Role of repair protein Rad51 in regulating the response to gefitinib in human non-small cell lung cancer cells

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Abstract

Gefitinib (Iressa, ZD1839) is a selective epidermal growth factor receptor tyrosine kinase inhibitor that can block growth factor-mediated cell proliferation and extracellular signal-regulated kinases 1/2 (ERK1/2) activation. High-level Rad51 expression has been reported in chemoresistant or radioresistant carcinomas. In this study, we examined the role of Rad51 in regulating the response to gefitinib among different human lung cancer cell lines. The H520 line (human squamous cell carcinoma) was less sensitive to gefitinib compared with the H1650 (human adenocarcinoma) or A549 (human bronchioloalveolar carcinoma) lines. In H1650 and A549 cells but not in H520 cells, gefitinib decreased cellular levels of phospho-ERK1/2 and Rad51 protein and message levels. Moreover, gefitinib decreased Rad51 protein levels by enhancing Rad51 protein instability through 26S proteasome-mediated degradation. Inhibition of endogenous Rad51 levels by si-Rad51 RNA transfection significantly enhanced gefitinib-induced cytotoxicity. In contrast, transfection with constitutively active MKK1 vector could restore both Rad51 protein levels and cell survival inhibited by gefitinib. The MKK1/2-ERK1/2 signaling pathway constitutes the upstream signaling for maintaining Rad51 message and protein levels. Rad51 protein can protect lung cancer cells from cytotoxic effects induced by gefitinib. Suppression of Rad51 may be a novel lung cancer therapeutic modality to overcome drug resistance to gefitinib. [Mol Cancer Ther 2008;7(11):3632–41]

Introduction

Lung cancer is one of the most commonly occurring malignancies in the United States and is the leading cause of cancer deaths among both men and women. Non-small cell lung cancer (NSCLC) accounts for ~85% of all lung cancers and has an overall survival at 5 years of <15% (1). Current treatment options for lung cancer include surgery, chemotherapy, and radiation therapy. Surgery is recommended, but complete surgical resection is not always possible because the majority of patients with NSCLC present with locally advanced inoperable or metastatic disease (2). The use of cytotoxic chemotherapies has resulted in improvements in both median overall survival and 1-year survival rates compared with best supportive care (3). The prognosis is especially poor for patients with advanced NSCLC who have not responded to multiple prior chemotherapy regimens. The goals of therapy for advanced lung cancer include symptom improvement, disease stabilization, and improved quality of life.

The epidermal growth factor receptor (EGFR) is a member of the family of erbB receptors. The structure of the receptor includes extracellular ligand binding, transmembrane, and intracellular tyrosine kinase domains (4), which are involved in multiple cellular functions, including cell proliferation, survival, and migration (5). According to previous studies, activation of the EGFR pathway results in activation of downstream signaling pathways, including the Ras-Raf-MKK-extracellular signal-regulated kinase (ERK) and lipid kinase phosphatidylinositol 3-kinase/Akt pathways (6, 7). These pathways have been implicated in the inhibition of cell apoptosis and the promotion of tumor cell growth and motility (8). Dysregulation of these pathways can result in oncogenesis and cancer progression.

EGFR is commonly expressed at high levels in NSCLC (9) and is most commonly reported in squamous cell followed by large cell and adenocarcinoma (10). Several studies have indicated that the level of EGFR expression correlates with poor disease prognosis and reduced survival (11, 12). In view of the importance of the EGFR pathway in human cancer, several approaches to inhibit EGFR have been developed with the aim of blocking cancer cell proliferation and promoting cell apoptosis. These strategies include antibodies blocking the receptor-ligand interaction and small molecules blocking dimerization of the intracellular kinase domain and the activation of downstream signaling pathways that regulate tumor cell proliferation and survival (13, 14).

Gefitinib (Iressa, ZD1839) is a quinazoline derivative that inhibits EGFR tyrosine kinase activity by competitively inhibiting the ATP-binding site of the catalytic domain in EGFR (15, 16). This tyrosine kinase inhibitor (TKI) can block the downstream signaling pathways of EGFR, such as ERK1/2 and phosphatidylinositol 3-kinase/AKT, which
regulate cell growth and survival (17), resulting in the antitumor effect on NSCLC (18). Previous studies have shown that good objective tumor responses to gefitinib occur more frequently in women than in men, are higher with adenocarcinoma than other histologic types, and occur more often in those patients who have never smoked (19). The molecular mechanisms accounting for the different responses to gefitinib among different types of human lung cancer still remain unclear.

Human Rad51 is a protein with a structural homology to Escherichia coli RecA recombinase, and its role in DNA repair has been shown from several studies (20, 21). When cells are exposed to genotoxic agents or irradiation, such as mitomycin C, UV, and ionizing radiation, Rad51 protein is recruited to sites of DNA damage where it mediates the search for a homologous sequence during homologous recombination (22, 23). It has been revealed that the Rad51 nuclear foci are the sites of DNA repair of DNA damage (24). Blastocytes of mouse embryos with a Rad51 gene deletion are highly sensitive to ionizing radiation (25). In addition, conditional Rad51 mutants of chick DT40 cells accumulate broken chromosomes (26). Rad51 is also essential for tumor progression and is associated with resistance to radiation or chemotherapeutic agents (27, 28).

Elevated expression of wild-type Rad51 protein is correlated with histologic grading of invasive breast carcinoma (29), and overexpression of Rad51 has been observed in several tumor cells (29, 30) with elevated rates of homologous recombination (23). The role of Rad51 in chemoresistance and radioresistance still remains unknown, especially in the case of drug resistance to TKI. In addition, the role of Rad51 in regulating the response to gefitinib among different cell types of human lung cancer also still remains to be elucidated.

In the present study, we found that gefitinib decreased cellular levels of both phospho-ERK1/2 and Rad51 protein and message levels in human lung adenocarcinoma but not in lung squamous cell carcinoma. Knockdown of endogenous Rad51 levels by si-Rad51 RNA transfection significantly enhanced gefitinib-induced cytotoxicity in both adenocarcinoma and squamous cell carcinoma. These results show that suppression of Rad51 may be a novel and additive therapeutic modality in NSCLC, especially in gefitinib-resistant lung cancer cells.

Materials and Methods

Materials

Cycloheximide was purchased from Sigma-Aldrich. N-acetyl-Leu-Leu-norleucinal (ALLN), MG132, and U0126 were purchased from Calbiochem-Novabiochem. Gefitinib was purchased from AstraZeneca. Gefitinib, ALLN, MG132, and U0126 were dissolved in DMSO. Cycloheximide was dissolved in MilliQ-purified water (Millipore). Plasmid expressions of MKK1-CA (a constitutively active form of MKK1; \Delta N3/S218E/S222D) and MKK2-CA (a constitutively active form of MKK2; \Delta N4/S222E/S226D) were achieved as described previously (31).

Cell Culture

Human lung adenocarcinoma H1650 cells (CRL-5883; American Type Culture Collection), human lung carcinoma A549 cells derived from human alveolar type 2 cells (CCL-185; American Type Culture Collection), and lung squamous cell carcinoma H520 (HTB-182; American Type Culture Collection) were cultured at 37°C in a humidified atmosphere containing 5% CO2 in RPMI 1640 supplemented with 2.2% (w/v) sodium bicarbonate, 0.03% (w/v) t-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% FCS.

Western Blot

After different treatments, equal amounts of proteins from each set of experiments were subjected to Western blot analysis as described previously (31). The specific phospho-ERK1/2 (Thr202/Tyr204) antibody was from Cell Signaling. Rabbit polyclonal antibodies against ERK2 (C-14; sc-154), Rad51 (H-92; sc-8349), HA (F-7; sc-7392), and actin (I-19; sc-1616) were from Santa Cruz Biotechnology.

Treatments

Exponentially growing human lung cells were plated for 18 h before exposure to gefitinib for 24 h in a RPMI 1640 containing 10% fetal bovine serum. To determine the effects of gefitinib on de novo protein synthesis, cycloheximide (0.1 mg/mL), an inhibitor of de novo protein synthesis, was coadded with gefitinib for 3 to 9 h. To determine the effect of MKK1/2-ERK1/2 signaling on Rad51, MKK1/2-CA expression vectors were transfected into H1650 or A549 cells before gefitinib treatments.

Transfection

The sense-strand sequences of small interfering RNA duplexes used for Rad51 and scrambled (as a control) were 5'-UGUAGCAUAUGCUAGGAGC-3' and 5'-GGCUGG-UUUGUAGGATTGG-3' (Dharmacon Research). Small interfering RNA duplexes (200 nmol/L) were transfected into cells using Lipofectamine 2000 (Invitrogen).

Colony-Forming Ability Assay

Immediately after the gefitinib treatments, cells were washed with PBS and trypsinized for the determination of cell numbers. The cells were plated at a density of 200 to 1,000 on a 60 mm diameter Petri dish in triplicate for each treatment. The cells were cultured for 10 to 14 days, and the cell colonies were stained with 1% crystal violet solution in 30% ethanol. Cytotoxicity was determined by the number of colonies in the treated cells divided by the number of colonies in the untreated control.

Reverse Transcription-PCR

RNA was isolated from cultured cells using TRIzol (Invitrogen) as detailed by the manufacturer. Reverse transcription-PCR was done on 2 μg total RNA using random hexamers following the MMLV reverse transcriptase cDNA synthesis system (Invitrogen). The final cDNA was used for subsequent PCRs. Rad51 was amplified by using the primers with the sequence 5'-CCTTGGCCCA-CAACCCATTTC-3' (forward) and 5'-ATGGCCTTTCCTT-CACCTCCAC-3' (reverse) in conjunction with a thermal cycling program consisting of 26 cycles of 95°C for 30 s,
61°C for 30 s, and 72°C for 60 s. Glyceraldehyde 3-phosphate dehydrogenase was amplified as an internal control. The glyceraldehyde 3-phosphate dehydrogenase primers were 5'-CTACATGGTTTACATGTTCC-3' (forward) and 5'-GTGAGCTTCCCGTTCAGCTCA-3' (reverse). The samples were loaded in triplicate, and the results of each sample were normalized to glyceraldehyde 3-phosphate dehydrogenase.

Cell Viability Analysis
The cell viability of H1650, A549, or H520 cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, the cells (1 x 10^4) were plated in 96-well cell culture plates in RPMI 1640 containing 10% fetal bovine serum in a final volume of 0.2 mL. When the cells reached 50% confluence, they were treated with gefitinib (0.5-20 μmol/L) for 24 h. Cell survival was assessed by directly adding 100 μL MTT (500 μg/mL) to the medium for another 3 h, and cells were solubilized in DMSO (100 μL/well) on a shaker at room temperature for 15 min before reading the absorbance at 562 nm using a Bio-Rad Technologies Microplate Reader.

Determination of Cell Growth
H1650, A549, or H520 cells (1 x 10^5) were treated with gefitinib (20 μmol/L) for 1 to 4 days. The growth of cells was determined by exclusion of trypan blue staining, and the numbers of cells were counted by a hemocytometer. The stain is excluded from living cells but will penetrate dead cells. The proportion of dead cells was determined by counting the cells stained by trypan blue using a hemocytometer.

Statistical Analysis
For each protocol, three or four independent experiments were done. Results were expressed as mean ± SE. Statistical calculations were done using the SigmaPlot 2000 software (Systat Software). Differences in measured variables between experimental and control groups were assessed using an unpaired t test. P < 0.05 was considered statistically significant.

Results
Gefitinib Variably Inhibits Cell Growth in Different Human Lung Cancer Cell Lines
We first compared the cytotoxic effect of gefitinib on different NSCLC cell types, including adenocarcinoma (H1650), bronchioloalveolar cell carcinoma (A549), and squamous cell carcinoma (H520), by MTT assay. MTT assay dose-response curves to gefitinib for these three NSCLC cell lines are shown in Fig. 1A. The results showed that both H1650 and A549 cells were sensitive to gefitinib in a dose-dependent manner. In contrast, H520 cells were more resistant to gefitinib than H1650 or A549 cells. Moreover, assessment of NSCLC cell death after gefitinib treatment using the trypan blue exclusion assay also revealed that H1650 and A549 cells were more sensitive to gefitinib than H520 cells (Fig. 1B). In addition, growth inhibition induced by gefitinib in NSCLC cell lines is shown in Fig. 1C; gefitinib significantly suppressed cell growth in H1650 and A549 cells but not in H520 cells.

Gefitinib Decreases Phospho-ERK1/2 and Rad51 Protein Levels
To evaluate the molecular mechanisms of the different responses to gefitinib among different NSCLC cell types, three different human NSCLC cell lines were exposed to various concentrations of gefitinib (2-20 μmol/L) for 24 h. Phosphorylation of ERK1/2 was determined by Western
blot analysis using an antibody specific to phospho-ERK1/2. Gefitinib treatment decreased cellular phospho-ERK1/2 levels in both H1650 and A549 cells, whereas endogenous ERK2 protein levels did not change in each cell extract (Fig. 2A and B). In contrast, gefitinib did not affect the phospho-ERK1/2 levels in gefitinib-resistant H520 cells (Fig. 2C). Similarly, gefitinib decreased cellular Rad51 protein levels in a dose-dependent manner in H1650 and A549 cells lines. Interestingly, cotreatment with cycloheximide, an inhibitor of de novo protein synthesis, and gefitinib has additive effects in decreasing Rad51 protein levels (Fig. 2A and B). However, gefitinib alone or cotreatment with cycloheximide did not affect the protein levels of Rad51 in gefitinib-resistant H520 cells (Fig. 2C).

**Gefitinib Decreases Rad51 Message Levels in H1650 and A549 Cells**

To elucidate whether the down-regulation of Rad51 protein levels induced by gefitinib occurred at the transcriptional levels, various concentrations of gefitinib were added to NSCLC cell lines for 24 h. Total RNA was isolated and subjected to reverse transcription-PCR analysis for Rad51. Rad51 message levels were suppressed by various concentrations of gefitinib in H1650 and A549 cells (Fig. 2D). In contrast, gefitinib did not affect the Rad51 message levels in gefitinib-resistant H520 cells (Fig. 2D).

**Influence of Gefitinib Treatment on Rad51 Protein Instability in NSCLC Cell Lines**

To investigate whether the regulation of Rad51 protein levels affected by gefitinib also occurred at the post-translational levels, cells were cotreated with cycloheximide and gefitinib for 3 to 9 h. The Rad51 protein levels decreased gradually with time in the presence of cycloheximide. In addition, gefitinib cotreatment significantly enhanced Rad51 degradation on cycloheximide treatment in H1650 or A549 cells (Fig. 3A). After treatment with cycloheximide for 9 h, ~45% of the Rad51 still remained in DMSO-treated cells, whereas only 9% of the Rad51 remained in gefitinib-treated H1650 cells (Fig. 3A), indicating that Rad51 protein was less stable after gefitinib treatment. In contrast, treatment with gefitinib had no significant effect on Rad51 protein stability in H520 cells (Fig. 3A). Therefore, gefitinib cotreatment significantly enhanced Rad51 instability in the H1650 and A549 but not the H520 cells.

**Gefitinib Influences Rad51 Protein Stability through the 26S Proteasome**

To investigate the role of proteasome-mediated degradation of Rad51 protein induced by gefitinib, the 26S proteasome inhibitors MG132 and ALLN were added in the final 4 h of gefitinib treatment before harvesting the H1650 or A549 cells. As shown in Fig. 3B and C, both MG132 and ALLN restored the Rad51 protein levels, which had been decreased by gefitinib alone or gefitinib and cycloheximide cotreatment. Thus, the mechanism by which gefitinib enhanced Rad51 protein instability in H1650 or A549 cells involved 26S proteasome-mediated proteolysis.

**MKK1/2-ERK1/2 Signaling Pathway Controls Rad51 Message and Protein Levels in NSCLC Cells**

To determine whether inactivation of the ERK1/2 signaling pathway was involved in down-regulation of Rad51 protein levels in NSCLC cells, these three cell lines were treated with various concentrations of the MKK1/2 inhibitor U0126. As shown in Fig. 4A to C, the ERK1/2 phosphorylation and Rad51 protein levels significantly decreased in U0126-treated NSCLC cells. Moreover, to examine whether the down-regulation of Rad51 protein levels induced by U0126 occurred at message levels, various concentrations of U0126 were added to NSCLC cells for 24 h and total RNA was isolated and subjected to reverse transcription-PCR analysis for Rad51. The Rad51 message levels were down-regulated at various concentrations of U0126 treatment in H1650, A549, or H520 cells.

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**Figure 2.** Gefitinib treatment decreased phospho-ERK1/2 and Rad51 protein and message levels in H1650 and A549 cells, but these results are not observed in H520 cells. A to C, H1650, A549, or H520 cells (1 x 10⁶) were cultured in complete medium for 18 h and then exposed to gefitinib (2-20 μmol/L) alone or cotreated with cycloheximide (50 μg/mL) for 24 h. After treatment, cells extracts were examined by Western blot for the determination of phospho-ERK1/2, Rad51, actin, and ERK2 protein levels. D, H1650, A549, or H520 cells were cultured in complete medium for 18 h and then exposed to gefitinib (2-20 μmol/L) for 24 h. After treatment, the RNA was isolated and subjected to reverse transcription-PCR analysis for Rad51.
Taken together, these data show that the MKK1/2-ERK1/2 signaling pathway is the upstream signal for maintaining Rad51 protein and message levels in NSCLC cells.

MKK1/2-ERK1/2 Signaling Pathway Acts to Maintain Rad51 Protein Stability

To determine whether the ERK1/2 signaling pathway was involved in regulation of Rad51 protein levels in A549 or H1650 cells, these cell lines were transiently transfected with plasmids carrying MKK1-CA or MKK2-CA, a constitutively active form of MKK1/2. Overexpression of MKK1-CA or MKK2-CA could increase cellular ERK1/2 phosphorylation and Rad51 protein levels in gefitinib-treated or gefitinib and cycloheximide cotreated H1650 or A549 cells (Fig. 5A; Supplementary Fig. S1). Moreover, to determine whether the ERK1/2 signaling pathway was involved in regulation of Rad51 protein stability, H1650 and A549 cells transiently transfected with MKK1-CA vectors and then treated with gefitinib were investigated by cycloheximide chase analysis to determine if the stability of Rad51 was affected by the MKK1-ERK1/2 signaling pathway. MKK1-CA plasmid transfection increased Rad51 protein stability in H1650 or A549 cells treated with gefitinib compared with that in pcDNA3-transfected cells (Fig. 5B). These findings reveal that the MKK1/2-ERK1/2 signaling pathway is the upstream signal for maintaining the protein stability of Rad51, which is decreased by gefitinib.

Blockage of ERK1/2 Activation by MKK1/2 Inhibitor Decreases Rad51 Protein Levels and Protein Stability in H520 Cells

Next, we propose that blockage of ERK1/2 activation by U0126 in gefitinib-resistant H520 cells will decrease Rad51 protein levels. Interestingly, U0126 could down-regulate the Rad51 protein levels in gefitinib alone or gefitinib and cycloheximide coexposed H520 cells (Fig. 5C). Both ALLN and MG132 could rescue the decreased Rad51 protein levels induced by U0126 and gefitinib in H520 cells (Fig. 5D, top). Moreover, cotreatment with U0126 and gefitinib could remarkably decrease Rad51 protein stability compared with that in cells treated with gefitinib alone, indicating that the Rad51 was less stable under U0126 coexposure (Fig. 5D, bottom). Therefore, MKK1/2-ERK1/2 inactivation is required for the down-regulation of Rad51 protein levels and for promoting Rad51 protein instability in gefitinib-treated H520 cells.

Knockdown of Rad51 Enhances the Cytotoxicity and Cell Death in Gefitinib-Treated NSCLC Cells

To determine the effect of Rad51 down-regulation on augmentation of cytotoxicity in gefitinib-treated NSCLC cells,
cells, Rad51 was knocked down using specific small interfering RNA duplexes. As shown in Fig. 6A, transfection of si-Rad51 RNA duplex suppressed Rad51 protein levels without affecting ERK1/2 phosphorylation in gefitinib-treated NSCLC cells. The cytotoxicity and cell death induced by gefitinib and si-Rad51 RNA transfection in NSCLC cell lines were determined by colony-forming ability assay and MTT assay (Fig. 6B and C). Interestingly, suppression of Rad51 by si-Rad51 RNA transfection resulted in enhanced cytotoxicity and cell death caused by gefitinib compared with that by si-scramble RNA transfection (as control) in NSCLC cells (Fig. 6B and C).

**MKK1-CA Expression Reduces Cytotoxicity Induced by Gefitinib in H1650 and A549 Cells**

To evaluate the effects of ERK1/2 activation on cytotoxicity induced by gefitinib, H1650 and A549 cells were transfected with MKK1-CA vectors followed by treatment with various concentrations of gefitinib. The levels of cytotoxicity were assessed by colony-forming ability assay. Transfection with MKK1-CA vector could enhance ERK1/2 activation and cell survival previously suppressed by gefitinib in H1650 and A549 cells (Fig. 6D).

**MKK1/2 Inhibitor Increases Cytotoxicity Induced by Gefitinib in H520 Cells**

To evaluate the effects of ERK1/2 inactivation on cytotoxicity induced by gefitinib in H520 cells, these cells were treated with U0126 (5 μmol/L) followed by treatment with various concentrations of gefitinib (2-10 μmol/L); the levels of cytotoxicity were assessed by colony-forming ability assay. The MKK1/2 inhibitor U0126 could decrease the cell survival in gefitinib-treated H520 cells (Supplementary Fig. S2).

In conclusion, these results are summarized and depicted in Supplementary Fig. S3; this diagram illustrates the molecular mechanisms involved in the different responses to gefitinib in NSCLC cells. In gefitinib-sensitive cells, the ERK1/2 activation and Rad51 protein and message levels can be suppressed by treatment with gefitinib; however, in gefitinib-resistant cells, the ERK1/2 activation and Rad51 protein and message levels cannot be suppressed by treatment with gefitinib.

**Discussion**

EGFR is frequently dysregulated in carcinomas, and high levels of EGFR expression have been detected in a wide range of human malignancies including breast, lung, esophageal, ovarian, head and neck, colon, and prostate carcinomas. EGFR-positive tumors often exhibit higher proliferation and tumor grade and a worse survival than EGFR-negative tumors (11). EGFR activation can initiate signal transduction cascades, such as the mitogen-activated protein kinase and AKT pathways that promote tumor cell proliferation and survival (32). Overexpression of EGFR in carcinoma is associated with poor prognosis (10). A recent study has shown that EGFR protein overexpression was observed in 62% of all NSCLCs, more frequently in SCC (squamous cell carcinoma) than in non-SCC (82% versus 44%), and in 80% of the bronchioloalveolar carcinomas. However, EGFR overexpression or high gene copy numbers had no significant influence on prognosis of NSCLC.

![Figure 4](https://mct.aacrjournals.org/mct/article-pdf/2008/7/11/3637/3637)

**Figure 4.** U0126 (MKK1/2 inhibitor) decreased phospho-ERK1/2 and Rad51 protein and message levels in NSCLC cells. A to C, H1650, A549, or H520 cells (1 x 10⁶) were cultured in complete medium for 18 h. Then cells were exposed to U0126 (2-10 μmol/L) for 24 h. After treatment, the cell extracts were examined by Western blot for the determination of phospho-ERK1/2, Rad51, and ERK1/2 protein levels. D, H1650, A549, or H520 cells were exposed to U0126 (2-10 μmol/L) for 24 h. After treatment, RNA was isolated and subjected to reverse transcription-PCR analysis for Rad51.
In addition, efficacy, toxicity, and symptom outcome in patients with NSCLC treated with gefitinib do not seem to be related to HER2 expression (34).

In recent years, a substantial research effort has aimed at developing new anticancer therapies that target specific components of the EGFR signal transduction pathway. TKIs have been developed that target either the extracellular ligand-binding region of the EGFR or the intracellular tyrosine kinase region, resulting in interference with the signaling pathways that modulate mitogenic and other cancer-promoting responses (e.g., cell motility, cell adhesion, invasion, and angiogenesis; ref. 12). Gefitinib, a quinazoline-derived TKI (35), shows antitumor activity in mouse xenograft models (36) and tumor cell lines (37) through blockage of downstream signaling pathways such as ERK1/2 and phosphatidylinositol 3-kinase/AKT, which regulate cell growth and survival (17). In addition, gefitinib can prevent cell survival and induce cell apoptosis by activating the proapoptotic protein BAD (38). In Taiwanese patients with advanced NSCLC, gefitinib has clinical antitumor activity and good tolerability, a higher response rate than that seen in Europe or in European heritage.

Figure 5. MKK1-ERK1/2 signaling pathway constitutes the upstream signaling for maintaining Rad51 protein levels. A, MKK1-CA expression vectors (2 µg) were transfected into H1650 or A549 cells using Lipofectamine 2000. After expression for 24 h, the cells were treated with various concentrations of gefitinib alone or cotreated with cycloheximide (50 µg/mL) and gefitinib for 24 h. B, after MKK1-CA transfection, the cells were treated with gefitinib (10 µM/L) for 9 h followed by addition of cycloheximide (0.1 mg/mL) for 3 to 9 h. C, H520 cells were pretreated with U0126 (5 µM/L) and then cotreated with various concentrations of gefitinib alone or cotreated with gefitinib and cycloheximide (50 µg/mL) for 24 h. D, top, gefitinib (10 µM/L) and U0126 (5 µM/L) were coadded to H520 cells for 20 h and cells were cotreated with MG132 (25 µM/L) or ALLN (10 µM/L) for 4 h. Bottom, H520 cells were treated with U0126 and gefitinib for 9 h followed by addition of cycloheximide (0.1 mg/mL) for 3 to 9 h. After treatment, the cell extracts were examined by Western blot for the determination of Rad51, phospho-ERK1/2, ERK1/2, actin, and HA-MKK1 protein levels.
Americans, and a better response in chemonaive patients responded versus patients with prior chemotherapy (39).

Daily gefitinib treatment has high activity, is well tolerated, and provides very good survival in Taiwanese NSCLC patients who have failed previous chemotherapy. The median survival was 9.5 months and the 1-year survival rate was 45.1% (40). However, the molecular mechanisms of different responses to gefitinib among different cell types of human lung cancer still remained unclear.

Previous studies have shown that certain lung cancers are caused by activating mutations in EGFR, such as L858R (41) or exon 19 deletion (42, 43), and that these EGFR mutations preferentially involve a subset of lung cancers, which have better response to TKIs (44) and which are clinicopathologically characterized by female sex, non-smoking, adenocarcinoma histology, and East Asian ethnicity (43). Unfortunately, whereas adenocarcinoma cancer cells are initially responsive to small molecule TKIs

Figure 6. Rad51 can protect lung cancer cells from cytotoxic effects induced by gefitinib. A, H1650, A549, or H520 cells were transfected with small interfering RNA duplexes (200 nM) specific to Rad51 or scrambled (control) for 24 h before treatment with gefitinib (2-10 μM) for 24 h. Whole-cell extracts were collected for Western blot analysis. After treatment, the cytotoxicity was determined by colony-forming ability (B) and the cell viability was determined by MTT assay (C). *, P < 0.05; **, P < 0.01, for comparison between the cells treated with gefitinib plus si-Rad51 RNA or si-scramble RNA (Student’s t test). D, H1650 or A549 cells were transfected with MKK1-CA vectors for 24 h and then treated with gefitinib (5-20 μM) for 24 h. The cytotoxicity affected by MKK1-CA vector transfection was determined by colony-forming ability. Mean ± SE of three independent experiments. **, P < 0.01, for comparison between the cells transfected with MKK1-CA or pcDNA3 vectors (Student’s t test).
Rad51 Regulate the Response to Gefitinib

(42), the efficacy of these agents is often limited because of the emergence of drug resistance conferred by a second mutation, such as T790M (45). A recent report has revealed that MET amplification occurs independently of EGFR (T790M) mutations and that MET may be a clinically relevant therapeutic target for some patients with acquired resistance to small-molecule TKIs (46). However, there are still limited reports concerning the role of DNA repair proteins in resistance to TKIs in NSCLC, especially in squamous cell carcinoma.

In this study, we have shown that the DNA repair protein Rad51 is important in regulating the response to gefitinib among different human lung cancer cell lines. The H520 cell line (human squamous cell carcinoma) was less sensitive to gefitinib compared with H1650 cells (human adenocarcinoma) or A549 cells (human bronchioloalveolar carcinoma). Gefitinib decreased cellular levels of phospho-ERK1/2 and Rad51 protein and message levels in H1650 and A549 cells but not in H520 cells. Moreover, depletion of endogenous Rad51 by si-Rad51 RNA transfection significantly enhanced gefitinib-induced cytotoxicity. In contrast, transfection of MKK1-CA vector could rescue the protein levels of Rad51 and cell survival inhibited by gefitinib. U0126 (a MKK1/2 inhibitor) decreases phospho-ERK1/2 and Rad51 message and protein levels in H520 cells treated with gefitinib. In conclusion, Rad51 protein can protect lung cancer cells from cytotoxic effects induced by gefitinib. Suppression of Rad51 may be a novel lung cancer therapeutic modality to overcome drug resistance to gefitinib, especially in squamous cell carcinoma.

Abnormal expression of Rad51 has been reported in various malignant tumors (29). Overexpression of Rad51 protein in mammalian cells confers resistance to ionizing radiation, and Rad51 participates in the repair of double-strand breaks by homologous recombination involving sister chromatids formed after the S phase (23). High levels of Rad51 are correlated with resistance to chemotherapeutic agents and poor prognostic outcome (29, 47). In NSCLC, high expression of Rad51 is associated with an unfavorable prognosis and resistance to platinum agents (48, 49). In addition, our previous study has shown that suppression of Rad51 expression by small interfering RNA transfection can augment the cytotoxic effect of gefitinib (50).

The present study represents the first evaluation of the molecular mechanisms of different responses to gefitinib among different cell types of human NSCLC, especially between adenocarcinoma and squamous cell carcinoma. Our results reveal that gefitinib can decrease cellular levels of phospho-ERK1/2 and Rad51 protein and message levels in human adenocarcinoma cells but not in squamous cell carcinoma. Knockdown of endogenous Rad51 by si-Rad51 RNA transfection or blockage of the MKK1/2-ERK1/2 signaling pathway using U0126 can significantly enhance gefitinib induced cytotoxicity in both adenocarcinoma and squamous cell carcinoma. These results show that suppression of Rad51 may be a novel and additive therapeutic modality in NSCLC, especially in gefitinib-resistant lung cancer cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
20. Haaf T, Golub EI, Reddy G, Radding CM, Ward DC. Nuclear foci of phospho-ERK1/2 and Rad51 protein and message levels in H520 cells treated with gefitinib. In conclusion, Rad51 protein can protect lung cancer cells from cytotoxic effects induced by gefitinib. Suppression of Rad51 may be a novel lung cancer therapeutic modality to overcome drug resistance to gefitinib, especially in squamous cell carcinoma.

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cytotoxic agents against human tumor xenografts is markedly enhanced.


Role of repair protein Rad51 in regulating the response to gefitinib in human non-small cell lung cancer cells

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