EGF Receptor Inhibition Radiosensitizes NSCLC Cells By Inducing Senescence In Cells Sustaining DNA Double-Strand Breaks

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EGFR inhibition causes DSB inducible senescence

ABSTRACT

The mechanisms by which inhibition of the epidermal growth factor receptor (EGFR) sensitizes non-small cell lung cancer (NSCLC) cells to ionizing radiation remain poorly understood. We set out to characterize the radiosensitizing effects of the tyrosine kinase inhibitor erlotinib and the monoclonal antibody cetuximab in NSCLC cells that contain wild-type p53. Unexpectedly, EGFR inhibition led to pronounced cellular senescence but not apoptosis of irradiated cells both in-vitro and in-vivo. Senescence was completely dependent on wild-type p53 and associated with a reduction in cell number as well as impaired clonogenic radiation survival. Study of ten additional NSCLC cell lines revealed that senescence is a prominent mechanism of radiosensitization in 45% of cell lines and occurs not only in cells with wild-type p53 but also in cells with mutant p53 where it is associated with an induction of p16. Interestingly, senescence and radiosensitization were linked to an increase in residual radiation-induced DNA double-strand breaks irrespective of p53/p16 status. This effect of EGFR inhibition was at least partially mediated by disruption of the MEK-ERK pathway. Thus, our data indicate a common mechanism of radiosensitization by erlotinib or cetuximab across diverse genetic backgrounds. Our findings also suggest that assays that are able to capture the initial proliferative delay that is associated with senescence should be useful for screening large cell line panels to identify genomic biomarkers of EGFR inhibitor-mediated radiosensitization.
Introduction

It has become clear that molecular-targeted cancer therapies can only reach their full potential through appropriate patient selection. However, the substantial genetic heterogeneity inherent to human cancers makes the identification of patients most likely to benefit from a given anticancer agent challenging (1). Cancer-derived cell lines are increasingly being used to model the genetic heterogeneity encountered in patients. Recent technological advances have facilitated the parallel analysis of large panels of cell lines, in order to test the efficacy of novel agents and discover genomic biomarkers that are predictive of treatment response (2, 3).

There has also been great interest in the combination of targeted agents with radiation therapy to improve cure rates in many disease sites, including non-small cell lung cancer (NSCLC) which is the leading cause of cancer death in the United States (4). The “gold standard” for measurement of the effects of radiation on cells, without and with drug interactions, has long been the clonogenic cell survival assay because this assay is felt to best mimic the desired clinical outcome of decreasing tumor cell clonogenicity (5). However, clonogenic assays are not suitable for the large scale and high-throughput cell line screens that are needed to identify subsets of tumors with sensitivity to radiation/drug combinations. Screening cell line panels for evaluating cytotoxic or cytostatic effects of anticancer drugs is usually based on various short-term cell proliferation, survival, or viability assays (6-8). These assays, which may reflect apoptotic responses or cell growth rate, are generally poor predictors of clonogenic survival after irradiation, and therefore have been regarded as unsuitable for the study of cellular radiosensitivity in epithelial malignancies (5, 9). However, it is likely that situations exist in which a given agent enhances the sensitivity of cells to radiation based on both short-term survival/proliferation and clonogenic survival endpoints. A better understanding of the underlying
EGFR inhibition causes DSB inducible senescence mechanisms will be critical for overcoming barriers to the use of short-term assays in pre-clinical testing and clinical translation of combinations of radiation with targeted agents.

The epidermal growth factor receptor (EGFR) initiates diverse biological responses including enhanced cell proliferation and survival (10, 11). Inhibition of the EGFR by small molecule tyrosine kinase inhibitors (TKI), such as erlotinib, or monoclonal antibodies (mAb), such as cetuximab, has been shown to radiosensitize a limited number of NSCLC cell lines in-vitro and in-vivo (12-14). However, the molecular and cellular mechanisms by which EGFR TKI and mAb may cause radiosensitization across genetically diverse cell lines have remained largely elusive. While a variety of signaling pathways downstream of EGFR has been implicated in radioresistance, including PI3K-AKT, MEK-ERK, and PLC-PKC, no pathway has emerged as a common effector in more than any one cell line (15-17).

Exposure of the cellular DNA to ionizing radiation inflicts various types of damage (18). It is established that the creation of DNA double-strand breaks (DSB) represents the principal damage that, if not adequately repaired, leads to loss of cell clonogenicity via the generation of lethal chromosomal aberrations or the direct induction of apoptosis. While exogenous DSBs can be induced by radiation, endogenous DSBs arise as byproducts of normal intracellular metabolism. Misrepair of or failure to close DSB can cause genomic instability, which may promote carcinogenesis. Two principal DSB repair pathways have been recognized, homologous recombination and non-homologous end-joining (NHEJ) (18, 19). DSB caused by are predominantly repaired by the latter, which operates mainly in G1 but also in the other cell cycle phases.

Cellular senescence is an irreversible cell-cycle arrest, which limits the proliferative capacity of cells exposed to stress signals (20, 21). An inducible form of senescence may act in response to oncogenic signaling as a natural barrier to interrupt carcinogenesis at a premalignant level. How senescence programs can be reactivated in human tumors that have overcome this barrier is currently of great interest (21). With regard to EGFR inhibitors, it has been shown that these agents can inhibit G1-
EGFR inhibition causes DSB inducible senescence S cell cycle transition and have cytostatic effects (13, 22-25). However, whether EGFR signaling can suppress cellular senescence programs has remained unknown.

The tumor suppressor p53 is a transcription factor with pleiotropic cellular functions, including regulation of apoptosis, cell-cycle arrest, and various DNA repair pathways (26). In primary human fibroblasts, p53 mediates radiation-induced cellular senescence and decreases colony forming ability (27-29), but it is unclear under which conditions p53 might be able to mediate similar effects in cancers which generally have suppressed senescence programs.

Here, we demonstrate that EGFR inhibition induces cellular senescence in response to DSB produced by radiation, thereby affecting both short-term cell survival/proliferation and clonogenic survival endpoints. In a panel of NSCLC cell lines with or without wild-type p53, radiosensitization by EGFR inhibitors is dependent on an increase in the levels of non-repairable DSB and disruption of the MEK-ERK pathway, suggesting a common mechanism of radiosensitization across diverse genetic backgrounds.
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Materials and Methods

Cell lines

Cell lines were selected from a previously published panel located in the Center for Molecular Therapeutics (CMT) at Massachusetts General Hospital (6), except for A549 and Calu-6 which were purchased directly from ATCC. Cell lines were obtained during 2009-10 and cultures passaged for < 3 months after thawing a given frozen vial. The identity of each of the cell lines in the panel was tested using a set of 16 short tandem repeats (STR) (AmpFLSTR Identifier KIT, ABI). In addition, single nucleotide polymorphism (SNP) profiles based on a panel of 63 SNPs assayed using the Sequenom Genetic Analyzer was used for in-house identity checking whenever a cell line was propagated and confirmed uniqueness of cell lines for the ones without available STR. No cell line was ever treated for mycoplasma and all lines tested mycoplasma free prior to the experiments (MycoAlert, Lonza).

A549 cells were maintained in DMEM, Calu-6 in α-MEM, ABC1 and EBC1 in DMEM/F12, and LU99B, NCI-H23, NCI-H3122, HCC44, NCI-H460, EPLC-272, and NCI-H1869 in RPMI1640 (all Sigma-Aldrich). All cell lines were maintained in a humidified incubator at 37°C and 5% CO2, with HEPES (Sigma-Aldrich) added to the medium at 10%. A549 clones with varying p53 status were generated by transfection with linearized expression vectors for the p53-273L or -179Q missense mutant (30) or an empty cDNA3/neo vector using standard methods. Stably transfected clones were selected with 500 μg/ml G418 (Mediatech) and maintained at 200 μg/ml G418.

Xenograft experiments

A549 xenografts were grown in nude mice (nu/nu; 7-14 weeks old) under published conditions in the Experimental Centre of the Medical Faculty Carl Gustav Carus, Technology University of Dresden, Germany (31). The animal facilities and the experiments were approved according to...
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institutional guidelines and animal welfare regulations. Erlotinib was given orally at a standard dose of 50 mg/kg on days 0-4 after tumors reached 7 mm diameter. X-ray irradiations (200 kV, 0.5 mm Cu, ~1 Gy/min) were performed on days 1-3 with 2 Gy/day. An additional group of animals was treated with erlotinib alone using the same schedule as described above. Control tumors were either not treated or irradiated according to the schedule above. Tumors were harvested on day 7, snap frozen, and subjected to β-galactosidase staining.

Treatments

Irradiation was performed using a Siemens Stabilipan 2 X-ray generator operated at 250 kVp and 12 mA, at a dose rate of 1.98 Gy/minute. Erlotinib and U0126 (LC Laboratory) were dissolved in DMSO to generate 100 mM stocks, and cetuximab (Bristol-Myers Squipp) was obtained at 2 mg/ml. Drugs were added 1 hour prior to irradiation and maintained for the duration of the respective experiment.

Cell proliferation and survival assays

Clonogenic survival assays were performed as previously published (32). Determination of cell numbers 72 hours after irradiation was performed by manual counting or by using a fluorescent nucleic acid stain Syto60 as described (6, 33).

Senescence staining

Cells were seeded onto 8-well chamber slides and treated after 24 hours. After 7 days, staining for senescence-associated β-galactosidase was performed using a commercial kit (Cell Signaling).

Flow cytometry

Cell cycle distributions were determined using standard ethanol fixation and propidium iodide (Sigma-Aldrich) staining followed by flow cytometry (33). For quantification of γ-H2AX by flow
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cytometry, cells were counterstained with propidium iodide and anti-γ-H2AX antibody (ab18311, Abcam) followed with staining with an Alexa-488-conjugated secondary antibody (A11029, Invitrogen) using a standard approach.

**Immunofluorescence microscopy**

Staining and visualization of γ-H2AX foci was performed as described (34). For visualization of p21 expression, the same protocol was used. Following fixing and permeabilization, cells were incubated with anti-p21 antibody (OP64, Calbiochem) followed by incubation with an Alexa-488-conjugated secondary antibody (A11029, Invitrogen). All slides were counterstained with DAPI and examined by fluorescence microscopy (Olympus BX51).

**Western blotting**

Proteins in whole cell lysates from exponentially growing cell cultures were detected using standard methods. Specific antibodies against phosphorylated Akt, phosphorylated Erk, total Akt, and total Erk (all Cell Signaling, #4058, 9101, 9272, 9102, respectively), trimethylated histone H3 lysine 9 (H3K9me3) (ab8898, Abcam), and horseradish peroxidase–conjugated secondary antibody (Santa Cruz) were used. Protein bands were visualized with enhanced chemiluminescence (Invitrogen) followed by autoradiography.

For additional materials and methods, see Supplementary Figure Legends. For an overview of experimental endpoints, see model in Fig. S11.
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Results

EGFR inhibition sensitizes irradiated NSCLC cells to p53-dependent senescence

We sought to determine how the mechanisms of radiosensitization by erlotinib or cetuximab might affect not only clonogenic cell survival, but also short-term survival/proliferation which is typically measured in cell line screens of molecular targeted drugs (6, 8). We initially employed A549 cells known to exhibit EGFR-dependent radioresistance (35). EGFR phosphorylation induced by irradiation was blocked by pharmacologically achievable concentrations of erlotinib (2 μM) and cetuximab (100 nM) (data not shown). Erlotinib or cetuximab radiosensitized A549 cells to a similar extent in a clonogenic survival assay (Fig. 1A). EGFR inhibition also decreased the number of cells present at 72 hours after irradiation with a clinically relevant dose (2 Gy) compared to radiation alone (Fig. 1A, Fig. S1A,B). While we did not detect apoptosis (Fig. S2), there was a sustained increase in the fraction of cells in the G1 phase of the cell cycle following combined irradiation and EGFR inhibition compared to radiation alone (Fig. S1C).

A549 cells harbor wild-type p53, which triggers G1 cell cycle arrest in response to radiation and is also involved in aspects of the radiation-induced G2/M block (36-38). Given the observed G1 cell cycle arrest, we hypothesized that wild-type p53 may mediate radiosensitization through cellular senescence, which is preceded by cell cycle or proliferative delay (20). Indeed, we observed a prolonged induction of the cdk inhibitor p21 in irradiated cells with wild-type p53 that were treated with erlotinib or cetuximab (Fig. S3A). Consistent with this observation, we noticed downregulation of the E2F1 transcription factor (Fig. S3B). Examination of p53 wild-type cells revealed several features of senescence, including morphological characteristics indicating premature differentiation and expression of senescence-associated β-galactosidase (Fig. 1B) as well as increased levels of trimethylated histone H3K9 (Fig. 1C). Senescence impaired the ability of irradiated cells to continue proliferation and form colonies (Fig. S3C). Importantly, senescence was detectable within 3 days of
EGFR inhibition causes DSB inducible senescence and thus likely contributed to the reduction in cell number seen at that time point (Fig. 1A). Irradiated A549 xenografts exhibited intense β-galactosidase staining in the presence of erlotinib, thus confirming the senescence phenotype in-vivo (Fig. 1D). Of note, erlotinib or cetuximab alone caused neither p21 induction nor senescence in cell culture although some baseline senescence was observed in the xenograft setting (Fig. S3D, and data not shown).

To determine the p53 dependence of the observed senescence phenotype, we stably expressed dominant-negative mutant forms of p53, i.e., p53-273L or -179Q, in A549 cells (Fig. S3E). Upon treatment of p53-mutant cells with erlotinib or cetuximab cells, there was neither an increase in p21 expression (Fig. S3A) nor a reduction in cell numbers at 72 hours post-irradiation (Fig. 2A). Strikingly, the p53-273L mutant completely abrogated radiosensitization in a colony formation assay (Fig. 2A), while the disruptive effect of the p53-179Q mutant was only slightly less pronounced (data not shown). Consistent with these findings, cellular senescence induction was dependent on wild-type p53 function (Fig. 2B).

**Cellular senescence is a dominant mechanism of radiosensitization in NSCLC cell lines**

Our data suggested that EGFR inhibition sensitizes NSCLC cells with wild-type p53 to radiation via senescence, which can be measured not only in a clonogenic assay but also in a 72-hour proliferation/survival assay. We next asked whether this phenomenon can be observed in different genetic backgrounds, particularly in cells with endogenous mutant p53 which potentially have the ability to induce senescence through p53-independent mechanisms (20).

We screened 10 additional NSCLC cell lines using a previously established high-throughput platform which utilizes a fluorescent nucleic acid stain, Syto60, to determine the number of cells present 72 hours after treatment initiation (Fig. S1A,B) (6). Cell lines were ranked by the ability of erlotinib to radiosensitize (Tab. 1, Fig. S4A). The ability of erlotinib alone to impair cell proliferation did not correlate with the rank order of radiosensitization (Fig. S4B). We next confirmed that the
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presence of relative radiosensitization seen in the Syto60-based assay predicted radiosensitization using colony formation (Tab. 1). This was done by calculating dose enhancement factors (DEF) to quantify the degree of radiosensitization based on clonogenic survival differences (Fig. S5). Six cell lines including A549 (55%) were radiosensitized by erlotinib with DEF ranging from 1.15 to 1.46 (mean, 1.28), and strikingly in 5 of these (83%), radiosensitization was associated with cellular senescence (Fig. S6, Tab. 1). Importantly, as expected, there was no correlation between the Syto60 and colony formation assays with regard to the absolute radiosensitivity of individual cell lines (Fig. S4C). Altogether, the data indicate that cellular senescence is a dominant mechanism of radiosensitization associated with EGFR inhibition.

With regard to effector pathways of the erlotinib-induced senescence phenotype, we sought to determine whether the p53 dependence that was observed for A549 cells could be extended to other cell lines. We therefore expressed the dominant-negative p53-273L mutant in LU99B cells and, similar to A549 cells, found an abrogation of erlotinib-mediated radiosensitization and senescence (Fig. S7A-C). In contrast, NCI-H460 cells, which also harbor wild-type p53, could not be radiosensitized by erlotinib, possibly due to another mutation in a downstream pathway, though this was not investigated further. With regard to the mechanism of senescence induction in cell lines with endogenous mutant p53, we observed an induction of p16 expression upon erlotinib and radiation treatment (Fig. S7D), consistent with the known role of this protein in p53-independent senescence (20).

Senescence induction is associated with increased levels of unrepaired DSB

Cellular senescence can be triggered by DSB, and it has been suggested that inhibition of EGFR signaling impairs the removal of radiation-induced DSB (20, 39). We first used A549 cells to assess the levels of non-repaired DSB at 1-7 days post-irradiation by staining for the phosphorylated histone variant γ-H2AX which accumulates in foci at DSB. γ-H2AX foci accumulated in p21-expressing cells suggesting a link to senescence (Fig. S8A). Correspondingly, we detected a 13.4% increase in the
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fraction of cells with non-repaired DSB upon EGFR inhibition which was dependent on wild-type p53 (Fig. 3A). Furthermore, the presence of wild-type p53 was associated with an EGFR inhibitor-mediated increase in γ-H2AX staining intensity primarily in the G1 phase of the cell cycle and to a lesser extent in G2 (Fig. 3B, Fig. S8B).

We also asked whether senescence can be induced by simply increasing the levels of DSB even in the absence of EGFR inhibition. Strikingly, when we disrupted DSB repair with inhibitors of DNA-PKcs or ATM kinases, we observed senescence after 2 Gy that was not seen with irradiation alone (Fig. S8C). A similar result was observed when we merely increased the dose of radiation (Fig. S8D). Our data also demonstrate that neither ATM nor DNA-PKcs kinase is required for execution of p53-mediated DSB inducible senescence in irradiated cells treated with cetuximab or erlotinib (Fig. S9A).

Next, in order to elucidate a common mechanism of radiosensitization in p53 wild-type A549 cells and the other NSCLC cell lines, we investigated the ability of erlotinib to increase radiation-induced γ-H2AX foci levels in a subset of lines. We observed a strong correlation (p=0.01) between erlotinib-mediated radiosensitization and levels of non-repaired DSB at 24 hours (Fig. 3C, Fig. S9B,C).

DSB-mediated radiosensitization is dependent on the MEK-ERK pathway

Because the MEK-ERK pathway promotes NHEJ in A549 cells (15) and erlotinib abrogates ERK phosphorylation (Fig. 4A), we asked whether MEK-ERK may prevent the persistence of lethal DSB and constitute a suppressor pathway of radiation-induced senescence in NSCLC cells. Using A549, ABC1, and HCC44 cells as representative examples we observed that treatment with a MEK inhibitor indeed increased the fraction of cells with residual γ-H2AX foci by 8.2-18.1% (Fig. 4B), which was comparable to the effects of EGFR inhibition in A549 cells (Fig. 3A). MEK inhibition also caused p21 induction (Fig. S10A,B), radiosensitization (Fig. 4C), cellular senescence (Fig. 4D), and did not further enhance the radiosensitizing effects of erlotinib (Fig. S10C), implicating the MEK-ERK
EGFR inhibition causes DSB inducible senescence pathway as one common effector pathway of radioresistance downstream of EGFR. In contrast, disruption of the PI3K–AKT or JAK/STAT pathways did not recapitulate the effects of EGFR or MEK inhibition (Fig. S10D-F).
EGFR inhibition causes DSB inducible senescence

Discussion

We report here that inhibition of the EGFR triggers cellular senescence in response to DSB produced by ionizing radiation in 5 of 11 (45%) NSCLC cell lines (Tab. 1). EGFR inhibition induced senescence following relatively low doses of radiation (2 Gy) that are associated with ~50% clonogenic cell survival. Importantly, EGFR inhibition alone using a dose of 2 μM did not cause senescence or even significantly suppress proliferation in this set of cell lines known to be resistant to erlotinib in monotherapy (6).

The EGFR is expressed in 65-90% of NSCLC (40). In approximately 10% of patients, EGFR acts as a cancer-driving oncogene due to activating mutations in its tyrosine kinase domain. However, its cancer promoting functions in the remaining cases are poorly understood. Our data lend support to the hypothesis that EGFR may suppress cellular senescence programs responding to low levels of endogenous DSB that cause or are associated with genomic instability, thereby promoting tumor progression. Our and other data imply that genetic events such as loss of p53 or p16 function that suffice to overcome an oncogene-induced senescence barrier as a prerequisite of tumor formation may not necessarily co-disrupt the availability of therapy-inducible senescence (21, 41, 42).

What are the mechanisms by which EGFR suppresses DSB inducible senescence? An analysis of radiobiological parameters determining clonogenic survival revealed an increase in the α/β ratio as described by the linear-quadratic formula (43) in 4 of the 5 cell lines undergoing senescence, i.e., A549, H3122, ABC1, HCC44 (Fig. S5B). These data suggest a reduction in the contribution of so-called sublethal damage to the observed loss of clonogenicity, i.e., reduced β component indicating repairable DSB (44), and a concomitant increase in lethal lesions, i.e., increased α component consistent with non-repairable DSB. We postulate that prolonged cell-cycle arrest and subsequent senescence are a logical cellular response to the presence of non-repairable DSB. In support of this mechanism, EGFR...
EGFR inhibition causes DSB inducible senescence inhibition increased the levels of residual γ-H2AX foci after irradiation in several cell lines (Fig. 3C, Fig. S9B,C). Altogether, these data suggest that EGFR commonly promotes the removal of repairable DSB from the genome.

Recently, investigators reported that EGFR can translocate into the nucleus upon irradiation where it may promote NHEJ through an interaction with DNA-PKcs (35, 45, 46). Other data indicate that MEK-ERK signaling may stimulate NHEJ in NSCLC (A549) and glioma cells (15, 47). Our data provide evidence for a common role of MEK-ERK in modulating the levels of radiation-induced DSB in NSCLC cells (Fig. 4B-D) (model Fig. S11A). A possible mechanism is suggested by the interesting observation that ERK signaling can activate PARP-1 which has a role in NHEJ (48, 49). However, we believe it is unlikely that EGFR-MEK-ERK suppresses DSB inducible senescence through only a single mechanism, i.e., by reducing the number of persistent DSB.

A prerequisite for p53-mediated senescence is the arrest of cells in the G1 phase following the induction of DSB (29). Interestingly, ERK has been shown to promote G1/S transition through multiple mechanisms, and nuclear translocation is required for S-phase entry (50). Thus, loss of ERK signaling may cooperate with p53 to halt cells in G1. However, ERK has also been shown to contribute to p53 activation through serine 15 phosphorylation, at least after UV irradiation (51). Thus, the functional interaction of ERK signaling with p53, or with the p16 pathway in the absence of p53 (29), in the regulation of senescence is likely complex. The genes encoding p53 and p16 are among the most commonly mutated tumor suppressors in human cancers. Our data suggest that in cancers that have mutated either of these genes, the presence of the other unaltered gene product can be therapeutically exploited for DSB inducible senescence. For example, p16-mutant A549 cells undergo p53-mediated DSB inducible senescence while p16-mediated senescence may be activated in p53-mutant ABC1 cells (Tab. 1, Fig. S11A).

Other genomic determinants of radiosensitization are likely to exist but are not readily apparent from the cell line profile data available (Tab. 1 and data not shown). Much larger cell line panels are
EGFR inhibition causes DSB inducible senescence needed to establish genotypes that correlate with radiosensitization. With regard to the importance of histological cancer subtype, the three squamous cell cancer cell lines in our panel could not be radiosensitized by erlotinib (Tab. 1) or cetuximab (Wang et al., unpublished data). However, there is at least one published lung squamous cell cancer cell line that can be radiosensitized by erlotinib (13). Thus, it remains to be determined whether any histological subtype exists, such as adenocarcinoma versus squamous cell carcinoma, that can be preferentially radiosensitized.

Lastly, our data suggest that the use of short-term cell survival/proliferation as a readout for the efficacy of low dose radiation and EGFR inhibitors accurately predicts radiosensitization in a colony formation assay because the senescence response underlying radiosensitization is associated with a cell proliferative delay that is captured with the short-term assay (Fig. S11B). In other words, even though short-term survival/proliferation assays may not provide a surrogate for “absolute” radiosensitivity (as illustrated in Fig. S4C), they can provide a measure of “relative” radiosensitization in a given cell line when radiation is combined with a potentially radiosensitizing and senescence-inducing drug, at least in the case of EGFR or MEK/ERK inhibitors. Thus, assays able to capture the proliferative delay that is associated with senescence should be useful for screening large cell line panels in order to identify genomic biomarkers of EGFR inhibitor-mediated radiosensitization. Future studies should also be directed at determining the contribution of different cellular responses to radiation/drug combinations by using multiplex assessments of apoptosis, autophagy, and necrosis, in addition to senescence (52), all of which may contribute to the cytotoxic effects of radiation plus a targeted agent in patients.
Acknowledgements

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References


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43. Das, A. K., Chen, B. P., Story, M. D., Sato, M., Minna, J. D., Chen, D. J., and Nirodi, C. S. Somatic mutations in the tyrosine kinase domain of epidermal growth factor receptor (EGFR)
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Figure Legends

**Figure 1.** Radiosensitization of A549 NSCLC cells. A, Left panel shows clonogenic survival after exposure to ionizing radiation (IR) with or without erlotinib (2 μM) or cetuximab (100 nM) treatment. Data points represent means +/- standard error based on three independent repeats. Survival curves were fitted using the linear-quadratic formula and statistical comparisons were carried out by use of the F test (two-sided). Right panel shows impact of erlotinib or cetuximab on cell numbers 72 hours (h) after irradiation (2 Gy). Effects of the combination treatments were corrected for the effects of erlotinib or cetuximab alone (see also Fig. S1B). Bars represent means with standard error based on 3 independent repeats. Statistical comparisons were performed with the student’s T-test (two-sided). B, illustration of morphological changes consistent with cellular senescence following irradiation and erlotinib. C, for further confirmation of senescence, A549 cells with wild-type p53 were treated with 2 Gy or 8 Gy ionizing radiation (IR) with or without EGFR inhibitor (EGFRi) erlotinib or cetuximab. Seven days post-irradiation, whole cell lysates were subjected to Western blotting with a specific antibody against H3K9me3 or H3. Lower panel shows senescence-associated β-galactosidase (SA-β-gal) staining at 3 or 7 days post-irradiation in erlotinib-treated A549 cells. D, for confirmation of the senescence phenotype in-vivo, A549 was grown as a xenograft in nude mice. Representative images are from tumors harvested on day 7 following daily irradiation with 2 Gy on days 1-3 with or without erlotinib on days 0-4. Similar findings were obtained using daily irradiation with 2 Gy for 6 days (data not shown).

**Figure 2.** Role of p53 in radiosensitization and senescence of A549 cells. A, Upper panel, effect of stable expression of dominant-negative p53 mutants on short-term cell survival/proliferation, analogous to Fig. 1A. Cells were treated with 2 Gy ionizing radiation (IR), with or without cetuximab (100 nM)
EGFR inhibition causes DSB inducible senescence (C). Lower panel, effect of p53 mutants on clonogenic survival, analogous to Fig. 1A. D, staining for senescence-associated β-galactosidase 7 days following irradiation with or without erlotinib or cetuximab.

Figure 3. Effect of EGFR inhibition on residual DNA double-strand breaks (DSB) as measured by γ-H2AX staining in NSCLC cells. A, fraction of nuclei with ≥ 20 γ-H2AX foci at 24 hours after irradiation (8 Gy IR) of p53 wild-type or mutant (273L) A549 cells with or without cetuximab treatment. Data are based on two independent repeat experiments. C, fraction of A549 cells with high levels of γ-H2AX staining distributed over the G1 and G2/M cell cycles phases based on FACS analysis at 24 hours post-irradiation. See also Fig. S8B. Data are based on two independent repeat experiments. C, relative increase in the levels of residual γ-H2AX foci 24 hours after irradiation caused by EGFR inhibition (Fig. S9B) is plotted against relative radiosensitization based on short-term cell survival/proliferation (Fig. 4A) in a panel of NSCLC cell lines. Data are fitted by a linear regression line, and 95% confidence limits are depicted as dotted lines. *data point taken from A549 cells expressing mutant (mut) p53 treated with cetuximab (Fig. 2A).

Figure 4. Mechanism of radiosensitization of NSCLC cell lines. B, whole cell lysates of A549 cells with wild-type p53 treated with 2 Gy ionizing radiation (IR) with or without EGFR inhibitor (i), i.e., erlotinib (E) or cetuximab (C), were subjected to Western blotting with antibodies against kinases as shown. B, effect of the MEK inhibitor U0126 (10 μM) on the fraction of cells with ≥ 20 γ-H2AX foci 24 hours after irradiation. Data are based on two independent repeat experiments. C, effect of MEK inhibitor on short-term cell survival/proliferation after 2 Gy irradiation analogous to Fig. S4A. D, staining for senescence-associated β-galactosidase 7 days following irradiation of cells with or without MEK inhibitor. U0126 alone does not cause senescence in these cell lines (data not shown).
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Table 1. Radiosensitization of 11 NSCLC Cell lines by Erlotinib.

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*aRank based on relative radiosensitization seen in syto60 assay.

*bRelative radiosensitization derived from the ratio of cell density seen with 2 Gy radiation + erlotinib versus radiation alone as measured with the syto60 assay at 72 hours (Fig. S1A, S4A).

*cDose enhancement factors (DEF) were calculated as the ratio of the dose of radiation that achieved 10% survival to the dose of radiation required to achieve the same degree of survival when combined with erlotinib in a colony formation assay (CFA) (Fig. S5A).

*dSenescence determined by presence or absence of SA-β-galactosidase staining after 2 Gy radiation + erlotinib (Fig. S6).

*eGenomic information obtained from the Center for Molecular Therapeutics at Mass General Hospital (Cyril Benes, Jeff Settleman, unpublished), the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk) and The TP53 Website (http://p53.free.fr).

Abbreviations: adenoca, adenocarcinoma; NSCLC, non-small cell lung cancer not otherwise specified; squamous, squamous cell carcinoma; wt, wild-type; mut, mutant; cni, copy number increase <8; N/A, not available.
EGF receptor inhibition radiosensitizes NSCLC cells by inducing senescence in cells sustaining DNA double-strand breaks

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