Roles of MKK1/2-ERK1/2 and Phosphoinositide 3-Kinase–AKT Signaling Pathways in Erlotinib-Induced Rad51 Suppression and Cytotoxicity in Human Non–Small Cell Lung Cancer Cells

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Abstract

Erlotinib (Tarceva) is a selective epidermal growth factor receptor tyrosine kinase inhibitor in the treatment of human non–small cell lung cancer (NSCLC). In this study, we investigated the roles of ERK1/2 and AKT signaling pathways in regulating Rad51 expression and cytotoxic effects in different NSCLC cell lines treated with erlotinib. Erlotinib decreased cellular levels of phosphorylated ERK1/2, phosphorylated AKT, Rad51 protein, and mRNA in erlotinib-sensitive H1650, A549, and H1869 cells, leading to cell death via apoptosis, but these results were not seen in erlotinib-resistant H520 and H1703 cells. Erlotinib decreased Rad51 protein levels by enhancing Rad51 mRNA and protein instability. Enforced expression of constitutively active MKK1 or AKT vectors could restore Rad51 protein levels, which were inhibited by erlotinib, and decrease erlotinib-induced cytotoxicity. Knocking down endogenous Rad51 expression by si-Rad51 RNA transfection significantly enhanced erlotinib-induced cytotoxicity. In contrast, overexpression of Rad51 by transfection with Rad51 vector could protect the cells from cytotoxic effects induced by erlotinib. Blocking the activations of ERK1/2 and AKT by MKK1/2 inhibitor (U0126) and phosphoinositide 3-kinase inhibitor (wortmannin) suppressed the expression of Rad51 and enhanced the erlotinib-induced cell death in erlotinib-resistant cells. In conclusion, suppression of Rad51 may be a novel therapeutic modality in overcoming drug resistance of erlotinib in NSCLC.

Introduction

Epidermal growth factor (EGF) receptor (EGFR) is over-expressed in non–small cell lung cancer (NSCLC; ref. 1) and is most commonly reported in squamous cell carcinoma, followed by large cell carcinoma and adenocarcinoma (2). Several studies have indicated that activation of the EGFR signaling pathway in cancer cells can enhance antiapoptosis, cell proliferation, angiogenesis, and metastasis, leading to poor disease prognosis (3, 4). Efforts to develop novel target therapeutic strategies for lung cancer have led to the approvals of erlotinib and gefitinib, the small molecular EGFR tyrosine kinase inhibitors (TKI) in the treatment of NSCLC. EGFR-TKIs can block the Ras-Raf-MKK-ERK and lipid kinase phosphoinositide 3-kinases (PI3K)–AKT pathways (5), which have been implicated in the inhibition of cell apoptosis and the promotion of cell growth and motility (6). Erlotinib is a small molecule targeting the ATP-binding site of the EGFR, leading to inhibition of tyrosine kinase activity (7). Erlotinib is the first anti-EGFR agent approved by the U.S. Food and Drug Administration to prolong survival in patients with advanced NSCLC after first-line and second-line chemotherapies (8). Previous studies have shown that good objective tumor responses to EGFR-TKIs occur more frequently in women than in men, are higher in adenocarcinoma than other cancer types, and occur more often in patients who have never smoked (9). The increased activations of PI3K-AKT and MEK1/2-ERK1/2 signaling pathways have been found in the TKI-resistant NSCLC (10, 11). The molecular mechanism accounting for the different responses to EGFR-TKIs in different cell types of human lung cancer still remains unknown.

Human Rad51 is a protein with a structural homology to Escherichia coli RecA recombinase and is essential to homologous recombination repair for DNA damage (12, 13). When cells are exposed to genotoxic agents or irradiation, Rad51 protein is recruited to sites of DNA damage where it mediates the search for a homologous sequence during homologous recombination (14). Previous studies have indicated that a high level of Rad51 is associated with an increased rate of DNA recombination and resistance to radiation or chemotherapeutic agents (15, 16). On the contrary, the blastocytes of mouse embryos with Rad51 gene deletions are highly sensitive to radiation (17). Knocking down the
expression of Rad51 increased the radiosensitivity of malignant glioma and mouse cells (18, 19). However, the expression status of Rad51 protein does not affect the sensitivity to radiation in cancer cells (20, 21). Abnormal expressions of Rad51 have been reported in various carcinomas (22, 23). In lung cancer, high expression of Rad51 in tumor tissue is associated with an unfavorable prognosis (24). Our recent study has shown that the MEK1/2-ERK1/2 is the upstream signaling pathway in regulating the Rad51 expression (25, 26). However, the role of Rad51 in regulating the response to erlotinib in different cell types of human NSCLC also remains to be elucidated.

In the present study, we found that erlotinib could decrease cellular levels of phosphorylated ERK1/2, phosphorylated AKT, and Rad51 protein and mRNA in erlotinib-sensitive NSCLC cell lines. Knockdown of endogenous Rad51 levels by si-Rad51 RNA transfection significantly enhanced erlotinib-induced cytotoxicity. Moreover, MKK1/2-ERK1/2 and PI3K-AKT signaling pathways are the upstream signals in regulating the expression of Rad51, which is associated with tumor growth and survival. Suppression of Rad51 may be a novel and synergistic therapeutic modality in NSCLC, especially in erlotinib-resistant lung cancer cells.

Results
Erlotinib Variably Inhibits Cell Growth and Induces Apoptosis in Different Human Lung Cancer Cell Lines

We first compared the cytotoxic effect of erlotinib on different NSCLC cell lines, including adenocarcinoma (H1650), bronchioloalveolar cell carcinoma (A549), and squamous cell carcinoma (H1869, H520, and H1703) by MTT assay. The dose-response curves to erlotinib in these five NSCLC cell lines were shown in Fig. 1A. H1650, A549, and H1869 cells were sensitive to erlotinib in a dose-dependent manner. In contrast, the H520 and H1703 cells were more resistant to erlotinib than H1650, A549, and H1869 cells. Moreover, assessment of cell death after erlotinib treatment using the trypan blue exclusion assay also revealed that H1650, A549, and H1869 cells were more sensitive to erlotinib than H520 and H1703 cells (Fig. 1B). In addition, erlotinib significantly suppressed cell growth in H1650 and A549 cells but not in H520 and H1703 cells (Fig. 1C). The growth inhibition effect of erlotinib on H1650 or A549 cells were ~2.5-fold higher than H1703 or H520 cells. In erlotinib-sensitive A549 cells, cell cycle distribution analysis showed that the proportion of cells in the sub-G1 phase increased in a dose-dependent manner after erlotinib exposure for 72 hours (3.98-18.61%). In contrast, the treatment of erlotinib had no effect on the percentage of cells in the sub-G1 phase in erlotinib-resistant H1703 cells (Fig. 1D).

Erlotinib Decreases Phosphorylated ERK1/2, Phosphorylated AKT, and Rad51 Protein and mRNA Levels in Erlotinib-Sensitive NSCLC Cell Lines

To evaluate the molecular mechanisms of the different responses to erlotinib in different cell types of lung cancer, five human NSCLC cell lines were exposed to various concentra-

Influence of Erlotinib on Rad51 mRNA and Protein Instability in Different NSCLC Cell Lines

To examine whether the regulation of Rad51 mRNA by erlotinib occurred at the posttranscriptional level, the cells were cotreated with actinomycin D (an inhibitor for RNA synthesis) and erlotinib for 3 to 9 hours. The Rad51 mRNA levels decreased with time in the presence of actinomycin D and erlotinib in H1650 and A549 cells but not in H520 and H1703 cells, indicating that Rad51 mRNA was unstable upon treatment with erlotinib in erlotinib-sensitive cells (Fig. 2B, bottom).

In addition, to determine whether the regulation of Rad51 protein by erlotinib occurred at the posttranslational level, cells were cotreated with cycloheximide (an inhibitor of de novo protein synthesis) and erlotinib for 3 to 9 hours. The Rad51 protein levels decreased gradually with time in the presence of cycloheximide. Erlotinib cotreatment significantly enhanced Rad51 protein degradation in H1650 or A549 cells, indicating that the Rad51 protein was less stable after erlotinib treatment (Fig. 2C). In contrast, treatment with erlotinib had no significant effect on Rad51 protein stability in H520 and H1703 cells (Fig. 2C). Together, erlotinib treatment significantly enhanced Rad51 mRNA and protein instability in erlotinib-sensitive cells but not in erlotinib-resistant cells.

Erlotinib Enhances Rad51 Protein Instability through the Degradation by 26S Proteasome

To investigate whether the proteasome-mediated degradation of Rad51 protein was induced by erlotinib, the 26S proteasome inhibitors MG132 and N-acetyl-Leu-Leu-norleucinal (ALLN) were cotreated with erlotinib in H1650 or A549 cells. As shown in Fig. 2D, both MG132 and ALLN restored the Rad51 protein levels, which were decreased by erlotinib. Therefore, erlotinib-enhanced Rad51 protein instability is through 26S proteasome-mediated proteolysis in H1650 and A549 cells.

The PI3K-AKT Signaling Pathway Maintains Rad51 mRNA and Protein Levels in NSCLC Cells

Our previous studies indicated that MKK1/2-ERK1/2 signaling pathway is the upstream molecules to maintain the cellular Rad51 protein and mRNA levels in NSCLC cells (26). To determine whether activation of the PI3K-AKT signaling pathway was also involved in the maintaining of Rad51 protein and mRNA expression, the NSCLC cell lines were treated with various concentrations of PI3K inhibitors, wortmannin, or LY294002. As shown in Fig. 3A and C, the cellular phosphorylated AKT and Rad51 protein levels significantly decreased.
after treatment with wortmannin or LY294002 without affecting the cellular phosphorylated ERK1/2 levels in these NSCLC cell lines (Fig. 3A and C). In addition, the Rad51 mRNA levels were down-regulated by the treatment with wortmannin or LY294002 (Fig. 3B and D). Taken together, these results indicate that the PI3K-AKT signaling pathway is the upstream signal for maintaining Rad51 protein and mRNA expression in different cell types of NSCLC.

**FIGURE 1.** H520 and H1703 cells were less sensitive to the cytotoxic and growth inhibitory effects by erlotinib than H1650, A549, and H1869 cells. A. NSCLC cells lines were treated with various concentrations of erlotinib for 24 h. The cell survival of these cell lines was determined by MTT assay. B. Both unattached and attached cells were collected and stained with trypan blue for manual counting of the numbers of dead cells. These results (mean ± SEM) were obtained from three independent experiments. C. NSCLC cells (1 × 10⁵) were treated with erlotinib (20 μmol/L) for 1 to 4 d, and then the cell viability was determined by MTT assay. Growth inhibitory effects of a single administration of 20 μmol/L erlotinib on a panel of NSCLC cell lines using MTT assays (right). These results (mean ± SEM) were obtained from four independent experiments. D. Flow cytometry analyses of the cell cycle of A549 and H1703 cells. Cells were treated with 2 to 10 μmol/L of erlotinib for 72 h. The percentage of apoptotic cells (sub-G1 phase) was shown in each histogram.
Both PI3K-AKT and MKK1/2-ERK1/2 Signaling Pathways Maintain Rad51 Protein Expression in NSCLC Cells

To determine whether the PI3K-AKT signaling pathway was also involved in maintaining Rad51 protein expression in A549 or H1650 cells, these cell lines were transiently transfected with plasmids carrying myrAKT, a constitutively active form of AKT, harbored a consensus myristylation domain that replaces the amino acids 4 to 129 of wild-type AKT. Overexpression of myrAKT could rescue Rad51 protein expression in H1650 and A549 cells inhibited by erlotinib (Fig. 4A). In addition, myrAKT plasmid transfection restored Rad51 protein stability in H1650 and A549 cells treated with erlotinib compared with that in pcDNA3-transfected cells (Fig. 4C).

Next, 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 could rescue the cellular phosphorylated ERK1/2 and Rad51 protein levels, which were suppressed by erlotinib (Fig. 4B). MKK1-CA plasmid transfection also enhanced the Rad51 protein stability in erlotinib-treated H1650 and A549 cells compared with pcDNA3-transfected cells (Fig. 4D). Taken together, these findings reveal that both PI3K-AKT and MKK1/2-ERK1/2 signaling pathways are the upstream signals for maintaining the protein expression and stability of Rad51, which is inhibited by erlotinib.

Blockages of AKT and ERK1/2 Activations by PI3K and MKK1/2 Inhibitors Decrease Rad51 Protein Expression and Stability in Erlotinib-Resistant NSCLC Cells

Furthermore, we proposed that blockage of AKT and ERK1/2 activation by PI3K inhibitor (wortmannin) and MKK1/2 inhibitor (U0126) in erlotinib-resistant H520 and H1703 cells can effectively decrease Rad51 protein expression and enhance the cytotoxic effect of erlotinib. Interestingly, wortmannin or U0126 could suppress the expression of Rad51 proteins, which are not affected by erlotinib in H520 and H1703 cells (Fig. 5A and B). Moreover, cotreatment with wortmannin or U0126 and erlotinib could remarkably decrease the Rad51 protein stability compared with that treated with erlotinib alone, indicating that Rad51 was unstable and subjected to degradation more easily under cotreatment with erlotinib and wortmannin or U0126 (Fig. 5C and D). Therefore, the inactivation of PI3K-AKT and MKK1/2-ERK1/2 signaling pathways is important for down-regulation of Rad51 protein expression and for promoting Rad51 protein degradation in erlotinib-resistant H520 and H1703 cells. In addition, both ALLN and MG132 could rescue the decreased Rad51 protein levels induced by erlotinib and wortmannin or U0126 in erlotinib-resistant H520 and H1703 cells (data not shown).

Knockdown of Rad51 Expression Enhances Cytotoxicity and Cell Death in Erlotinib-Treated NSCLC Cells

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

Rad51 Overexpression Enhances the Cell Viability Which Is Suppressed by Erlotinib in H1650 and A549 Cells

To determine the effect of Rad51 overexpression on protection from cytotoxicity induced by erlotinib in NSCLC cells, Flag-Rad51 vectors was transfected into NSCLC cells. As shown in Fig. 6C, transfection of Flag-Rad51 vector prevented the cell death induced by erlotinib in H1650 and A549 cells determined by MTT assay. In contrast, Rad51 overexpression did not affect the cell viability in erlotinib-resistant H520 and H1703 cells.

Inactivation of AKT and ERK1/2 Enhances Erlotinib-Induced Cell Death in H520 and H1703 Cells

To determine whether the activations of AKT and ERK1/2 were associated with the resistance of erlotinib in H520 and H1703 cells, the H520 and H1703 cells were pretreated with wortmannin and/or U0126 and then cotreated with erlotinib to determine the cell viability by MTT assay. Inactivation of AKT and ERK1/2 synergistically enhanced the cell death in erlotinib-resistant H520 and H1703 cells (Fig. 6D, left).

Transfection with MKK1-CA and myrAKT Vectors Enhance the Cell Survival Decreased by Erlotinib in H1650 and A549 Cells

To examine the effect of ERK1/2 and AKT activation on the cell survival in erlotinib-sensitive H1650 and A549 cells, the cells were transfected with MKK1-CA and/or myrAKT vector to evaluate the viability of cells treated with erlotinib. Both MKK1-CA and myrAKT vector transfection can rescue the cell death induced by erlotinib in H1650 and A549 cells (Fig. 6D, right). Together, in erlotinib-sensitive cells, the activations of ERK1/2 and AKT and the expressions of Rad51 protein and mRNA can be suppressed by erlotinib, leading to cell death. On the contrary, the activation of ERK1/2 and AKT, Rad51 protein expression, and cell survival were not affected by erlotinib in erlotinib-resistant cells.

Discussion

The EGFR (ErbB1/HER-1) is activated by many ligands and belongs to EGFR super family of tyrosine kinase receptors, including ErbB2, ErbB3, and ErbB4. The known ligands for EGFR include EGF, transforming growth factor-α, amphiregulin, epiregulin, b-cellulin, and heparin-binding EGF. Ligand binding to EGFR induces the formation of receptor homodimers and heterodimers and the activation of the intrinsic kinase domain, resulting in phosphorylation of specific tyrosine residues within the cytoplasmic tail (5). The EGFR and its family members are deregulated in many human tumors, and EGFR amplification and mutations are detected at a high frequency...
in carcinomas and glioblastomas, leading to poor outcome in cancer patients (27, 28).

Among the main pathways activated downstream of EGFR are the Ras-Raf-MKK-ERK1/2, STAT3, and STAT5 pathways controlling mainly proliferation and differentiation and the PI3K-AKT-mTOR cascade acting as a prosurvival and antiapoptotic pathway (27). In general, ERK1/2 signal is activated by growth factors and mitogens (29), and activated ERKs are critical in the control of cell proliferation, survival, differentiation, growth arrest, apoptosis, cell transformation, and tumor invasion (30). Numerous reports indicate that the ERK1/2 signaling pathway plays a vital role in tumorigenesis and promoting tumor cell growth (31), and blockage of ERK pathway induces marked G1 cell cycle arrest and apoptosis in tumor cells (32).

Currently, two types of EGFR family inhibitors are in clinical use: small molecule TKIs like erlotinib (Tarceva), gefitinib (Iressa), or lapatinib, and antibodies such as trastuzumab (Herceptin) and cetuximab (Erbitux) directed against ErbB2 and EGFR, respectively (33, 34). Preclinical studies have shown that erlotinib blocks the growth of human NSCLC cell lines in vitro by inhibiting the EGFR and the downstream protein phosphorylation, such as MKK1/2-ERK1/2 and PI3K-AKT. Erlotinib is the first EGFR-TKI that has shown a survival benefit in NSCLC patients. In a randomized study conducted by the National Cancer Institute of Canada (BR.21) in second- and third-line NSCLC treatment, erlotinib significantly prolonged overall survival and decreased symptoms compared with placebo in patients with stage IIIB or stage IV NSCLC (7). These similar results are also seen in Taiwanese patients with proved stage IIIB/IV NSCLC who had received at least one line of standard chemotherapy or radiotherapy (35). Sensitivity to erlotinib has been associated with EGFR mutations, most commonly deletions of four to six amino acids in exon 19 or a point mutation (L858R) in exon 21. Increased EGFR gene copy number has also been pointed out as a good predictive marker for erlotinib response (36). Although the survival benefit from erlotinib was observed in varied subgroups of patients, the radiographic responses were more common in certain patient subgroups, such as female patients, patients with no

![Figure 2](http://example.com/figure2.png)

**FIGURE 2.** Erlotinib treatment decreased phosphorylated ERK1/2, phosphorylated AKT, Rad51 protein, and message levels in H1650 and A549 cells, but these results were not observed in H520 and H1703 cells. **A.** Cells were exposed to erlotinib (0.5-10 μmol/L) for 24 h. After treatment, the cell extracts were subjected to Western blot analysis. **B.** Top, cells were exposed to erlotinib (0.5-10 μmol/L) for 24 h. Bottom, cells were treated with erlotinib (10 μmol/L) for 9 h, followed by addition with actinomycin D (2 μg/mL) for 3 to 9 h. After treatment, the total RNA was isolated and subjected to reverse transcription-PCR analysis for Rad51.
history of smoking, patients with adenocarcinoma histology, patients of Asian ethnicity, and patients with the presence of EGFR tyrosine kinase domain mutations (37). One recent report has shown that one White male former smoker with advanced-staged squamous cell lung cancer without EGFR tyrosine kinase mutations had a good response to first-line erlotinib (38).

Human Rad51 is essential in homologous recombination repair for DNA damage, especially in double-strand breaks (12). When cells are exposed to genotoxic agents or irradiation, Rad51 protein is recruited to sites of DNA damage and associated with nuclear matrix (39) where it mediates the search for a homologous sequence during homologous recombination (14), and it has been shown to have antiapoptotic activity in tumor cells (40). A number of reports showed that Rad51 is not only involved in the progression of carcinogenesis but also affects resistance to platinum agents (41). Overexpression of Rad51 is associated with increased rate of DNA recombination and resistance to radiation or chemotherapeutic agents (42, 43). Rad51 has been reported to influence the outcome of patients treated with chemoradiotherapy (44). In lung cancer, high expression of Rad51 in tumor tissue is associated with an unfavorable prognosis (24). Our recent study has shown that the polycyclic hydrocarbon carcinogen benzo(a)pyrene can enhance the expression of Rad51 through the activation of ERK1/2 (45), and MEK1/2-ERK1/2 is the upstream signaling pathway to regulate the Rad51 expression, which is associated with the resistance to gefitinib, mitomycin C, and cisplatin in NSCLC (25, 26). In our previous studies, we have shown that gefitinib can inhibit ERK1/2 phosphorylation and the expression of Rad51 protein and mRNA (25, 26). From the results of the present study, we first showed that AKT and ERK1/2 signaling maintained the Rad51 gene and protein expression. Suppression of Rad51 expression through AKT and ERK1/2 inactivation sensitize NSCLCs to erlotinib. Therefore, knockdown of Rad51 can enhance the cytotoxicity and cell death in NSCLC cells treated with gefitinib and erlotinib. Consistence with our studies, erlotinib attenuate radiation-induced Rad51 expression and enhance the radiation-induced apoptosis in human NSCLC cells (46). Imatinib (Gleevec) reduce Rad51 protein levels and action for chemosensitization and radiosensitization of H1299 human lung carcinoma cell line with imatinib (47). Rad51 is also involved in the sensitivity of small cell lung cancer to etoposide (VP16; ref. 48). In our other study, we have found the excision repair cross-complementation 1, a protein component of the nucleotide excision repair complex, was also involved in the sensitivity of erlotinib. However, this study was under

**FIGURE 2.** C. Cells were treated with erlotinib (10 μmol/L) for 9 h, followed by addition of cycloheximide (0.1 mg/mL) for 3 to 9 h. D. Erlotinib (2-10 μmol/L) was added to H1650 or A549 cells for 20 h, and then the cells were cotreated with MG132 (10 μmol/L) or ALLN (10 μmol/L) for 4 h. Whole cell extracts were collected for Western blot analysis for Rad51 protein expression.
investigation. Moreover, we used the two EGFR activating cell lines U-87 MG (human glioblastoma) and HRT-18 (colorectal cancer cell line) to analyze whether Rad51 knockdown could enhance the sensitivity of erlotinib. Interestingly, down-regulation of Rad51 by specific siRNA transfection could lead to sensitization to erlotinib in U-87 MG and HRT-18 cells (Supplementary Fig. S1). Suppression of Rad51 also notably increased the sensitivity of erlotinib in NSCLC cell line H1975 (L858R/T790M) with the EGFR mutation and a resistance mutation (Supplementary Fig. S2). However, the detailed mechanism was under our next investigation.

In this study, we used the EGFR mutant cell line (H1650) and EGFR wild-type cell lines (A549, H520, and H1703) to examine the role of Rad51 in regulating different responses of erlotinib. The detailed description of status of EGFR, K-Ras, p53, RB, and INK4a/ARF of these cell lines was studied (Supplementary Table S1). Moreover, the H1650 and A549 had a high expression of EGFR, whereas H520 cell showed nearly no expression of EGFR (49). Activating mutations of EGFR have been related to an increased response rate and survival in patients treated with EGFR TKIs (50). It will be interesting to compare the status of these genes of NSCLC cells in more detail in association with Rad51 expression, and those previous studies are in progress in our laboratory. In Fig. 6B, erlotinib caused the ~20% to 30% of the cell death in H520 and H1703 cell lines. Interestingly, these cells become more sensitive to erlotinib when these cells were treated with wortmannin and/or U0126. However, there are still ~50% of cells that survived even when Rad51 was down-regulated with wortmannin and U0126. Therefore, erlotinib has both Rad51-dependent and Rad51-independent mechanisms of cytotoxicity.

This study is the first report to reveal that PI3K-AKT signaling pathway is also the upstream signal and acts together with MKK1/2-ERK1/2 signal to control the Rad51 expression and maintain its stability. It is interesting to note that, in the erlotinib-resistant H520 and H1703 lung cancer cell lines, depletion of Rad51 by siRNA or inactivation of AKT and ERK1/2 could restore sensitivity to erlotinib. Previous studies have also shown that the PI3K inhibitor, wortmannin, can prevent the radiation-induced Rad51 foci formation in NSCLC (42). Together, Rad51 is essential to maintain cell survival when NSCLC cells are treated with TKIs, radiation, and chemotherapeutic agents.

In conclusion, suppression of Rad51 expression by blocking MKK1/2-ERK1/2 and PI3K-AKT pathways may be a novel therapeutic modality in enhancing the cytotoxic effect of TKIs.

**FIGURE 3.** Wortmannin or LY294002 decreased phosphorylated AKT, Rad51 protein, and mRNA levels in NSCLC cells. A and C. NSCLC cells were exposed to wortmannin (2-10 μmol/L) or LY294002 (5-20 μmol/L) for 24 h. After treatments, the cell extracts were subjected to Western blot analysis. B and D. After treatments as above, total mRNA was isolated and subjected to reverse transcription-PCR analysis for Rad51.
and overcoming the drug resistance of TKIs in NSCLC in the future.

Materials and Methods

**Materials**

Cycloheximide and actinomycin D were purchased from Sigma-Aldrich (St. Louis). ALLN, MG132, wortmannin, LY294002, and U0126 were purchased from Calbiochem-Novabiochem. Erlotinib was purchased from Genentech. erlotinib, ALLN, MG132, actinomycin D, wortmannin, LY294002, 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, ΔN3/S218E/S222D) and MKK2-CA (a constitutively active form of MKK2, ΔN4/S222E/S226D) were achieved as

**FIGURE 4.** The PI3K-AKT and MKK1-ERK1/2 signaling pathways constituted the upstream signals for maintaining Rad51 protein expression. A and B, myrAKT and MKK1/2-CA expression vectors were transfected into H1650 and A549 cells using Lipofectamine. After transfection, the cells were treated with erlotinib for 24 h. C and D, After myrAKT or MKK1/2-CA transfection, the cells were treated with erlotinib (10 μmol/L) for 9 h, followed by addition with cycloheximide (0.1 mg/mL) for 3 to 9 h. The cell extracts were subjected to Western blot analysis.
previously described (25). The constitutively active AKT expression plasmid (pcDNA3-myrAKT) harbored a consensus myristylation domain that replaces the amino acids 4 to 129 of wild-type AKT.

Cell Lines

Human lung adenocarcinoma H1650 cells (CRL-5883), bronchioloalveolar carcinoma A549 cells (CCL-185), lung squamous cell carcinoma H520 (HTB-182), lung squamous cell carcinoma H1703 (CRL-5889), and lung squamous cell carcinoma H1869 (CRL-5900) were obtained from the American Type Culture Collection and were cultured at 37°C in a humidified atmosphere containing 5% CO₂ in RPMI 1640 complete medium supplemented with sodium bicarbonate (2.2%, w/v), L-glutamine (0.03%, w/v), penicillin (100 units/mL), streptomycin (100 μg/mL), and FCS (10%).

Western Blot

After different treatments, equal amounts of proteins from each set of experiments were subjected to Western blot analysis.
as previously described (25). The specific phosphorylated ERK1/2 (Thr202/Tyr204) and phosphorylated AKT (Ser473) antibodies were purchased from Cell Signaling. Rabbit polyclonal antibodies against ERK2 (C-14), Rad51 (H-92), AKT (H-136), HA (F-7), and actin (I-19) were purchased from Santa Cruz Biotechnology.

**Flow Cytometric Analysis for Cell Cycle Distribution**

Human lung cancer cell lines were plated in 60-mm dishes, cultured for 24 h, and then treated with or without 2 to 10 μmol/L erlotinib for 72 h. The floating and adherent cells were collected by trypsinization, fixed for 24 h in 70% ethanol, and resuspended in propidium iodide (25 μg/mL) supplemented with...
0.1% RNase A and 0.1% Triton X-100. The DNA contents and the cell cycle distribution of cancer cells were measured with a FACScan flow cytometer (BD Biosciences). These experiments were repeated thrice for statistical analysis.

**Transfection**

The sense-strand sequences of siRNA duplexes for Rad51 and scrambled (as a control) were 5′-UGU AGC AUA UGC UCG AGC G-3′ and 5′-GCG CGC UUU GUA GGA TTC G-3′ (Dharmacon Research). siRNA duplexes (200 nmol/L) were transfected into cancer cells using Lipofectamine 2000 (Invitrogen).

**Colony-Forming Ability Assay**

Immediately after the erlotinib treatments for 24 h, the cancer cells were trypsinized to determine the cell numbers. The cells were plated at a density of 200 to 1,000 cells on a 60-mm-diameter Petri dish in triplicates for each treatment. The cells were cultured for 10 to 14 d, and the cell colonies were stained with 1% crystal violet solution in 30% ethanol. The cytotoxicity of erlotinib 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 from total RNA (2 μg) using random hexamers from the Moloney murine leukemia virus reverse transcriptase cDNA synthesis system (Invitrogen). The final cDNA was used for subsequent PCRs. Rad51 was amplified by using the primers with the sequence of 5′-ATG GCC TTT -GTG AGC TTC CCG TTC-3′ (forward) and 5′-ATG GCC TTT CCT TCA CCT CCA C-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. The glyceraldehyde-3-phosphate dehydrogenase was amplified as an internal control. The sequence of glyceraldehyde-3-phosphate dehydrogenase primers were 5′-CTA CAT GGT TTA CAT-3′ (forward) and 5′-GTG AGC TTC CCG TTC AGC TCA-3′ (reverse). The samples were loaded in triplicates, and the results of each sample were normalized to glyceraldehyde-3-phosphate dehydrogenase.

**Cell Viability Analysis**

The cell viability of H1650, A549, H520, or H1703 cells was evaluated by MTT assay. In brief, the cells (1 × 10^4) were plated in 96-well cell culture plates in RPMI containing 10% fetal bovine serum in a final volume of 0.2 mL. When the cells reached 50% confluence, they were treated with erlotinib (0.5-20 μmol/L) for 1 to 4 d. Cell survival was assessed by directly adding 100 μL of MTT (500 μg/mL) to the medium for another 3 h, and then the 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.

**Statistical Analysis**

For each protocol, three or four independent experiments were done. Results were expressed as mean ± SEM. 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.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Dr. Jia-Ling Yang for providing MKK1/2-CA plasmids, Dr. Hong-Yo Kang (Chang Gung University and Chang Gung Memorial Hospital, Kaohsiung Medical Center, Kaohsiung, Taiwan) for providing pcDNA3-mlvAKT plasmids, and Dr. Yi-Rong Chen (National Health Research Institutes, Division of Molecular and Genomic Medicine, Taiwan) for providing the H1975 cell line.

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doi:10.1158/1541-7786.MCR-09-0051

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