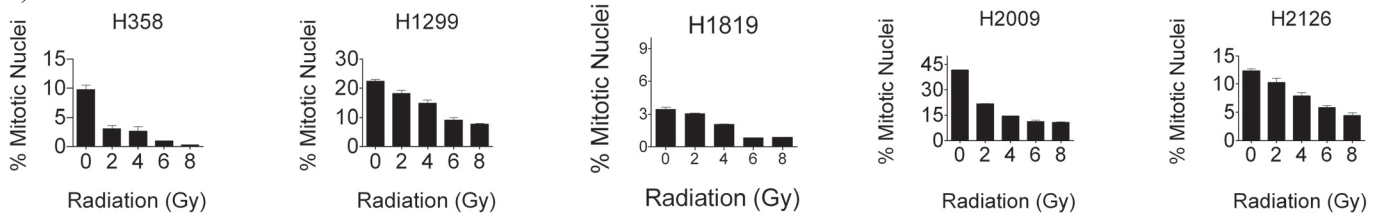
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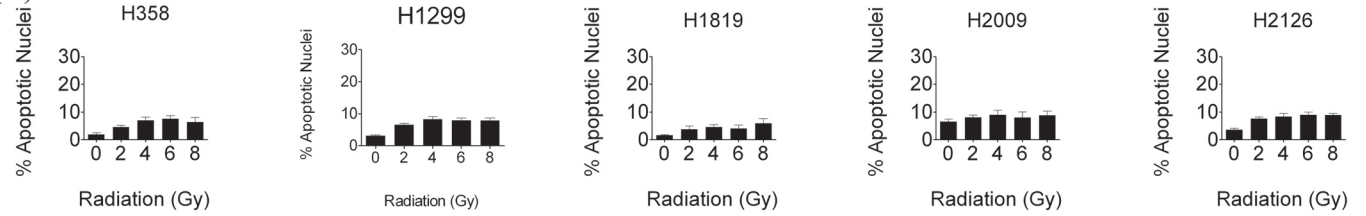
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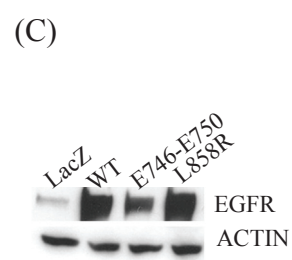
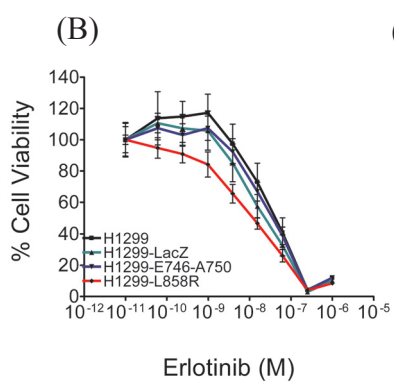
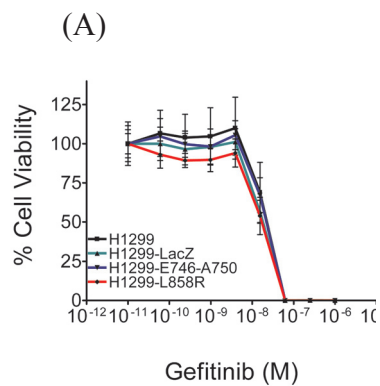


(B)



(C)





## Figure Legends for Supplemental Section

**Supplemental Figure 1:** DNA synthesis, mitosis and apoptosis in wild-type EGFR NSCLCs in response to radiation. (A) BrDU positive nuclei were counted at 18 hours following IR at indicated doses in 4 wild-type EGFR expressing cell lines by immunocytochemistry with anti-BrDU primary and Cy-5 labeled secondary antibody. Mean values and standard deviations were calculated from 15 different fields, in triplicate wells, representing > 8000 nuclear events (B) Mitotic fractions were identified in 5 wild-type NSCLC cell lines in response to indicated doses of IR by immunocytochemistry with anti-phospho histone H3 antibody and Cy5-labeled secondary antibody. Mean and standard deviation values (error bars) in the figure are representative of > 5000 nuclear events from 24 fields in duplicate wells. (C) Apoptotic nuclei were detected in 5 wild-type EGFR expressing cell lines by the presence of high intensity apoptotic bodies. Percent apoptotic nuclei were plotted as a function of radiation dose. Mean values (solid bars) and standard deviations (error bars) are a representative of > 5000 nuclear events from 60 fields in 6 different wells.

**Supplemental Figure 2.** *Radiation induces micronuclei formation in mutant EGFR but not wild-type expressing NSCLC cell lines.* Twenty-four hours following IR, medium was replaced and cells were maintained in cytochlasin B containing medium for 28 hours and then fixed and stained with DAPI. Images were acquired at 20x magnification on the In Cell Analyzer 1000 and micronuclei were scored using Developer Toolbox software on the basis of the following criteria (a) 3-7  $\mu\text{m}$  diameter (b) < 10  $\mu\text{m}$  distance from mitotic binuclei (c) intensity of DAPI fluorescence equal that of mitotic nuclei. Micronuclei (arrows) per 100 cells was plotted as a function of radiation dose.

(A) Images of DAPI stained nuclei from 2 representative cell lines, A549 and H820 are shown. (B) Micronuclei index was calculated as number of micronuclei per 100 nuclei and plotted as a function of radiation dose. Mean values (solid bars) and standard deviations (error bars) are a representative of > 3000 nuclear events from 30 fields in 6 different wells.

**Supplemental Figure 3.** Ectopic expression of mutant EGFR does not sensitize H1299 cells to gefitinib or erlotinib. Cell viability was examined in H1299 NSCLC cells stably expressing a LacZ vector, wild-type EGFR, L858R or  $\Delta$ E746-E750 mutant EGFR in response to a dose range of (A) gefitinib or (B) erlotinib by MTS (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI) assays according the manufacturer's instructions. Inset shows levels of EGFR expression in various transfectants. Cells were plated 24 hrs prior to addition of gefitinib or erlotinib. Absorbance measurements were determined at 490 nm for MTS 96 hrs after addition of drug. Assays were performed in duplicate 96 well plates until a minimum of three plates produced a standard deviation smaller than the mean Assays were performed in duplicate 96 well plates until a minimum of three plates produced a standard deviation (error bars) smaller than the mean. Insets in A-E reveal assessment of EGFR expression by western blot analysis with anti-EGFR antibody.