Biology Contribution

Short-Course Treatment With Gefitinib Enhances Curative Potential of Radiation Therapy in a Mouse Model of Human Non-Small Cell Lung Cancer

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Summary

We show that an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (gefitinib) increases clonogenic cell killing by radiation in non-small cell lung cancer (NSCLC) cell lines that are highly sensitive to gefitinib alone. Furthermore, in a mouse xenograft model of human EGFR mutation-positive NSCLC, a short course of gefitinib treatment markedly enhanced the antitumor effects of a single dose of radiation, producing effective cures in a model of NSCLC, suggesting that this combination may be of clinical interest for stereotactic radiation.

Purpose: To evaluate the combination of radiation and an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) in preclinical models of human non-small cell lung cancer.

Methods and Materials: Sensitivity to an EGFR TKI (gefitinib) or radiation was assessed using proliferation assays and clonogenic survival assays. Effects on receptor signal transduction pathways (pEGFR, pAKT, pMAPK) and apoptosis (percentage of cleaved PARP Poly (ADP-ribose) polymerase (PARP)) were assessed by Western blotting. Radiation-induced DNA damage was assessed by γH2AX immunofluorescence. Established (≥100 mm³) EGFR-mutated (HCC287) or EGFR wild-type (A549) subcutaneous xenografts were treated with radiation (10 Gy, day 1) or gefitinib (50 mg/kg, orally, on days 1-3) or both.

Results: In non-small cell lung cancer (NSCLC) cell lines with activating EGFR mutations (PC9 or HCC827), gefitinib treatment markedly reduced pEGFR, pAKT, and pMAPK levels and was associated with an increase in cleaved PARP but not in γH2AX foci. Radiation treatment increased the mean number of γH2AX foci per cell but did not significantly affect EGFR signaling. In contrast, NSCLC cell lines with EGFR T790M (H1975) or wild-type EGFR (A549) were insensitive to gefitinib treatment. The combination of gefitinib and radiation treatment in cell culture produced additive cell killing with no evidence of synergy. In xenograft models, a short course of gefitinib (3 days) did not significantly increase the activity of radiation treatment in wild-type EGFR (A549) tumors (P = .27), whereas this combination markedly increased the activity of radiation (P < .001) or gefitinib alone (P = .002) in EGFR-mutated HCC827 tumors, producing sustained tumor regressions.

Conclusions: Gefitinib treatment increases clonogenic cell killing by radiation but only in cell lines sensitive to gefitinib alone. Our data suggest additive rather than synergistic interactions between gefitinib and radiation and that a combination of short-course gefitinib and high-dose/fraction radiation may have the greatest potential against the subsets of lung cancers harboring activating mutations in the EGFR gene. © 2014 Elsevier Inc.
Introduction

Lung cancer is the leading form of cancer in terms of both incidence and cancer-related deaths, accounting for 1.3 million deaths per year (1). Although smoking is widely acknowledged to be the main cause of lung cancer, a significant proportion of cases occur in non- or never-smoking patients, of whom approximately half have activating mutations in the epidermal growth factor receptor (EGFR) gene (2). These oncopgenic mutations in EGFR are associated with a better overall prognosis in advanced disease (2), and, furthermore, EGFR mutation-positive tumors are highly responsive to EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib (3-5).

Surgical resection is standard therapy for early stage non-small cell lung tuberculosis (NSCLC), although high-dose/fraction stereotactic ablative body radiation therapy (SABR) is considered a therapeutic option for patients with inoperable peripheral early stage NSCLC. SABR can achieve excellent clinical outcomes with local control rates of >80%, although for a significant proportion of patients, further improvements in reducing local, regional, and distant recurrences are required (6-8). In addition, there is growing interest in the use of SABR to treat oligometastatic disease and for use in NSCLC patients with synchronous brain metastasis (9), although further studies are still required to identify which patient subgroups are likely to gain the most benefit from these approaches. Of interest, a recent phase 2 study suggested that EGFR mutation-positive NSCLC patients may be more prone to developing brain metastases (10).

Interactions between EGFR signaling and radiation have been described that strongly suggested that radiation can directly activate EGFR signaling to promote cell survival and activate DNA double-strand break (DSB) repair (11-13). However, to date, little attention has been given to preclinical evaluation of potentially beneficial interactions between radiation and EGFR TKIs in NSCLC with activating mutations in the EGFR gene.

In this study we evaluated the combination of high-dose/fraction radiation and short-course exposure to an EGFR TKI (gefitinib) in human NSCLC cell lines with mutated and/or activated, or wild-type EGFR to determine whether there are positive interactions between the 2 treatment modalities that might have potential clinical utility.

Methods and Materials

Cell culture

Cell lines HCC827, A549, and NCI-H1975 were acquired from American Type Culture Collection. Cell line PC9 was kindly provided by Dr. Kazuto Nishio, Kinki University, Osaka, Japan. Cell lines were routinely maintained in advanced Dulbecco modified Eagle medium plus F-12 medium (Invitrogen) supplemented with 5% fetal bovine serum (Invitrogen), GlutaMAX (Invitrogen), and penicillin-streptomycin (Sigma-Aldrich). Cells were cultured at 37°C in a humidified environment containing 7.5% CO₂.

Cell proliferation

Cells (10⁷/well) were seeded into 96-well plates (Corning) and treated with gefitinib (0-3 µM; Selleck) or radiation (0-6 Gy; Gamma Services GSR DI, with a cesium-137 source at a dose rate of 1.69 Gy/min). Following incubation (5 days), resazurin (12.5 µg/mL; Sigma-Aldrich) was added to each well for 4 hours, and fluorescence intensity was determined (544-nm excitation, 590- to 10-nm emission; POLARstar; BMG Labtech, Ortenberg, Germany) (14). Half-maximal inhibitory concentration (IC₅₀) values were determined using CalcuSyn (Biosoft, Cambridge, UK).

Clonogenic survival

Sensitivity to gefitinib (0-3.0 µM) or radiation therapy (0-6 Gy) was assessed using clonogenic survival assays. Controls were treated with dimethyl sulfoxide (0.1%) or were mock irradiated. For combination studies, the cells were seeded into 6-well plates (Corning) and left to adhere before being exposed to a single dose of radiation (0-10 Gy) and treatment with gefitinib (0, 0.1, or 1.0 µM) for 24 hours. Following treatment, cells were washed twice with phosphate-buffered saline (PBS) before being replenished with fresh drug-free medium and incubated for approximately 14 days to allow colonies to form. Colonies were fixed, stained (0.5% [w/v] crystal violet in 5% acetic acid, 20% H₂O, 75% methanol), and (≥50 cells) counted. Replicate dishes usually containing 50 to 500 colonies per well were manually counted for each treatment. Experiments were carried out in triplicate. Clonogenic survival data were fitted to a linear-quadratic model to calculate the dose at which 37%, 50%, or 90%, cell killing was achieved (D37, D50, D90) (OriginPro 8.5.1, OriginLab Corp, MA, USA) (15).

Electrophoresis and Western blot analysis

Cells were irradiated (0 or 3 Gy) before treatment with gefitinib (0, 0.1, or 1.0 µM) for 0, 2, 4, or 24 hours. Cells were then lysed on ice in lysis buffer (50 mM Tris-HCl, pH 7.6, 137 mM NaCl, 10% glycerol, 0.1% octylphenoxypolyethoxyethanol [Igepal], 0.1% sodium dodecyl sulfate, 50 mM NaF; protease inhibitor [Roche]), and the proteins were separated on a 10% precast TGX polyacrylamide gel (Biorad) and transferred to nitrocellulose membranes (Biorad). After blocking (LI-COR blocking buffer), membranes were incubated overnight with primary antibodies to β-actin (1:5000 dilution; code A1978; Sigma-Aldrich), EGFR (1:1000 dilution; code 2232), pEGFR (1:2000 dilution; code 44788G; Invitrogen), AKT (1:2000 dilution; code 9272), pAKT (1:1000 dilution; code 2232), pEGFR (1:2000 dilution; code; 44788G; Invitrogen), AKT (1:2000 dilution; code 9272), pAKT (1:1000 dilution; code 2232), ERK1/2 (1:1000 dilution; code 4696), pERK1/2 (1:1000 dilution; code 4376), and Poly (ADP-ribose) polymerase (PARP) (1:2000 dilution; code 9542). After being washed, membranes were incubated for 1 hour with goat anti-mouse (1:25,000 dilution; code 926-68,070) and goat anti-rabbit (1:5000 dilution; code A1978) secondary antibodies (LI-COR Biosciences), and the resulting protein-antibody complexes were detected using an Odyssey infrared imager (LI-COR Biosciences). All antibodies used were purchased from Cell Signaling Technology, unless otherwise stated.

γH2AX foci

Cells (10⁷/well) were seeded into 96-well plates and incubated overnight before being irradiated (0, 10 Gy) and/or treated with gefitinib (0-1.0 µM) for 24 hours. Cells were washed in PBS and then fixed (4% paraformaldehyde, 10 minutes at room temperature)
and permeabilized (0.25% Triton X-100 in PBS for 10 minutes at room temperature). After being washed twice in PBS, cells were blocked in 3% bovine serum albumin in PBS for at least 30 minutes and then treated overnight with an anti-phospho-histone H2AX (Ser139) primary antibody (1:1500 dilution; clone JBW301 [code 05-636]; Millipore). Following PBS washing (3×), wells were incubated with Alexa 488-conjugated rabbit anti-mouse secondary antibody (1 hour at room temperature; code; A11011; Invitrogen) in the dark and then with DNA stain (1 mg/mL 4′,6-diamidino-2-phenylindole for 5 minutes). Following PBS washing (3×), DNA γH2AX foci were counted (IN cell analyzer 1000; GE Healthcare).

Response of NSCLC xenografts to gefitinib and radiation

Animal procedures were carried out after local ethical committee review under a project license issued by the UK Home Office under the UK Animals (Scientific Procedures) Act of 1986 and performed according to national guidelines (16). HCC827 (0.1 mL, 5.0 × 10⁶) or A549 (0.1 mL, 2.5 × 10⁶) cells in 50% gelatinous protein mixture (Matrigel) were subcutaneously injected in a single site on the backs of anesthetized BALB/c female nude mice (CAnN.Cg-Foxn1nu/Crl; 6-8 weeks old; n = 64). Mice were weighed, and their tumors were measured 3 times per week with calipers (tumor volume = [W × H × L]/2). When tumors reached an average size of 100 mm³, the mice were randomized before the mice were irradiated. Control animals received 1% polysorbate 20, days 1-3), or radiation (10 Gy, day 1, 1.82 Gy/7-8 animals per group) to receive gefitinib (50 mg/kg orally in 0.1 mL, 5.0 × 10⁶) or A549 (0.1 mL, 2.5 × 10⁶) cells. Moreover, in cell proliferation assays, radiation treatment (0-6 Gy) had no additional effect on cell proliferation in the presence of increasing doses of gefitinib (0-3 μM) (see Supplementary Fig. 1).

Statistics

Statistical analyses were carried out using SPSS software (IBM). Curves were fitted using OriginPro, version 8.5.1, software. Univariate analysis of variance was carried out in order to determine whether there was any significant effect of treatments or interactions between the treatments in cell proliferation assays. Clonogenic survival data were fitted to a linear-quadratic equation, where lnS = 2D + βD². S = surviving fraction, D = dose, alpha and beta are constants.

Results

Effects of gefitinib on NSCLC cells

Gefitinib potently inhibited the growth of NSCLC cell lines with activating EGFR mutations (mean IC₅₀ ± SEM was 70 ± 0.8 nM for PC9 and 30 ± 0.08 nM for HCC827), whereas NSCLC cell lines with wild-type EGFR (A549) or the EGFR T790M “gate-keeper” mutation (H1975) were insensitive to gefitinib, with IC₅₀ values of >3 μM (Fig. 1A, and see Supplementary Table 1). PC9 and HCC827 cells were also highly sensitive to gefitinib in clonogenic survival assays, with 50% cell killing at drug concentrations of 52 ± 7 nM for PC9 and 14 ± 4 nM for HCC827, respectively, whereas A549 and H1975 cells did not reach 50% cell killing even at the highest concentration of gefitinib tested (3 μM) (Fig. 1B).

Effect of radiation on NSCLC cells

EGFR has been reported to play a role in the DNA damage response to radiation (13,17). In clonogenic survival assays, PC9 and HCC827 cells were most resistant to the effects of radiation, with mean ± SEM surviving fraction values at 4 Gy (SF4) of 0.58 ± 0.015 μM and 0.48 ± 0.07 μM, respectively. In contrast, A549 and H1975 cells were more radiation sensitive, with SF4 values of 0.23 ± 0.018 μM and 0.28 ± 0.005 μM, respectively (Fig. 2, Table 1). These clonogenic survival assays were carried out in triplicate using 3 different methods: treatment before seeding or in suspension (data not shown) or treatment at 6 hours after seeding (as shown). Regardless of the method used, the results were similar.

Effect of combined gefitinib and radiation treatment on cell survival and proliferation

For clonogenic assays, based on the gefitinib survival and proliferation curves (Fig. 1), we selected a dose of 0.1 μM of gefitinib for combination studies with radiation with the gefitinib-sensitive PC9 and HCC827 cells, and a 10-fold higher dose (1 μM) for the gefitinib-insensitive A549 and H1975 cells. Although gefitinib-treatment significantly reduced clonogenic survival in PC9 and HCC827 cells (P<.01), there was no significant interaction between radiation and gefitinib treatment (P=.5), suggesting each treatment acts independently (Fig. 2, Table 1). There was no significant treatment effect of gefitinib in A549 (P =.086) or H1975 (P=.198) cells. Moreover, in cell proliferation assays, radiation treatment (0-6 Gy) had no additional effect on cell proliferation in the presence of increasing doses of gefitinib (0-3 μM) (see Supplementary Fig. 1).

Effect of gefitinib and radiation on downstream EGFR signaling

In HCC827 cells, gefitinib treatment markedly reduced pEGFR, pAKT, and pMAPK levels compared with those in controls, whereas radiation treatment increased pAKT levels, most prominently after 24 hours (Fig. 3A). Combined treatment also led to reduced pEGFR, pAKT, and pMAPK levels, although the reductions in pEGFR and pAKT appeared delayed compared with those with gefitinib treatment alone. In H1975 cells, gefitinib treatment did not affect pEGFR levels but did lead to reduced levels of pAKT after 24 hours (Fig. 3B). Radiation treatment did not affect EGFR, AKT, or MAPK levels in H1975 cells (Fig. 3B).
cleavage in HCC827 but not in H1975 cells, whereas radiation treatment had no effect on cleaved PARP levels in either cell line, either alone or in combination with gefitinib (Fig. 3C).

**Effect of gefitinib on radiation-induced residual DNA damage**

The effects of gefitinib on DNA repair were assessed by measuring residual DNA DSBs remaining 24 hours after radiation treatment by using a γH2AX immunofluorescence assay (Fig. 4) (19). As anticipated, radiation treatment markedly increased the proportion of cells with residual DNA damage in each of the cell lines tested (P < .01). Gefitinib treatment alone led to an increase in residual γH2AX foci in the HCC827 cells, which was statistically significant at the highest doses tested (P < .01, at ≥0.1 μM), possibly due to drug-induced apoptosis (Fig. 3C) (20). For 3 of the cell lines (PC9, A549, and H1975), there was no effect of gefitinib on residual DNA DSBs following radiation (Fig. 4). However, for HCC827 cells, the combination treatment significantly increased residual DNA DSBs compared with radiation alone (P < .01 for all gefitinib concentrations).

**Effect of combining gefitinib and radiation on NSCLC xenografts**

Compared with the vehicle-treated controls, tumor growth inhibition (TGI) with radiation (10 Gy) alone was 61.1% (P < .001) and 55.6% (P < .001) in HCC827 and A549 xenografts, respectively.
respectively (Fig. 5A and B). Gefitinib treatment was modestly effective in A549 (TGI, 34.8%, $P < .01$) but highly effective in HCC827, inducing marked tumor regression within 2 days of starting treatment (TGI, 91.1%, $P < .001$), although the tumors began to regrow by day 11 (Fig. 5A and B). In A549 xenografts, combination therapy was more effective than gefitinib ($P = .02$) or radiation alone, although in the latter case, this did not reach statistical significance ($P = .27$). In contrast, in HCC827 cells, combination therapy was markedly more effective than either radiation ($P < .001$) or gefitinib ($P = .002$) alone, and tumor regression was sustained for the duration of the experiment (42 days after treatment), with no evidence of regrowth (Supplementary Figs. 2 and 3).

**Discussion**

NSCLC patients with EGFR mutations can gain clinical benefit from EGFR TKIs, but resistance almost invariably emerges (21). EGFR signaling has been shown to play an important role in the radiation response of tumor cells (13); therefore, combining gefitinib with radiation in EGFR-mutated NSCLC patients seems an attractive treatment approach.

In contrast to a previous study (22), we did not show that NSCLC cell lines with activating EGFR mutations were more sensitive to radiation than NSCLC cells with wild-type EGFR. After radiation treatment, we saw no clear associations between residual DNA DSBs (as assessed by γH2AX foci) or induction of apoptosis (as measured by cleaved PARP) and

**Table 1** Radiation sensitivity of NSCLC cell lines in the presence or absence of gefitinib

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>$\alpha^a$</th>
<th>SF4 (SF)</th>
<th>$\beta$ (SF)</th>
<th>SER (Gy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC9</td>
<td>DMSO (0.1%)</td>
<td>0.052</td>
<td>0.069</td>
<td>0.58</td>
<td>-</td>
</tr>
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<td></td>
<td>Gefitinib (0.1 μM)</td>
<td>0.021</td>
<td>0.026</td>
<td>0.5</td>
<td>1.13</td>
</tr>
<tr>
<td>HCC827</td>
<td>DMSO (0.1%)</td>
<td>0.113</td>
<td>0.018</td>
<td>0.48</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Gefitinib (0.1 μM)</td>
<td>0.243</td>
<td>0.007</td>
<td>0.34</td>
<td>1.12</td>
</tr>
<tr>
<td>A549</td>
<td>DMSO (0.1%)</td>
<td>0.341</td>
<td>0.258</td>
<td>0.23</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Gefitinib (1 μM)</td>
<td>0.005</td>
<td>0.017</td>
<td>0.28</td>
<td>0.98</td>
</tr>
<tr>
<td>H1975</td>
<td>DMSO (0.1%)</td>
<td>0.016</td>
<td>0.076</td>
<td>0.28</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Gefitinib (1 μM)</td>
<td>0.160</td>
<td>0.051</td>
<td>0.23</td>
<td>1.01</td>
</tr>
</tbody>
</table>

*Abbreviations: DMSO = dimethyl sulfoxide; NSCLC = non-small-cell lung cancer.

Fig. 3. Effect of gefitinib and radiation treatment is shown on EGFR downstream signaling and apoptosis. HCC827 (A) and H1975 (B) cells were irradiated (3 Gy) and then treated with 0.1 μM gefitinib for 2, 4, or 24 hours. (C) HCC827 and H1975 cells were irradiated (3 Gy) before being treated with 0.1 μM gefitinib for 24 hours. Blots are representative of 3 independent repeats.
EGFR mutation status in NSCLC cells. Importantly, in xenograft studies, both wild-type and EGFR-mutated NSCLC tumors showed very similar responses to radiation treatment alone.

As expected, NSCLC cells with wild-type EGFR were insensitive to gefitinib in vitro, whereas cells with EGFR mutations were highly sensitive, unless a T790M “gatekeeper” mutation was also present (23). Our clonogenic survival assays suggested that gefitinib and radiation treatments were additive and independent factors in determining cell survival. This was supported by our in vitro studies in which we saw no evidence for significant effects of radiation treatment on EGFR signaling, gefitinib induced inhibition of cell proliferation, or apoptosis. Additionally, we saw no effect of gefitinib on radiation-induced residual DNA DSBs.

Several groups have combined EGFR TKIs with radiation, although most of those studies focused on wild-type EGFR NSCLC cells (24-26). Combining radiation with gefitinib treatment in vitro in our wild-type EGFR cell line A549 showed no significant increase in radiation sensitivity, in agreement with Park et al (27). However, our results showed that combination treatment in gefitinib-sensitive EGFR-mutated NSCLC lines produced additive effects in terms of clonogenicity and apoptosis. A previous in vitro study combining gefitinib with radiation in EGFR-mutated NSCLC cells did not result in

Fig. 4. Effect of gefitinib on radiation-induced residual DNA damage. (A) Immunofluorescence staining of γH2AX foci in A549 cells is shown. Arrows point to γH2AX foci. (B-E) Cells were irradiated (10 Gy) and treated with gefitinib (0-1 μM) for 24 hours. DNA double-strand break repair was assessed by counting residual γH2AX foci. Graphs represent data from 3 independent repeats. Error bars represent SEM.
decreased clonogenic survival or increased γH2AX foci following irradiation in HCC827 cells, but higher doses of gefitinib were administered, and prior to irradiation rather than concurrently, as in the present study (28).

Because of our interest in evaluating strategies to improve the effectiveness of high-dose/-fraction radiation in lung cancer, we studied the impact of a short course (3 days) of EGFR TKI during the most critical phases of DNA double-strand damage and repair following a single dose of radiation (29). In the A549 (wild-type EGFR) xenografts, combination therapy was more effective than gefitinib treatment alone but not radiation alone. Similarly, Chinnaiyan et al (24) observed that combining erlotinib and fractionated radiation (6 Gy, 2 Gy) in NCI-H226 NSCLC xenografts enhanced tumor growth inhibition compared with either treatment alone. Most significantly, our data showed that combination treatment was more effective than either radiation or gefitinib treatment alone in the HCC827 (mutated EGFR) xenografts. The combination treatment appeared particularly effective in vivo perhaps because of effects on pathways involved in angiogenesis and tumor cell invasion (24). However, as only a small number of cell lines were used in this study, further investigation is required to determine whether this type of treatment is more widely applicable to EGFR mutation positive or wild-type NSCLC.

Several small clinical studies have combined radiation and EGFR TKIs in NSCLC, with the greatest benefit seen in patients with EGFR mutations (30-32). Chang et al (32) treated 25 NSCLC patients with radiation therapy administered concurrently with EGFR TKI treatment. The results showed that this combination was effective only in patients who responded to EGFR TKI treatment (32). Another study showed that of the 9 patients treated with gefitinib and definitive radiation therapy, 2 patients with EGFR mutations both achieved a partial response and had an overall survival of longer than 5 years (31). A recent phase 2 study evaluating erlotinib and concurrent whole-brain radiation therapy in NSCLC patients with brain metastases suggested that patients with EGFR mutation-positive tumors had longer overall survival rates (10). In addition, there are several ongoing clinical trials that are combining gefitinib with radiation in the treatment of EGFR mutation-positive NSCLC patients (NCT00588445, NCT01391260, NCT01091376; clinicaltrials.gov).

Conclusions

In conclusion, our data now provide a strong preclinical rationale for the combination of high-dose/fraction radiation treatment with EGFR TKIs, and suggest that future clinical trials should be focused on patients with EGFR mutation-positive NSCLC.

References


