Down-Regulation of Rad51 Expression Overcomes Drug Resistance to Gemcitabine in Human Non–Small-Cell Lung Cancer Cells

Min-Shao Tsai, Ya-Hsun Kuo, Yu-Fan Chiu, Ying-Chen Su, and Yun-Wei Lin
Molecular Oncology Laboratory, Department of Biochemical Science and Technology, National Chiayi University, Chiayi, Taiwan
Received July 20, 2010; accepted September 17, 2010

ABSTRACT

Gemcitabine (2′,2′-difluorodeoxycytidine), a deoxycytidine analog, and erlotinib, an epidermal growth factor receptor-tyrosine kinase inhibitor, are used clinically to treat patients with non–small-cell lung cancer (NSCLC). However, the molecular mechanisms for the drug resistance of gemcitabine in NSCLC cells are poorly understood. In this study, we used constructs containing human Rad51 cDNA or specific Rad51 small interfering RNA (siRNA) to examine the role of Rad51 in chemoresistance of gemcitabine in three different human NSCLC cell lines. Exposure of human NSCLC cell lines to gemcitabine increased the phosphorylation levels of mitogen-activated protein kinase kinase (MKK) 1/2-extracellular signal-regulated kinase (ERK) 1/2 and AKT in a time- and dose-dependent manner, which was accompanied by an induction of Rad51 mRNA and protein expression. Gemcitabine increased the expression of Rad51 by increasing its mRNA and protein stability. Blockage of ERK1/2 or AKT activation by 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene (U0126; MKK1/2 inhibitor) or 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002; phosphatidylinositol 3-kinase inhibitor), respectively, decreased the gemcitabine-induced Rad51 expression. Gemcitabine-induced cytotoxicity was significantly increased upon siRNA depletion of Rad51 or blockage of ERK1/2 and AKT activation. Erlotinib enhanced the gemcitabine-induced cytotoxicity via the inactivation of ERK1/2 and AKT and the down-regulation of Rad51. Enforced expression of constitutively active MKK1/2 or AKT recovered cell viability and Rad51 protein levels that were decreased by the combination of erlotinib and gemcitabine. Suppression of Rad51 expression or the inactivation of ERK1/2 or AKT signaling may be considered potential therapeutic modalities for gemcitabine-resistant lung cancer.

Introduction

Non–small-cell lung cancer (NSCLC) remains the leading cause of cancer death in the world (Jemal et al., 2007). The majority of patients with NSCLC present with locally advanced inoperable or metastatic diseases (Silvestri and Spiro, 2006), and the use of cytotoxic chemotherapies can improve the median overall survival (Schiller et al., 2002). Gemcitabine, a pyrimidine analog, has been clinically used for patients with NSCLC (Martoni et al., 2005). The antiproliferative action of gemcitabine in pancreatic cancer cells results from the induction of apoptosis mediated by the activation of p38 mitogen-activated protein kinase (Koizumi et al., 2005). On the other hand, abrogation of gemcitabine-activated extracellular signal-regulated kinase (ERK) 1/2 in hepatocellular and cholangiocellular carcinomas accelerates cell death (Matsumoto et al., 2008). However, the molecular mechanisms for the growth inhibition and cytotoxicity of gemcitabine-treated NSCLC cells are poorly understood. Many tumors, particularly NSCLC, have increased or altered expression of epidermal growth factor receptor (EGFR)
or its ligands. Overexpression of EGFR occurs in 40 to 80% of NSCLC cases (Salomon et al., 1995). Several studies have indicated that high expression levels of EGFR correlate with a poor prognosis and reduced survival (Veale et al., 1993). Erlotinib (OSI-774; Tarceva; Roche, Basel, Switzerland) is an EGFR-tyrosine kinase inhibitor (TKI) approved for the treatment of advanced NSCLC (Silvestri and Rivara, 2005) that blocks EGFR activity by competing with ATP for binding to the tyrosine kinase pocket of the receptor (Mendelsohn, 2001). Therefore, erlotinib could inhibit the phosphorylation of ERK1/2 and AKT in NSCLC cell lines (Ono et al., 2004). The ERK1/2 signaling pathway has a key role in cancer progression, DNA synthesis, cell cycle progression, cell proliferation, differentiation, and apoptosis (Brunet et al., 1999). AKT, or protein kinase B, plays an important role in the control of cell survival. The up-regulation of AKT is implicated in NSCLC carcinogenesis (Balsara et al., 2004), with resistance to chemotherapy and radiation (Brognaud et al., 2001), and is associated with reduced patient survival (David et al., 2004).

Homologous recombination (HR) is an error-free DNA repair pathway for double-strand breaks that occur when cells are exposed to exogenous mutagens and during normal metabolic processes (Wymann and Kanaar, 2004; San Filippo et al., 2008). Defects in HR repair lead to genomic instability and tumor development (Richardson et al., 2004). Rad51 is a strand transferase that forms a nucleoprotein filament by polymerizing onto single-stranded DNA at the processed DNA break. The filament promotes DNA strand exchange with the undamaged homologous chromatid (San Filippo et al., 2008). Rad51 is involved in tumor progression by destabilizing the genome; for example, up-regulation of Rad51 expression results in an increased number of recombination events and chromosomal rearrangements in mouse embryonic stem cells (Richardson et al., 2004). In addition, the overexpression of Rad51 increases the spontaneous recombination frequency in mammalian cells (Vispé et al., 1998; Arnaudeau et al., 1999) and correlates with resistance to chemotherapeutic drugs in tumor cells (Bello et al., 2002; Slupianek et al., 2002). PD-321852 [4-(2,6-dichloro-phenyl)-9-hydroxy-6-(3-methylamino-propyl)-6H-pyrrolo[3,4-c]carbazole-1,3-dione], a small-molecule checkpoint kinase 1 (Chk1) inhibitor, sensitizes pancreatic cancer cells to gemcitabine by the inhibition of Rad51 protein levels and a Rad51-mediated DNA damage response (Parsels et al., 2009). In addition, gemcitabine interferes with Rad51 foci formation after irradiation and induces radiosensitization (Wachters et al., 2003). Therefore, Rad51 must be carefully regulated to maintain genomic stability in cells responding to various types of DNA damage. However, whether Rad51 could be a novel target for overcoming gemcitabine resistance in NSCLC cells needs further examination.

Clinical studies have shown that gemcitabine plus erlotinib is better than gemcitabine alone for advanced pancreatic cancer (Moore et al., 2007). Therefore, we designed a study to evaluate the role of Rad51 in cell survival in human lung cancer cells exposed to gemcitabine and erlotinib. We hypothesized that the ERK1/2 and AKT signaling pathways were responsible for gemcitabine resistance and that gemcitabine combined with erlotinib may enhance gemcitabine-mediated cytotoxicity by decreasing the activation of ERK1/2 and AKT and the expression of Rad51 in human NSCLC cell lines. Based on our data, combinatorial treatment with gemcitabine and inhibitors to ERK and AKT or the inhibition of Rad51 expression could provide new tools for treating patients with NSCLC, although the clinical relevance of these findings needs confirmation.

Materials and Methods

Cell Lines and Reagents. The human NSCLC cell lines A549, H520, and H1703 were obtained as described previously (Ko et al., 2009). Gemcitabine was obtained from Lilly France (Fegersheim, France). Erlotinib was purchased from Genentech (South San Francisco, CA). Cycloheximide and actinomycin D were purchased from Sigma-Aldrich (St. Louis, MO). 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), 1,4-diamo-2,3-dicyano-1,4-bis(methyl-thio)butadiene (U0126), N-benzoylloxycarbonyl (Z)-Leu-Leu-levulinic acid (MG132), and N-acetyl-Leu-Leu-norleucin (ALLN) were purchased from Calbiochem-Novabiochem (San Diego, CA).

Immunoblot Analysis. Immunoblot analysis was carried out as described previously (Ko et al., 2009). The specific phospho-ERK1/2 (Thr202/Tyr204), phospho-MKK1/2 (Ser217/Ser221), and phospho-AKT (Ser473) antibodies were obtained from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal antibodies against ERK2 (C-14), Rad51 (H-92), ubiquitin (P4D1), hemagglutinin probe (F-7), and actin (I-19) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The relative fold under each blot was calculated by averaging the results of three independent experiments and normalized by arbitrarily setting the densitometry of control cells to 100.

Immunoprecipitation. After treatment, equal amounts of proteins were immunoprecipitated using an anti-ubiquitin antibody and collected with protein G-Sepharose beads (GE Healthcare). The immunoprecipitate was then washed three times with ice-cold lysis buffer and subjected to immunoblot analysis.

Transfection with Small Interfering RNA. 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'-GGC CGC UUU GUA GGA TTC G-3' (Dharmacon RNA Technologies, Lafayette, CO), respectively. siRNA duplexes (200 nM) were transfected into cells using Lipofectamine 2000 (Invitrogen). After 24 h, the siRNA-transfected cells were treated with gemcitabine for 24 h, and Rad51 protein levels were determined by immunoblot analysis.

Transfection of Expression Plasmids into NSCLC Cells. The constitutively active expression plasmid (pCDNA3-myr-AKT) harbored a consensus myristoylation domain that replaces the 4 to 129 amino acids of wild-type AKT. Expression of MKK1/2-CA (constitutively active form of MKK1/2) plasmid was achieved as described previously (Ko et al., 2009). Exponentially growing human lung cancer cells (106) were plated for 18 h in RPMI 1640 complete medium (Invitrogen) before transfection. The myr-AKT and MKK1/2-CA expression vectors were transfected into cells using Lipofectamine 2000 (Invitrogen) before treatment with gemcitabine and erlotinib.

Cell Viability Analysis and Combination Index Analysis. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction method (Ko et al., 2009). Cytotoxicity induced by the combined treatment of gemcitabine and erlotinib was compared with the cytotoxicity induced by each drug individually with the use of a combination index (CI): CI values less than 0.9, equal to 0.9 to 1.1, and greater than 1.1 indicated synergistic, additive, and antagonistic effects, respectively (Peters et al., 2000). The CI analysis was performed using CalcuSyn software (Biosoft, Oxford, UK). CI values at a fraction affected of 0.5, 0.75, and 0.9 were averaged for each experiment, and the value was used to calculate the mean of the three independent experiments.
Reverse Transcriptase-Polymerase Chain Reaction. RNA was isolated from cultured cells using TRIzol (Invitrogen) according to the manufacturer’s instructions. RT-PCR was performed on 2 μg of total RNA using random hexamers according to the Moloney murine leukemia virus reverse transcriptase cDNA synthesis system (Invitrogen). The final cDNA was used for the subsequent polymerase chain reactions (Ko et al., 2009).

Determination of Cell Death. After treatment, unattached and attached cells were collected and stained using trypan blue solution (Sigma-Aldrich). 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.

Colony-Forming Ability Assay. Immediately after the si-Rad51 RNA transfection and gemcitabine treatment, cells were washed with phosphate-buffered saline and trypsinized for the determination of cell numbers. The cells were plated at a density of 200 to 500 cells 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% cresyl 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.

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

Results

Gemcitabine Stimulates Rad51 Expression and Increases the Phosphorylation Levels of MKK1/2, ERK1/2, and AKT In Human NSCLC Cell Lines. First, we examined the effects of gemcitabine on signaling molecules in human lung cancer cells. Three human NSCLC cell lines, A549, H520, and H1703, were exposed to 20 μg/ml gemcitabine for different periods of time, and the activation of MKK1/2, ERK1/2, and AKT was determined using immunoblot analysis. As shown in Fig. 1A, gemcitabine increased the levels of phospho-MKK1/2, phospho-ERK1/2, and phospho-AKT during different exposure times (3–24 h). Gemcitabine treatment also increased Rad51 protein levels, and this increase was accompanied by the activation of MKK1/2-ERK1/2 and AKT (Fig. 1A). Moreover, these three NSCLC cell lines were treated with various concentra-

![Fig. 1.](https://example.com)
tions of gemcitabine (2–20 µg/ml) for 24 h, resulting in the up-regulation of the protein levels of Rad51 and the phosphorylation levels of M KK1/2, ERK1/2, and AKT in a dose-dependent manner (Fig. 1B). To confirm the transcriptional regulation of Rad51 in the gemcitabine-treated NSCLC cell lines, mRNA levels were examined using the reverse transcriptase-polymerase chain reaction. Rad51 mRNA levels were increased under various concentrations of gemcitabine from 3 to 24 h (Fig. 1C and D).

**M KK-ERK and PI3K-AKT Maintain the Gemcitabine-Induced Rad51 mRNA Levels.** To examine whether the induction of Rad51 mRNA by gemcitabine was a result of the activation ERK1/2 and AKT, RT-PCR was performed to assess Rad51 gene expression. Cells treated with U0126 (M KK1/2 inhibitor) or LY294002 (PI3K inhibitor) had decreased basal and gemcitabine-induced Rad51 mRNA levels (Fig. 2A). We then examined whether gemcitabine could increase the stability of Rad51 mRNA in H1703 and H520 cells treated with actinomycin D (an inhibitor of RNA synthesis) and gemcitabine for 3 to 9 h. Figure 2B shows that the mRNA stability of Rad51 was increased after treatment with gemcitabine. However, blocking the activation of AKT using LY294002 decreased the stability of Rad51 mRNA in these two NSCLC cell lines treated with gemcitabine (Fig. 2C). In contrast, U0126 did not affect the stability of Rad51 mRNA that was induced by gemcitabine (Fig. 2D).

**Blockage of ERK1/2 and AKT Activation Decreases Rad51 Protein Levels and Protein Stability Induced by Gemcitabine.** Furthermore, we determined whether the ERK1/2 and AKT signaling pathways were involved in gemcitabine-induced Rad51 protein expression in the previously mentioned NSCLC cell lines. U0126 decreased the levels of Rad51 protein and phosphorylated ERK1/2 induced by gemcitabine (Fig. 3A). In addition, LY294002 caused the inactivation of AKT and a significant decrease of Rad51 protein levels in gemcitabine-treated NSCLC cells (Fig. 3A). To explore further whether gemcitabine affected the Rad51 protein levels at the translational level, 60 µg/ml cycloheximide, an inhibitor of de novo protein synthesis, was added during the final 3 to 9 h of the exposure of cells to gemcitabine. In H1703 cells, only ~45% of Rad51 remained in the cells treated with cycloheximide for 6 h compared with untreated cells; however, approximately ~78% of Rad51 remained in cells treated with gemcitabine and cycloheximide compared with control cells, indicating that gemcitabine remarkably enhanced the stability of the Rad51 protein (Fig. 3B). The cells were then cotreated with gemcitabine and U0126 or LY294002 to clarify whether ERK1/2 and AKT signaling was involved in the up-regulation of Rad51 protein expression. U0126 and LY294002 generated a significant inhibitory effect on gemcitabine-elicited Rad51 protein stability in H1703 and A549 cells (Fig. 3C). These results suggested that the up-regulation of Rad51 protein levels by gemcitabine re-

![Fig. 2. U0126 or LY294002 decreases Rad51 mRNA levels in NSCLC cells.](https://jpet.aspetjournals.org/)

A. NSCLC cells were exposed to 10 µg/ml gemcitabine and 5 µM U0126 (U0) or 10 µM LY294002 (LY) for 24 h. B to D, cells were exposed to 10 µg/ml gemcitabine in the presence or absence of 10 µM LY294002 or 5 µM U0126 for 9 h, followed by the addition of 2 µg/ml actinomycin D for 3 to 9 h. After treatment, total RNA was isolated and subjected to RT-PCR for Rad51. The relative fold under each experiment was calculated by averaging the results of three independent experiments and was normalized by arbitrarily setting the densitometry of control cells to 100. bp, base pairs; gapdh, glyceraldehyde-3-phosphate dehydrogenase.
sulted from the increase in its protein stability via the activation of ERK1/2 and AKT.

**ERK1/2 and AKT Signaling Protect Rad51 from Ubiquitin-26S Proteasome-Mediated Degradation.** Next, we hypothesized that ERK1/2 and AKT signaling are involved in the regulation of Rad51 stability by preventing the ubiquitin-26S proteasomal degradation pathway. As shown in Fig. 3D, whereas U0126 or LY294002 attenuate the gemcitabine-elicited Rad51 protein levels, both MG132 and ALLN, common 26S proteasomal inhibitors, restore the Rad51 protein levels. In addition, we examined the levels of ubiquitin conjugates on Rad51. Inhibition of ERK1/2 or AKT signaling by U0126 or LY294002 significantly increased the levels of ubiquitin-conjugated Rad51 in gemcitabine-treated NSCLC cell lines (Fig. 3E). The results revealed that ERK1/2 and AKT signaling increase Rad51 protein stability by protecting Rad51 from the ubiquitin-mediated degradation pathway in gemcitabine-treated NSCLC cells.

**Inactivation of ERK1/2 and AKT Enhance the Cytotoxicity of Gemcitabine.** We investigated the roles of ERK1/2 and AKT activation induced by gemcitabine in the lung cancer cells. In MTT and trypan blue exclusion assays, the inhibition of ERK1/2 or AKT by U0126 or LY294002, respectively, enhanced the cytotoxicity of gemcitabine (Fig. 4, A and B). Furthermore, we examined the antiproliferative effects for the combination of gemcitabine and U0126 or gemcitabine and LY294002 using an MTT assay. The results demonstrated that U0126 or LY294002 also enhanced the gemcitabine-induced inhibition of cell growth in the three previously mentioned NSCLC cell lines (Supplementary Fig. S1). These results indicate that ERK1/2 and AKT have protective effects against the induction of cytotoxicity by gemcitabine in NSCLC cell lines.
Induction of ERK1/2 and AKT Phosphorylation by Gemcitabine Can Be Prevented by Erlotinib. Erlotinib can decrease the activation of ERK1/2 and AKT in NSCLC cells (Ko et al., 2009); therefore, we tested whether erlotinib could enhance the cytotoxicity of gemcitabine via the inactivation of ERK1/2 and AKT. The gemcitabine-induced activation of ERK1/2 and AKT was decreased by erlotinib (Fig. 5A). In addition, under gemcitabine-treated lung cancer cells, the

Fig. 4. U0126 and LY294002 enhance the gemcitabine-induced cytotoxicity. A, A549, H1703, and H520 cells were treated with gemcitabine (5–20 μg/ml) and U0126 (10 μM) or LY294002 (10 μM) for 24 h. Cell viability was determined using an MTT assay. The results (mean ± S.E.M.) were obtained from three independent experiments. B, after treatment, the unattached and attached cells were collected and stained with trypan blue dye. The number of stained cells (dead) was counted manually. Columns, percentage of trypan blue-positive cells, representing the population of dead cells; bar, S.E. from three independent experiments. The results from different cell lines treated with gemcitabine alone or cotreated with U0126 or LY294002 were compared. **, P < 0.01 (Student’s t test).

Fig. 5. Erlotinib decreases the gemcitabine-induced levels of phospho-ERK1/2, phospho-AKT, and Rad51. A, H1703 and A549 cells were exposed to erlotinib (50 μM) and gemcitabine (5–20 μg/ml) for 24 h. B, cells were treated with 10 μg/ml gemcitabine and 50 μM erlotinib for 9 h, and were then treated with 60 μg/ml cycloheximide (CHX) for 3 to 9 h. The cell extracts were subjected to immunoblot analysis. C, cells were exposed to 50 μM erlotinib and 10 μg/ml gemcitabine for 24 h, and total RNA was isolated and subjected to RT-PCR for Rad51. D, MKK1/2-CA and myr-AKT expression vectors were transfected into A549 and H1703 cells using Lipofectamine 2000. After transfection, the cells were treated with 50 μM erlotinib and 20 μg/ml gemcitabine for 24 h. The cell extracts were subjected to immunoblot analysis. bp, base pair(s); gapdh, glyceraldehyde-3-phosphate dehydrogenase.
Rad51 protein levels were inhibited by erlotinib, with a decrease in the stability of the Rad51 protein (Fig. 5, A and B). Treatment with erlotinib or erlotinib plus gemcitabine downregulated the levels of Rad51 mRNA compared with untreated cells (Fig. 5C). Next, we determined whether ERK1/2 and AKT inactivation was involved in the down-regulation of Rad51 protein expression after treatment with gemcitabine plus erlotinib; the MKK1/2-CA vectors were transfected into human lung cancer cells. MKK1/2-CA recovered the levels of phospho-ERK1/2 and Rad51 inhibited by treatment with erlotinib and gemcitabine (Fig. 5D). Overexpression of a constitutively active form of AKT (myr-AKT) also rescued the Rad51 protein levels that were suppressed by treatment with erlotinib and gemcitabine (Fig. 5D). Furthermore, transfection of MKK1-CA or myr-AKT restored Rad51 protein stability in H1703 and A549 cells treated with erlotinib and gemcitabine compared with pcDNA3-transfected control cells (Supplementary Fig. S2). In conjunction with the previous findings, the inactivation of ERK1/2 and AKT by erlotinib decreased the gemcitabine-induced expression of Rad51 by increasing the instability of the Rad51 protein.

Combined Treatment with Erlotinib Enhances the Gemcitabine-Induced Cytotoxicity in NSCLC Cells. We next examined whether erlotinib could enhance the cytotoxicity of gemcitabine in the A549, H1703, and H520 cells. In Fig. 6A, erlotinib enhances the cytotoxicity of cells treated with gemcitabine. The combined effects of the drugs were analyzed as described under Materials and Methods. As seen in Fig. 6B, the mean values of CI were 0.0439, 0.1728, and 0.0876 after gemcitabine and erlotinib treatment of the A549, H1703, and H520 cells, respectively, indicative of a synergistic effect. Consistent with these observations, cell death was higher in cells that were cotreated with the two drugs than when they were treated with gemcitabine or erlotinib alone, as determined using the trypan blue exclusion assay (Fig. 6C).

Rad51 Expression Is Involved in Gemcitabine-Mediated Cell Growth Inhibition and Cytotoxicity. To determine whether Rad51 participated in the gemcitabine-elicited inhibition of cell growth and cytotoxicity, we knocked down Rad51 using a specific siRNA duplex (si-Rad51 RNA). As shown in Fig. 7A, si-Rad51 RNA effectively suppresses Rad51 protein expression induced by various concentrations of gemcitabine (5–20 µg/ml) and erlotinib (50 µM) for 24 h. The cell viability was determined using an MTT assay. The results (mean ± S.E.M.) were obtained from three independent experiments. B, the mean CI values for the gemcitabine-erlotinib combination treatment in A549, H1703, and H520 cells. CI values were averaged for each experiment, and the values were used to calculate the mean between experiments, as described under Materials and Methods. Points and columns, mean values obtained from three independent experiments; bars, S.E. C, unattached and attached cells were collected and stained with trypan blue dye. The number of stained cells (dead) was counted manually. Columns, percentage of trypan blue-positive cells, representing the population of dead cells; bar, S.E. from three independent experiments. The results from different cell lines treated with gemcitabine alone or cotreated with erlotinib were compared. **, P < 0.01 (Student’s t test).
of gemcitabine. On the other hand, si-Rad51 RNA did not interfere with the gemcitabine-induced activation of ERK1/2 and AKT. To examine further the role of Rad51 in the gemcitabine-mediated cell effects, si-Rad51 RNA-transfected cells were cultured in the presence or absence of gemcitabine, and the cytotoxicity was assessed using MTT and colony-forming ability assays (Fig. 7, B and C). It is interesting to note that suppression of Rad51 protein expression by si-Rad51 RNA markedly increased the sensitivity of cells to gemcitabine compared with si-control RNA-transfected cells (Fig. 7, B and C). In addition, blockage of Rad51 protein expression significantly enhanced the antiproliferative effects caused by gemcitabine, which were analyzed by a trypan blue exclusion assay (Fig. 7D). Taken together, induction of Rad51 resulted in anticytotoxicity effects in gemcitabine-exposed human lung cancer cells.

Overexpression of Rad51 Protects NSCLC Cells from Cytotoxicity Induced by Gemcitabine and Erlotinib. To assess the role of Rad51 on the combined effects of gemcitabine and erlotinib, the cells were transfected with FLAG-Rad51 and then incubated with gemcitabine in the presence or absence of erlotinib. Cell viability was analyzed using an MTT assay (Fig. 8A). Treatment of cells with gemcitabine and erlotinib decreased cell viability by ~70%. It is interesting to note that the induction of cytotoxicity by gemcitabine and erlotinib was significantly reduced (by ~55%) after overexpression of Rad51 (Fig. 8B). Therefore, Rad51 protects NSCLC cells from the cytotoxicity induced by gemcitabine and erlotinib.

Erlotinib Enhances Gemcitabine-Induced Cytotoxicity via the Inactivation of ERK1/2 and AKT. We determined whether activation of ERK1/2 and AKT could protect the cells from the cell death induced by gemcitabine and erlotinib. H520 and A549 cells were transfected with MKK1/2-CA and/or myr-AKT to evaluate the viability of cells treated with erlotinib and/or gemcitabine. Transfection of MKK1-CA and myr-AKT recovered the cell death induced by erlotinib and gemcitabine in H520 and A549 cells (Fig. 8C). Together, the activation of ERK1/2 and AKT and the expression of Rad51 protein and mRNA are suppressed by erlotinib, leading to the enhancement of gemcitabine-induced cytotoxicity.

Discussion
In this study, we first found that gemcitabine induced the expression of Rad51 via the activation of ERK1/2 and AKT.
Knockdown of Rad51 expression enhanced the gemcitabine-induced cytotoxicity. Consistent with the findings of our study, the inactivation of ERK1/2 by U0126 enhanced cell death in gemcitabine-treated hepatocellular and cholangiocellular carcinoma cells (Matsumoto et al., 2008). Therefore, these results demonstrate that the MKK1/2-ERK1/2 signaling pathway was responsible, in part, for the resistance to gemcitabine. In addition, nuclear factor-κB (Arlt et al., 2003) and bcl-2 (Okamoto et al., 2007) are associated with gemcitabine resistance. In our study, the overexpression of Rad51 enhanced the viability of cells treated with gemcitabine. In contrast, the down-regulation of Rad51 expression enhanced the gemcitabine-induced cytotoxicity and cell growth arrest. Therefore, Rad51 plays an important role in the chemoresistance to gemcitabine. Moreover, Rad51 siRNA enhances the sensitivity of cancer cells to cisplatin in vitro and in vivo (Ito et al., 2005). Low levels of BRCA1 also correlate with increased survival in patients with NSCLC treated with gemcitabine combined with cisplatin (Rosell et al., 2004; Taron et al., 2004). BRCA1 induces the translocation of Rad51 to single-stranded DNA (Wyman and Kanaar, 2004; San Filippo et al., 2008); however, further experiments are necessary to determine whether BRCA1 is involved in the gemcitabine-induced expression of Rad51.

EGFR-TKIs can block the Ras-Raf-MKK-ERK and PI3K-AKT pathways (Schlessinger, 2002) that are implicated in the inhibition of cell apoptosis and the promotion of cell growth and motility (Shibata et al., 1996). We have shown that the EGFR-TKI erlotinib enhances the gemcitabine-induced cytotoxicity via a decrease in the activation of ERK1/2 and AKT. Transfection of MKK1/2-CA or myr-AKT, to enhance the activity of ERK and AKT, respectively, decreased the gemcitabine- and erlotinib-induced cytotoxicity. Consistent with our study, increased activation of the PI3K-AKT and MKK1/2-ERK1/2 signaling pathways has been observed in TKI-resistant NSCLC (Janmaat et al., 2003; Kokubo et al., 2005). In pancreatic cancer, erlotinib in combination with gemcitabine and radiotherapy inhibited pAKT (Ser473) and increased the efficacy of gemcitabine and radiotherapy treatment (Morgan et al., 2008).

In preclinical models, the additive or synergistic activity of EGFR-TKIs and several chemotherapeutic drugs has been reported (Ciardiello et al., 2000). In this study, erlotinib blocked the activation of ERK1/2 and AKT in gemcitabine-treated NSCLC cell lines. It is interesting to note that erlotinib enhanced the gemcitabine-induced cytotoxicity and decreased the gemcitabine-elicted expression of Rad51. Overexpression of Rad51 rescued the gemcitabine- and erlotinib-induced cytotoxicity. Consistent with these observations, erlotinib attenuated the radiation-induced expression of Rad51 and enhanced the induction of apoptosis in radiation-treated human NSCLC (H226) cells (Chinnaiyan et al., 2005). Therefore, the Rad51
protein protects lung cancer cells from the synergistic cytotoxicity induced by gemcitabine and erlotinib.

In this study, the up-regulation of Rad51 expression via the ERK1/2 and AKT signaling pathways in gemcitabine-treated NSCLC cells was required for cell survival. High expression of Rad51 in tumor tissues is associated with an unfavorable prognosis in lung cancer (Qiao et al., 2005). Chronic hypoxia could decrease the levels of Rad51, leading to reduced levels of HR in hypoxic tumors and increased sensitivity to DNA cross-linking agents (Bristow and Hill, 2008; Chan et al., 2008). Our studies have shown that the down-regulation of Rad51 in lung cancer cells could increase the chemosensitivity of cells to gemcitabine.

This study is the first report, to our knowledge, that the PI3K-AKT signaling pathways control the expression of Rad51 and maintain its mRNA stability after exposure to gemcitabine. Previous studies have also shown that the PI3K inhibitor wortmannin can prevent radiation-induced apoptosis and the formation of Rad51 foci in NSCLC (Sak et al., 2005). In addition, there is evidence indicating that c-Abl is involved in increasing Rad51 transcription. The oncogenic fusion tyrosine kinase BCR/Abi enhances Rad51 expression (Slupianek et al., 2001); conversely, inmatinib (Gleevec; Novartis, Basel, Switzerland), a c-Abl inhibitor, reduces the elevated levels of Rad51 and sensitized tumor cells to experimental chemotherapy and radiotherapy (Choudhury et al., 2009). Taken together, these data indicate that Rad51 is essential for cell survival when NSCLC cells are treated with EGFR-TKIs, radiation, or chemotherapeutic agents.

In conclusion, we have clearly demonstrated that the combined inhibition of ERK1/2 and AKT by EGFR-TKI enhances the cytotoxicity of gemcitabine in three NSCLC cell lines. Suppression of Rad51 expression by blocking the MKK1/2-ERK1/2 and PI3K-AKT pathways may represent a novel therapeutic modality to enhance cytotoxicity and overcome the drug resistance of gemcitabine in NSCLC.

Acknowledgments

We thank Show-Mei Chuang, Chia-Chie Chang, and Jia-Ling Yang for providing the expression plasmids.

References

Arlt A, Gehrz A, Muë rkoë ster S, Vorndamm J, K ruse ML, Foë lsch UR, and Schaë fer H (2001); conversely, imatinib (Gleevec; Novartis, Basel, Switzerland), a c-Abl inhibitor, reduces the elevated levels of Rad51 and sensitized tumor cells to experimental chemotherapy and radiotherapy (Choudhury et al., 2009). Taken together, these data indicate that Rad51 is essential for cell survival when NSCLC cells are treated with EGFR-TKIs, radiation, or chemotherapeutic agents.

In conclusion, we have clearly demonstrated that the combined inhibition of ERK1/2 and AKT by EGFR-TKI enhances the cytotoxicity of gemcitabine in three NSCLC cell lines. Suppression of Rad51 expression by blocking the MKK1/2-ERK1/2 and PI3K-AKT pathways may represent a novel therapeutic modality to enhance cytotoxicity and overcome the drug resistance of gemcitabine in NSCLC.


**Address correspondence to:** Yun-Wei Lin, Department of Biochemical Science and Technology, National Chiayi University, Chiayi 600, Taiwan. E-mail: linyw@mail.nchu.edu.tw