Interaction of Radiation and Gefitinib on a Human Lung Cancer Cell Line with Mutant EGFR Gene *In Vitro*

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Abstract. Aim: To investigate the effect of gefitinib in combination with irradiation using HCC827 cells, a human lung cancer cell line bearing epidermal growth factor receptor (EGFR) mutation. Materials and Methods: The effects of treatment with radiation with and without gefitinib on HCC827 cells were assessed using a clonogenic assay. Apoptosis was measured by flow cytometry and EGFR signal transduction was evaluated by western blotting. Results: The Dq – quasi-threshold dose, the dose at which the straight portion of the survival curve, extrapolated backward, cuts the dose axis drawn through a survival fraction of unity – after radiation-alone and after combination treatment were 0.41±0.09 Gy and 0.08±0.11 Gy, respectively; thus indicating that combination treatment resulted in supra-additive effects of radiation. There was no significant difference on the D0 – final slope of the survival curve (the dose required to reduce the fraction of surviving cells to 37% of its previous value) – between-radiation alone and the combination treatment. Apoptosis significantly increased after the combination treatment in comparison to what was observed after radiation-alone. The expression of phosphorylated EGFR (pEGFR), phosphorylated ERK1/2 (pERK1/2) and phosphorylated AKT (pAKT) after the combination decreased in comparison to what was observed after radiation-alone. Conclusion: Gefitinib enhances radiosensitivity of supra-additively HCC827 cells by inhibiting the activation of the anti-apoptotic and proliferative signal transduction pathways.

Lung cancer remains the leading cause of cancer-related death worldwide (1). Stage III locally advanced non-small cell lung cancer (NSCLC) accounts for about 25% of all lung cancer cases (2). Combined chemoradiotherapy is the standard of care for patients with stage III NSCLC with good performance status (2, 3). Several randomized phase III trials have shown that concurrent chemoradiotherapy is superior to chemotherapy followed by radiotherapy in terms of response and survival of such patients (4, 5). On the other hand, NSCLC bearing mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) often exhibit a dramatic sensitivity to gefitinib, an EGFR tyrosine kinase inhibitor. Gefitinib is superior to carboplatin–paclitaxel as the initial treatment for pulmonary adenocarcinoma with a mutation of the *EGFR* gene (6). Therefore gefitinib with radiotherapy is thought to be a very attractive strategy for stage III NSCLC bearing an *EGFR* mutation. Several *in vitro* and *in vivo* studies suggest a synergism between gefitinib and radiation (7-11). However, the effect of gefitinib on the radiosensitivity of lung cancer cells bearing an *EGFR* mutation has not yet been sufficiently investigated. Signal transduction through the EGFR with irradiation activates several pathways that are thought to contribute to radiation resistance, including cell-cycle alterations, decreased apoptosis, and enhanced proliferation (12). The aim of this study was to investigate the sensitizing effect of gefitinib in combination with irradiation using a human lung cancer cell line bearing an *EGFR* mutation, HCC827. In addition, the mechanism of interaction between gefitinib and radiation was investigated in terms of EGFR signal transduction.

Materials and Methods

*Cell line and cell cultures.* The human lung cancer cell line HCC827, which has a base-pair deletion at exon 19 (delE746_A750) of *EGFR*, was used in this study. The cell line was kindly provided by Drs. John D. Minna and Adi F. Gazdar of University of Texas Southwestern Medical Center. The cells were maintained in RPMI-
1640 medium supplemented with 10% fetal calf serum at 37°C, in a humidified atmosphere, containing 5% CO₂ and 95% air.

**Radiosensitivity.** Exponentially growing HCC827 cells were seeded into 60-mm diameter petri dishes containing RPMI-1640 medium (Invitrogen Corporation, CA, USA) with 10% fetal bovine serum (BioWest, Nuaille, France) at appropriate concentrations for the assessment of clonogenic survival. Irradiation was started 24 h after incubation at 37°C to ensure sufficient time for recovery from cell damage induced by the trypsinization procedure. The cells were irradiated in air at room temperature by an X-ray machine (RX-650; FAXITRON-Xray Corporation, Lincolnshire, IL, USA) operated at 130 kV, 5 mA with a 0.5-mm Al filter. The dose rate, which was quantified by thermoluminescent dosimetry at the irradiation distance (30 cm), was 87 eGy/min. The effect of irradiation on cell survival was assessed with graded doses from 1 to 8 Gy by steps. Irradiated dishes were then incubated in a CO₂ incubator at 37°C for 14 days to allow for colony growth. The colonies were fixed and stained with a crystal violet solution and those of 50 cells or more were counted. The plating efficiency of the cells was determined for non-irradiated cells. The experiments were performed in triplicate. The slope of the radiation dose-response curve (D0) and the quasithreshold dose (Dq) were determined from the radiation dose-response curve.

**Cell viability assay of gefitinib.** Gefitinib was purchased from Tocris Bioscience (Ellisville, MO, USA) and supplied at a concentration of 10 mM. The drug was then divided into aliquots and stored at −20°C. The drug stock was freshly-diluted in culture medium before each experiment. The effect of gefitinib on the viability of HCC827 cells was evaluated using the WST-1 viability assay. The WST-1 assay was performed using the Cell Proliferation Reagent WST-1 allowed to adhere to the plate overnight. The experimental cultures were given a 72 h exposure at 37°C using graded concentrations of gefitinib ranging from 1 to 5000 nM. All experiments were performed in triplicate.

**Effect of gefitinib on radiation survival responses.** The IC₅₀ of gefitinib (50 nM/ml, see Results) was used to evaluate the sensitizing effect of gefitinib in combination with irradiation. The experiments were started 24 h after the cell inoculation using the procedures described above. The cells were irradiated with a graded dose from 1 to 6 Gy after 2 h exposure to gefitinib. The gefitinib-containing medium was changed under the conditions described above after 24 h. The dishes were then incubated for 14 days and the colonies were fixed and counted by the method described above. The experiments were performed in triplicate.

**Evaluation of EGFR signal transduction pathway.** The pEGFR, pERK1/2 and pAkt after irradiation, gefitinib exposure and the combination of irradiation and gefitinib, were investigated by western blot analysis as described previously (13). The cells were treated with 4 Gy irradiation-alone, the IC₅₀ of gefitinib-alone, or a combination of irradiation and gefitinib. Each treatment was performed as described above. Total cellular protein was isolated from cells 2 h after each treatment in lysis buffer containing proteinase inhibitors, and equal amount of protein were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% tris/glycine gels purchased from BioRad Laboratories, Hercules, CA, USA) and electroblotted to a nitrocellulose membrane. Immunoblotting was performed using a rabbit monoclonal antibody to recombinant human pEGFR (Thr669), pERK1/2 and pAkt (Cell Signaling Technology, Beverly, MA, USA), followed by a horseradish peroxidase (HRP)-conjugated goat-anti-rabbit, and goatanti-mouse secondary antibody (1:2000; Dakocytomation, Glostrup, Denmark). The proteins were detected using enhanced chemiluminescence, according to the manufacturer’s instructions (Amersham (now GE Healthcare), Buckinghamshire, England).

**Apoptosis estimation by flow cytometry and western blot analysis.** The induction of apoptosis was investigated after irradiation-alone, gefitinib-alone, and the combination of irradiation and gefitinib by flow cytometry. The 89-kDa cleaved product of poly ADP-ribose polymerase (cPARP) was investigated by a western blot analysis. The IC₅₀ dose of gefitinib was used. The radiation-alone and the combination groups were all exposed to 0-6 Gy X-ray, and all cells were harvested 24 h after irradiation. The percentage of apoptosis was estimated from the hypodiploid (sub-G₁) cell fraction. The sub-G₁ fraction was measured using flow cytometry. The cells were trypsinized, pelleted by centrifugation at 120 x g for 5 min at 4°C, and fixed in 70% ethanol at −20°C. The fixed cells were stained with propidium iodide (PI) and assayed at FL2 on a FACS Calibur instrument (Becton Dickinson). cPARP was investigated by a western blot analysis after irradiation-alone, gefitinib exposure and the combination of irradiation and gefitinib, using a mouse monoclonal antibody to recombinant human cPARP (Cell Signaling Technology). Each treatment was performed as mentioned above.

**Statistical analysis.** Statistical analyses were performed using the StatView J-5.0 Japanese version software package (HULINKS, Inc. Tokyo, Japan). D0 and Dq were compared using the Wilcoxon signed-rank test. Multiple-comparison analysis was performed by the Friedman test. Differences were considered to be significant at p<0.05.

**Results**

**Radiosensitivity.** The survival curve of HCC827 cells after irradiation is shown in Figure 1. The D0 and Dq were 1.43±0.15 Gy and 0.41±0.09 Gy, respectively.

**Gefitinib sensitivity.** The viability of HCC827 cells at different concentrations of gefitinib is shown in Figure 2. The IC₅₀ of gefitinib was 50 nM. Therefore, 50 nM of gefitinib were used as the dosage for the gefitinib and radiation combination treatment.

**Effect of gefitinib on radiation survival responses.** Figure 3 shows the survival curves for the HCC827 cells under radiation-alone and under treatment with IC₅₀ of gefitinib
combined with radiation at a dose range of 1 to 6 Gy. The D0 and Dq were 1.21±0.08 Gy and 0.08±0.11 Gy, respectively. The D0 for the combination therapy was not significantly different in comparison to that for radiation-alone, while the Dq value was significantly lower than that for radiation-alone (p=0.01). A supra-additive effect was observed in the combination treatment that was administered to the HCC827 cells.

**Evaluation of the EGFR signal transduction pathway.** A time course analysis showed the pEGFR protein expression of HCC827 cells increased from 0 to 6 h (peaking at 2 h) after 4 Gy irradiation-alone (data not shown). Hence, pEGFR and pERK1/2 were investigated at 2 h after irradiation-alone, gefitinib-alone and the combination of irradiation and gefitinib. Figure 4 demonstrates representative immunoblot patterns of the pEGFR, pERK1/2 and pAKT at 2 h after irradiation-alone, IC50 of gefitinib alone, and the combination of irradiation and gefitinib. The expression of pEGFR, pERK1/2 and pAkt after gefitinib alone and the combination of irradiation and gefitinib decreased in comparison to that of irradiation alone.

**Apoptosis estimation after radiation with and without gefitinib.** Figure 5 shows the percentage of apoptosis after irradiation, gefitinib, and the combination of irradiation and gefitinib. A significant increase in apoptosis was observed after the combination of irradiation and gefitinib in comparison to irradiation-alone. However, apoptosis after the combination of irradiation and gefitinib showed no radiation dose dependency and no significant difference in comparison to gefitinib-alone. An increase in cPARP expression was observed after gefitinib-alone and the combination of irradiation and gefitinib, while no obvious change of cPARP was recognized after radiation-alone (Figure 4).

**Discussion**

EGFR stimulates the phosphoinositide-3-kinase (PI3K)-AKT and RAS-mitogen activated protein kinase (MAPK) pathways through its tyrosine kinase activity. The PI3K-AKT and RAS-MAPK pathways play crucial roles in both anti-apoptosis and proliferation (14-16). EGFR stimulated by radiation is an important mechanism leading to resistance of tumor cells to radiotherapy (14-18), as is the...
overexpression of EGFR (19). EGFR tyrosine kinase inhibitors such as gefitinib are expected to sensitize radiation effects. Giocanti et al. reported that gefitinib did not impair DNA double-strand break rejoining and gefitinib and radiation acted additively to induce apoptosis of A-431 and HeLa cells after 24 h of contact with the drug (20). Taira et al. investigated the interaction of an EGFR inhibitor and irradiation for several esophageal cancer cell lines. Some cell lines exhibited a synergistic effect while the others showed an additive effect (21). Colquhouen et al. showed that the combination treatment of gefitinib and radiation for bladder carcinoma resulted in a significantly greater inhibition of colony formation than the respective treatments-alone (22). Unfortunately, the EGFR gene status of these cell lines was unclear. Many in vitro, in vivo and clinical studies have shown NSCLC bearing mutations in EGFR, which often exhibit dramatic sensitivity to gefitinib (7-11). Das et al. found that the majority of mutant EGFR-expressing NSCLCs exhibit characteristics consistent with a radiosensitive phenotype, which include delayed DNA repair kinetics, defective radiation-induced arrest in DNA synthesis or mitosis, and pronounced increases in apoptosis or micronuclei (23). It would therefore be of clinical interest to investigate whether gefitinib alters the radiosensitivity of EGFR-mutant cell lines. However, the effect of gefitinib on the radiosensitivity of lung cancer cells bearing an EGFR mutation has not yet been sufficiently investigated. The current study found that gefitinib had a supra-additive effect in combination with radiation in mutant EGFR-expressing NSCLC. Gefitinib eliminated the shoulder (Dq) of the dose response curve without changing the slope (D0). The Dq of the cell survival curve results from apoptosis (24). The extent of apoptosis of cells treated with the combination of radiation and gefitinib was significantly larger than that of those treated with radiation-alone. In addition, cPARP was detected after the combination of radiation and gefitinib, although cPARP was not detected following radiation-alone. These findings indicate that the combination of radiation and gefitinib induced apoptosis and eliminated the Dq.

Gefitinib produced a de-regulation of p-EGFR, ERK1/2 and AKT expression. Moreover, gefitinib reduced radiation-induced pEGFR, pERK1/2 and pAKT. Therefore, gefitinib was found to overcome radiation-induced EGFR signal transduction. A striking feature of AKT is its regulatory role in the down regulation of apoptosis (25). ERK1/2 translocates into the nucleus and stimulates various transcription factors responsible for the expression of genes controlling proliferation (26, 27). Therefore, the de-regulation of pAKT and pERK1/2 results in the up-regulation of apoptosis and the down-regulation of proliferation. Therefore, the supra-additive enhancement of radiosensitivity observed for gefitinib may be due to the activation of the antiapoptotic and anti-proliferative signal transduction pathways in human lung cancer cells bearing an EGFR mutation, such as the HCC827 cell line.

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References


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