Potentiation of the Effect of Erlotinib by Genistein in Pancreatic Cancer: The Role of Akt and Nuclear Factor-κB

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Abstract

The epidermal growth factor receptor (EGFR) is a target of new therapies in most nonhematologic cancers. EGFR blockade alone may not be sufficient for the control of growth and invasion of human pancreas cancer because of the independent activation of Akt and nuclear factor-κB (NF-κB). The expression of EGFR, Akt, and NF-κB was determined in six human pancreatic cancer cell lines. Selected cells for specific expression were treated with erlotinib, genistein, gemcitabine, or the combination. Growth inhibition was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and apoptosis was assayed by ELISA. EGFR, phosphorylated EGFR, phosphorylated Akt, and survivin expressions were determined by immunoblotting. Electrophoretic mobility shift assay was used to evaluate the DNA binding activity of NF-κB. Genistein significantly increased ($P < 0.05$) erlotinib-induced growth inhibition and apoptosis in BxPC-3, CAPAN-2, and AsPC-1 cells. In the BxPC-3 cell line, significant down-regulation of EGFR, phosphorylated Akt, NF-κB activation, and survivin was observed in the cells treated with the combination compared with the erlotinib-treated cells. In the HPAC and MIAPaCa cell line, no potentiation of the effects of erlotinib by genistein on cell growth or inhibition of the EGFR/Akt/NF-κB was observed. Genistein potentiated growth inhibition and apoptosis of the gemcitabine and erlotinib combination in COLO-357 cell line. Genistein potentiates the growth inhibition and apoptosis induced by erlotinib and gemcitabine in certain pancreatic cancer cells. Akt and NF-κB inhibition represents one of the mechanisms for the potentiation of erlotinib- and gemcitabine-induced cell death by genistein. (Cancer Res 2006; 66(21): 10553-9)

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related mortality in the United States (1). The 5-year survival of patients with pancreatic cancer is <5% without significant improvement over the past three decades (1). This poor prognosis is attributed to the high incidence of metastatic disease at diagnosis. Consequently, major improvement in the outlook of this disease will depend on the development of more effective drug therapies. The effect of newer cytotoxic chemotherapy on survival has been disappointing because of intrinsic drug resistance (2). Therefore, a rational strategy for future drug development is to specifically target the critical cellular pathways regulating proliferation, survival, and invasion.

The epidermal growth factor receptor (EGFR) is overexpressed and/or activated in a large proportion of human pancreatic cancers (3, 4). Activation of EGFR leads to phosphorylation and activation of phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (5). Akt in turn activates nuclear factor-κB (NF-κB) leading to the transcription of genes regulating growth, apoptosis, angiogenesis, and invasion (6). *Cyclooxygenase 2* (*COX-2*; ref. 7), *survivin* (8), *Bcl-xL*, and *Bcl-2* (9, 10) are among the genes that are transcriptionally regulated by NF-κB. In preclinical models, inhibition of the EGFR inhibits downstream signaling and sensitizes pancreatic cancer cells to the effects of certain cytotoxic agents, including gemcitabine (11–13). Moore et al. (14) reported recently that the addition of erlotinib to gemcitabine resulted in a very modest improvement in survival of patients with advanced pancreatic cancer. The very small benefit of EGFR blockade in pancreas and other common epithelial cancers is the EGFR-independent activation of Akt and NF-κB by a variety of mechanisms resulting from multigene mutations. It is therefore assumed that combining EGFR blockers, such as erlotinib, with an Akt/NF-κB inhibitor would be necessary to more effectively inhibit the EGFR signaling pathway and to sensitize the cancer cells to EGFR blockers and/or cytotoxics.

Genistein is a naturally occurring isoflavone present in soybeans (15). Isoflavones inhibit pancreatic cancer cell growth, induce apoptosis, and chemosensitize those cells by the inhibition of PI3K leading to inhibition of Akt and NF-κB (16–19). *In vivo* activity of genistein has been shown in healthy volunteers where NF-κB activation was inhibited in peripheral lymphocytes (20). Based on the *in vitro* and *in vivo* experimental data, we hypothesized that the addition of genistein to erlotinib will improve the effect of the inhibition of the EGFR/Akt/NF-κB signaling pathway on growth and survival of pancreas cancer cells. We have provided data in support of our hypothesis, which is summarized by a hypothetical mechanism of multitargeted blockade in cancer cells treated with erlotinib plus genistein (Fig. 1).

Materials and Methods

Cells, drugs, and reagents. Human pancreatic cancer cell lines AsPC-1, BxPC-3, CAPAN-1, CAPAN-2, COLO-357, HPAC, and MIAPaCa were used in this study. BxPC-3 and CAPAN-1 cells were grown in RPMI 1640 with 10% fetal bovine serum (FBS). AsPC-1, COLO-357, and MIAPaCa cells were grown as a monolayer cell culture in DMEM containing 4.5 mg/mL D-glucose and L-glutamine supplemented with 10% FBS. CAPAN-2 and HPAC cells were grown in McCoy's 5A and DMEM/F-12 (1:1) with 10% FBS. Erlotinib was a generous gift from OSI Pharmaceuticals (Melville, NY). Genistein was purchased from Toronto Research Chemicals (North York,

Note: B.F. El-Rayes and S. Ali contributed equally to this work.

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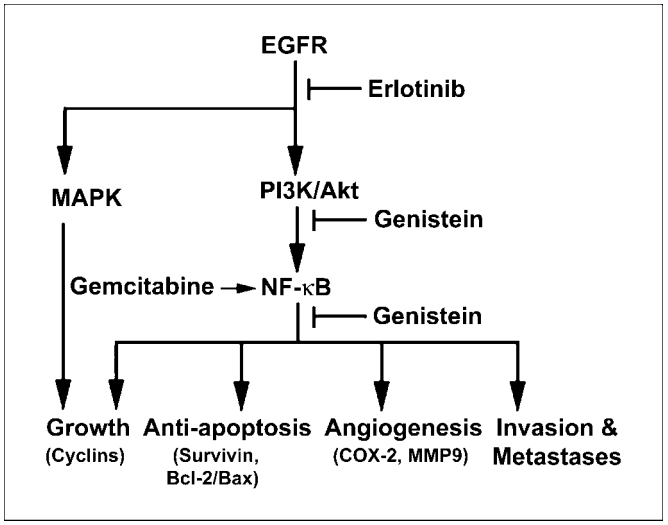

Figure 1. Schematic diagram of the EGFR pathway showing the EGFR activation of the Akt/NF-κB pathway and potential sites of blockade by drugs.

Ontario, Canada). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), isopropanol, and DMSO were acquired from Sigma Chemical (St. Louis, MO). Cell culture medium was purchased from Invitrogen (Carlsbad, CA).

Cell viability assay. The viability of treated cells was determined by the standard MTT reduction assay. Cells were plated (3,000-5,000 per well) in 96-well plate and incubated overnight at 37°C. Erlotinib was dissolved in DMSO and added to cell culture medium at a volume:volume concentration not exceeding 0.1%. Genistein was dissolved in 0.1 mol/L $NaHCO_3$, whereas gemcitabine was dissolved in water. The effects of genistein (25 μmol/L), erlotinib (2 μmol/L), and the combination in all six cell lines were studied. The effect of genistein (20 μmol/L), erlotinib (1 μmol/L), gemcitabine (10 nmol/L), and the dual and triple combination concomitantly for 72 hours was also studied in COLO-357 and MIAPaCa human pancreatic cancer cells. The MTT assay was done in triplicates for each drug concentration used. After the required drug treatment time, aliquots of 100 μL of MTT (1 mg/mL) were added to each well and incubated for 2 hours at 37°C. The supernatant was removed and isopropanol (100 μL) was then added. The color intensity was measured by Tecan microplate fluorometer (Tecan, Research Triangle Park, NC) at 595 nm. DMSO-treated cells were assigned a value of 100%. Linearity of the color intensity relative to cell number within the range expected in the study was determined at the outset.

Quantification of apoptosis by ELISA. The Cell Death Detection ELISA kit (Roche Applied Science, Indianapolis, IN) was used to detect apoptosis in treated BxPC-3, HPAC, MIAPaCa, and COLO-357 cells. The assay is based on a photometric enzyme immunoassay for the qualitative and quantitative determination of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes). The assay uses anti–histone-biotin antibodies that bind to H2A, H2B, H3, and H4 histones and anti–DNA peroxidase antibodies that react with ssDNA and dsDNA. Cells seeded in six-well plates were treated with genistein (25 μmol/L), erlotinib (2 μmol/L), or the combination. In addition, COLO-357 and MIAPaCa cells were also treated with genistein (20 μmol/L), erlotinib (1 μmol/L), gemcitabine (10 nmol/L), or the dual and triple combination. The cells were trypsinized and ~10,000 cells were added to 200 μL lysis buffer and incubated at room temperature for 30 minutes. The cells were centrifuged at 20,000 × *g* for 10 minutes and supernatant (100 μL) was transferred into anti-histone–coated microtiter plate and incubated at room temperature for 90 minutes. The plate was washed twice with 200 μL washing solution provided with the kit. A solution containing 100 μL anti–DNA peroxidase dissolved in incubation buffer was added to the same plate and incubated for 90 minutes. After removal of the unbound antibodies, the nucleosomes were quantified by the peroxidase reaction using 2,2′-azino-di-(3-ethylbenzthiazoline-sulfonate) as substrate. Tecan microplate fluorometer was used to measure color intensity at 405 nm.

Protein extraction and Western blot analysis. The expression of the survivin, Bcl-xL, Bcl-2, HERr-2/*neu*, COX-2, EGFR, and phosphorylated Akt proteins was determined in untreated AsPC-1, BxPC-3, CAPAN-1, CAPAN-2, COLO-357, HPAC, and MIAPaCa cells. BxPC-3 cells were treated with genistein with a range of concentrations (10-50 μmol/L) and also with erlotinib (2-10 μmol/L) to determine the effects of treatment on EGFR and phosphorylated EGFR. BxPC-3, HPAC, and MIAPaCa cells were treated with genistein (25 μmol/L), erlotinib (2 μmol/L), or the combination for 72 hours to determine the effects of treatment on phosphorylated Akt, EGFR, phosphorylated EGFR, survivin, and β-actin expression. In addition, COLO-357 and MIAPaCa cells were also treated with genistein (20 μmol/L), erlotinib (1 μmol/L), gemcitabine (10 nmol/L), or the dual and triple combination to determine the effects of treatment on EGFR, Bcl-xL, and survivin. Cells were harvested by scraping from culture plates and collected by centrifugation. Cells were resuspended in lysis buffer consisting of 250 mmol/L NaCl, 50 mmol/L Tris buffer (pH 7.5), 5 mmol/L EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L sodium fluoride, 1 mmol/L sodium orthovandate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL pepstatin, and protein inhibitor, which contains a broad spectrum of serine, cysteine, and metalloproteinases (Roche Applied Science) for 30 minutes on ice. Cell lysates were centrifuged for 20 minutes. Protein concentration was then measured using bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). The samples were loaded on 10% SDS-PAGE for separation and electrophoretically transferred to a nitrocellulose membrane. Each membrane was incubated with monoclonal antibody against survivin (R&D Systems, Inc. Minneapolis, MN), Bcl-xL (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-2 (1:200; Calbiochem, San Diego, CA), HER-2/*neu* (1:500; Oncogene, Cambridge, MA), COX-2 (1:1,000; Cayman Chemical Co., Ann Arbor, MI), EGFR (1:1,000; Neomarkers, Freemont, CA), EGFR-phosphorylated Tyr^{1173} (Upstate USA, Inc., Charlottesville, VA), phosphorylated AKT (1:1,000; Cell Signaling, Beverly, MA), and β-actin (1:2,000; Sigma). Blots were washed with phosphate buffer containing 0.05% Tween 20 and incubated with secondary antibodies conjugated with peroxidase. The signal intensity was then measured using chemiluminescent detection system (Pierce). Autoradiograms of the Western blots were scanned with Hewlett-Packard (Palo Alto, CA) scan jet and bands were quantitated using AlphaEaseFC software tool (Alpha Innotech Corp., San Leandro, CA).

Electrophoretic mobility shift assay for NF-κB activation. BxPC-3, HPAC, and MIAPaCa cells were treated with genistein (25 μmol/L), erlotinib (2 μmol/L), or the combination for 72 hours. COLO-357 and MIAPaCa cells were also treated with genistein (20 μmol/L), erlotinib (1 μmol/L), gemcitabine (10 nmol/L), or the dual and triple combination. The cells were suspended in 400 μL of ice-cold lysis buffer containing 1 mol/L HEPES (pH 7.9), 1 mol/L KCl, 0.5 mol/L EDTA, 0.1 mol/L EGTA, 0.1 mol/L DTT, 0.1 mol/L PMSF, 1 mg/mL aprotinin, 1 mg/mL leupeptin, and 250 mg/mL benzamidine for 15 minutes. About 12.5 μL of 10% NP40 were added to every 400 μL cell suspension, vortexed, and centrifuged for 1 minute at 4°C. The nuclear pellet was resuspended in 25 μL nuclear extraction buffer containing 1 mol/L HEPES (pH 7.9), 5 mol/L NaCl, 0.5 mol/L EDTA, 0.1 mol/L EGTA, 0.1 mol/L DTT, 0.1 mol/L PMSF, 1 mg/mL aprotinin, 1 mg/mL leupeptin, and 250 mg/mL benzamidine on ice for 30 minutes and then centrifuged at 10,000 × *g* for 20 minutes at 4°C. The supernatant was quantified using BCA protein assay kit.

Electrophoretic mobility shift assay (EMSA) was done using the Odyssey Infrared Imaging System (LI-COR, Inc., Lincoln, NE) with NF-κB IRDye-labeled oligonucleotide (LI-COR). The DNA-binding reaction was set up using 5 μg of the nuclear extract mixed with oligonucleotide and gel shift binding buffer consisting of 20% glycerol, 5 mmol/L $MgCl_2$, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 250 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), and 0.25 mg/mL poly(dI):poly(dC). The reaction was incubated at room temperature in the dark for 30 minutes. Orange loading dye (2 μL of 10×) was added to each sample and loaded on the prerun 8% polyacrylamide gel and ran at 30 mA for 1 hour. The gel was scanned using Odyssey Infrared Imaging System.

Statistical methods. Comparisons of survival, apoptosis, and EMSA for NF-κB between untreated and treated cells and the different treatment modalities were undertaken by the Student's *t* test. Statistical significance was assumed for a $P < 0.05$.

Results

EGFR signaling pathway in pancreatic cancer cells. The baseline expression and activation of the EGFR signaling proteins was determined in a panel of human pancreatic cancer cell lines that included AsPC-1, BxPC-3, CAPAN-1, CAPAN-2, COLO-357, HPAC, and MIAPaCa. The assays included the expression of membrane receptors (EGFR and HER-2/*neu*) activated downstream signaling proteins (phosphorylated Akt and NF-κB) and proteins that are transcriptionally regulated by NF-κB (COX-2, survivin, Bcl-xL, and Bcl-2). The results showed that the EGFR signaling pathway was frequently but differentially dysregulated in the different human pancreatic cancer cell lines (Fig. 2*A* and *B*).

The effects of genistein and erlotinib on the phosphorylation of EGFR in the BxPC-3 cells. The BxPC-3 cells were chosen to estimate the optimal concentrations of erlotinib and genistein in the inhibition of EGFR phosphorylation. There was a dose-dependent inhibition of EGFR phosphorylation in BxPC-3 cells treated with erlotinib at concentrations of 2, 5, and 10 μmol/L and by genistein at concentrations of 10, 25, and 50 μmol/L (Fig. 2*C*). The lowest erlotinib and genistein concentrations sufficient to inhibit the phosphorylation of the EGFR in BxPC-3 cell lines were selected for all subsequent experiments.

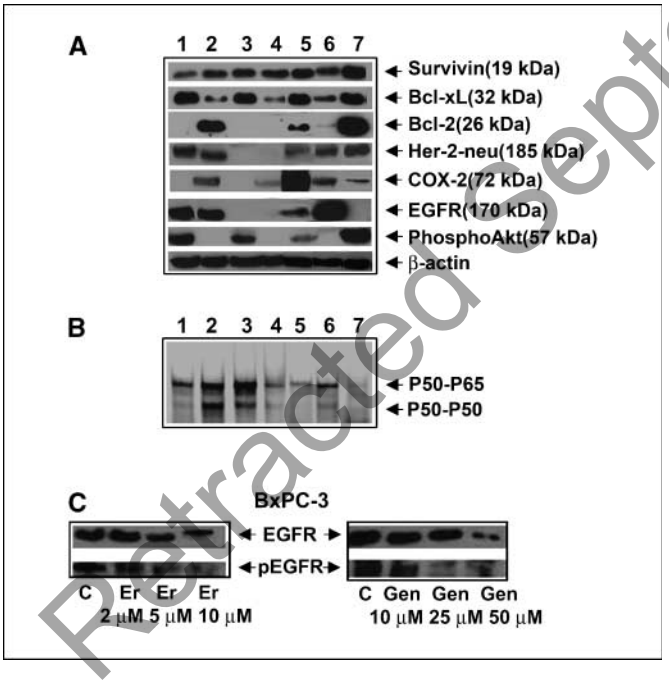

Figure 2. The level of survivin, Bcl-xL, Bcl-2, HER-2/*neu*, COX-2, EGFR and phosphorylated AKT (*PhosphoAkt*) expression, and NF-κB activation was compared between a panel of pancreatic cancer cell lines, including AsPC-1, BxPC-3, CAPAN-1, CAPAN-2, COLO-357, HPAC, and MIAPaCa cell lines (*lanes 1-7*, respectively). *A,* expression of protein was assayed by Western blot analysis. *B,* NF-κB activation was evaluated by the EMSA. *C,* the expression of EGFR and phosphorylated EGFR (*pEGFR*) in BxPC-3 human pancreatic cancer cells treated with 2, 5, and 10 μmol/L erlotinib (*Er*) and 10, 25, and 50 μmol/L genistein (*Gen*). Down-regulation of EGFR and phosphorylated EGFR protein expression was observed in cells treated with erlotinib and genistein in a dose-dependent manner (*C*).

The effects of genistein and erlotinib on the viability of pancreatic cancer cells. The viability of AsPC-1, BxPC-3, CAPAN-1, CAPAN-2, HPAC, and MIAPaCa, pancreatic cancer cells treated with genistein (25 μmol/L), erlotinib (2 μmol/L), or the combination, was determined by the MTT assay (Fig. 3). In the AsPC-1, BxPC-3, and CAPAN-2 cell lines, a significant potentiation of the growth inhibition of erlotinib by genistein was observed (Fig. 3). A trend toward potentiation of growth inhibition of erlotinib by genistein was observed in the CAPAN-1 and MIAPaCa cell lines. Erlotinib-inhibited growth of the HPAC cell line but no potentiation was observed with the addition of genistein. Therefore, the BxPC-3, HPAC, and MIAPaCa cells were selected to determine the mechanisms of enhanced growth inhibition.

Induction of apoptosis by erlotinib, genistein, and the combination. Apoptosis assays were done using BxPC-3, HPAC, and MIAPaCa cell lines to determine the mechanism of the observed growth inhibition. The effects of genistein (25 μmol/L) and erlotinib (2 μmol/L) individually and in combination were tested using the ELISA assay. Exposure of BxPC-3 and HPAC cells to either genistein or erlotinib for 72 hours significantly enhanced apoptosis (Fig. 4). The addition of genistein to erlotinib further increased apoptosis only in the BxPC-3 cell line. In the MIAPaCa cell line, no insignificant induction of apoptosis was seen by any treatment.

The effects of genistein, erlotinib, and the combination on the EGFR signaling pathway. The mechanisms contributing to enhancement of apoptosis by gensitein were studied in the BxPC-3, MIAPaCa, and HPAC cells. Specifically, the effects of the drugs on Akt and NF-κB in the context of EGFR inhibition were determined (Fig. 5). The down-regulation of EGFR and its phosphorylation together with Akt and NF-κB activation by erlotinib were potentiated by genistein in the BxPC-3 cell line (Fig. 5*A* and *B*). The combination treatment down-regulated the *survivin* and *EGFR* transcription through inhibition of NF-κB. Genistein down-regulated NF-κB activation in HPAC cells but no significant down-regulation of the expression of EGFR or survivin was observed in the HPAC cells treated with genistein or the combination (Fig. 5*C* and *D*). This suggested that transcription factors other than NF-κB are involved in the regulation of the expression of *survivin* and *EGFR* genes in HPAC cell lines. In the MIAPaCa cells, genistein inhibited NF-κB activation. However, neither genistein nor erlotinib inhibited Akt activation or down-regulated *survivin* expression (Fig. 5*E* and *F*).

The potentiation of growth inhibition and apoptosis of gemcitabine by genistein and erlotinib. The growth of COLO-357 and MIAPaCa cells treated with genistein (20 μmol/L), erlotinib (1 μmol/L), gemcitabine (10 nmol/L), or the combination was determined by MTT (Fig. 6*A*). A significant reduction in growth was observed in COLO-357 cells treated in triple combination compared with treatment with gemcitabine, erlotinib, genistein, or the combination of erlotinib and gemcitabine. In MIAPaCa cells, no potentiation in growth inhibition was observed in any drug combination that was tested. This observed growth inhibition with the triple combination was associated with a significant increase in apoptosis in the COLO-357 cells. In contrast, no change in apoptosis was observed in MIAPaCa cells (Fig. 6*B*).

The effects of genistein, erlotinib, gemcitabine, and the combination on the EGFR signaling pathway. In the COLO-357 cell line, the combination of genistein, erlotinib, and gemcitabine resulted in significant down-regulation of the EGFR, survivin, and Bcl-xL compared with cells treated with gemcitabine, erlotinib,

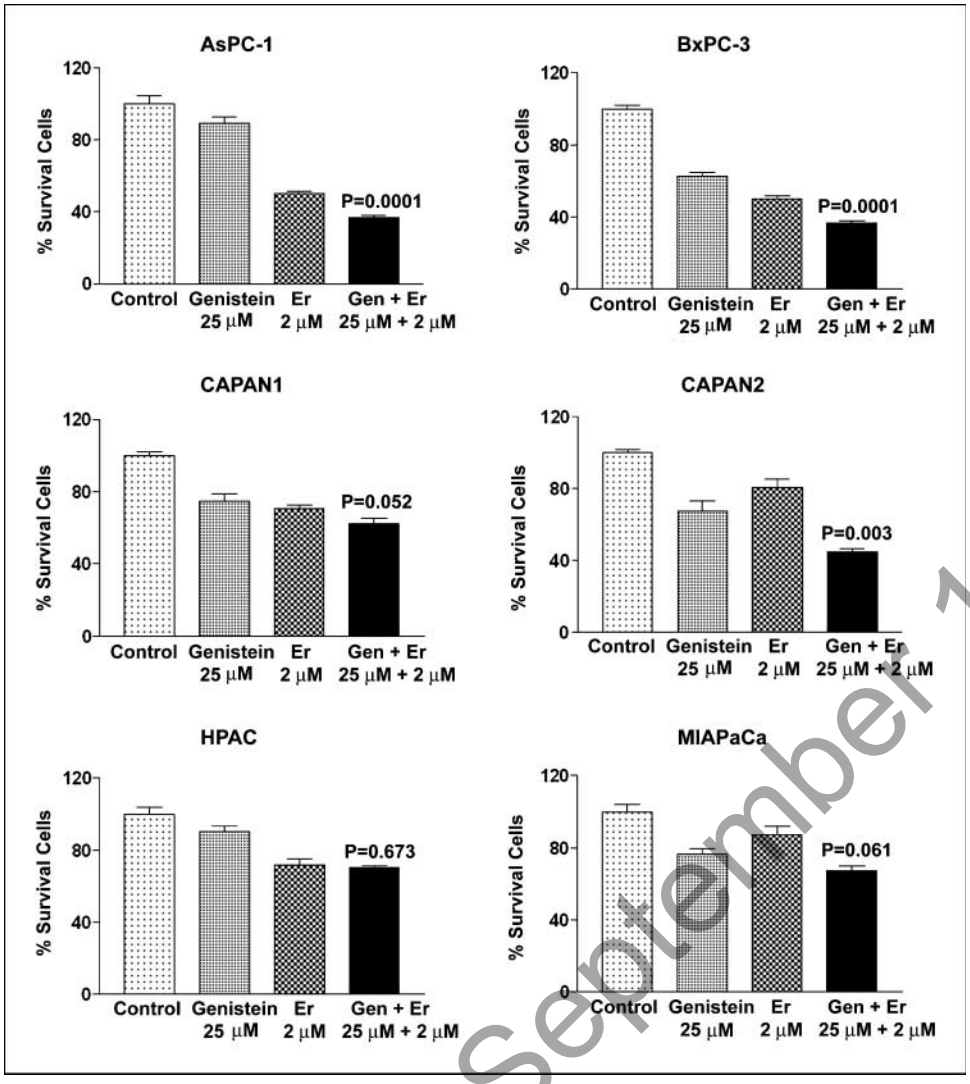

Figure 3. Growth inhibition of human pancreatic cancer cell lines treated with genistein, erlotinib, and the combination was evaluated by the MTT assay. AsPC-1, BxPC-3, CAPAN-1, CAPAN-2, HPAC, and MIAPaCa cells were treated with genistein (25 μmol/L), erlotinib (2 μmol/L), and the combination as described in Materials and Methods. There was a significant reduction in cell growth in the AsPC-1, BxPC-3, and CAPAN-2 cells treated with genistein and erlotinib compared with cells treated with erlotinib. A trend for potentiation of growth inhibition of erlotinib by genistein was observed in CAPAN-1 and MIAPaCa cells. Significant growth inhibition with erlotinib was observed in the HPAC cell line but no potentiation of the growth inhibition was observed with the combination. *P*s shown represent comparisons between erlotinib and the combination of both drugs using *t* test.

genistein, or the combination of erlotinib and gemcitabine (Fig. 6*C*). Gemcitabine treatment activated NF-κB (Fig. 6*D*). Treatment with genistein and erlotinib prevented the gemcitabine-induced NF-κB activation. No treatment effect on EGFR, survivin, Bcl-xL, or NF-κB was observed in the MIAPaCa cell lines (Fig. 6*C* and *D*).

Discussion

The existence of multigene abnormalities in pancreatic cancer forms the basis to develop combination therapies to those dysregulated gene products. Studying pancreatic cell lines based on variations in gene expression, such as those related to the EGFR pathway, may represent one approach to develop rational therapies of multitargeted agents. EGFR-independent activation of Akt in pancreatic cancer can occur through *Akt* gene amplification (21), activation of PI3K through aberrant expression of MMAC/PTEN (22), or activating mutations of the *Ras* oncogene (23). Inhibition of Akt by celecoxib (24) or PI3K inhibitors (LY294002 or wortamannin) sensitizes pancreatic cancer cells to the proapoptotic effects of cytotoxic agents (25, 26). EGFR-independent activation of Akt is associated with resistance to EGFR inhibitors in breast (27) and non–small cell lung cancer (28). The exact mechanisms by which Akt promotes growth and causes chemoresistance are unknown. One possible downstream target of Akt is IκB kinase (IKK; 29). Activated IKK phosphorylates IκB leading to the activation NF-κB, which regulates the transcription of key genes involved in growth, angiogenesis, and survival. Inhibition of NF-κB inhibits growth (29) and sensitizes pancreatic cancer cell lines to the proapoptotic effects of cytotoxic agents (18, 30, 31). The characterization of the human pancreatic cancer cells with respect to EGFR-related signaling proteins in this study indicated that EGFR expression and EGFR-independent activation of the Akt/NF-κB pathway were very frequent in pancreatic cancer.

A rational approach to enhance apoptosis by either EGFR blockers or cytotoxics is to combine EGFR blockers with agents that would also inhibit activation of Akt and NF-κB. Previous *in vitro* studies indicated that the growth-inhibitory effects of genistein were linked to the inhibition of PI3K leading to the inhibition of Akt and through the inhibition of IKK leading to the inhibition of NF-κB (17, 18, 32). This supported the basis of testing the combination of genistein with erlotinib as a multitargeted treatment for pancreatic cancer. In this study, genistein potentiated the growth inhibition and apoptotic effects of erlotinib in the

BxPC-3, CAPAN-2, and AsPAC-1 cell lines. The underlying molecular events for such growth inhibition confirm that genistein was able to potentiate the inhibition of Akt and NF-κB by erlotinib. These results support the hypothesis that targeting this pathway at multiple levels can enhance the inhibition of the EGFR pathway.

Jimeno et al. (33) showed recently that treatment of pancreatic cancer cell lines with erlotinib resulted in a compensatory up-regulation of the EGFR. The up-regulation of the EGFR contributed to the development of resistance to erlotinib. Inhibition of increased EGFR transcription by small-interfering RNA restored sensitivity to erlotinib. In this study, the genistein and erlotinib combination down-regulated EGFR expression. This effect is most probably mediated through the inhibition of NF-κB-dependent transcription of the *EGFR* gene. The down-regulation of EGFR transcription could represent an additional mechanism of synergy between erlotinib and genistein.

In the present study, we evaluated the effect of baseline activation of the EGFR pathway in relation to the combined effects of erlotinib and genistein. Although the HPAC cell line has high baseline expression of EGFR relative to other cell lines, the growth inhibition and apoptosis by erlotinib were not potentiated by genistein. This could be explained by the observation that the inhibition of Akt by erlotinib was not potentiated by genistein, indicating EGFR-dependent activation of Akt in HPAC. On the other hand, in the MIAPaCa cell line, although the baseline phosphorylated Akt was elevated relative to other cell lines, neither erlotinib nor genistein inhibited Akt activation and consequently no potentiation in apoptosis was observed. This suggests that Akt activation was independent of EGFR and PI3K.

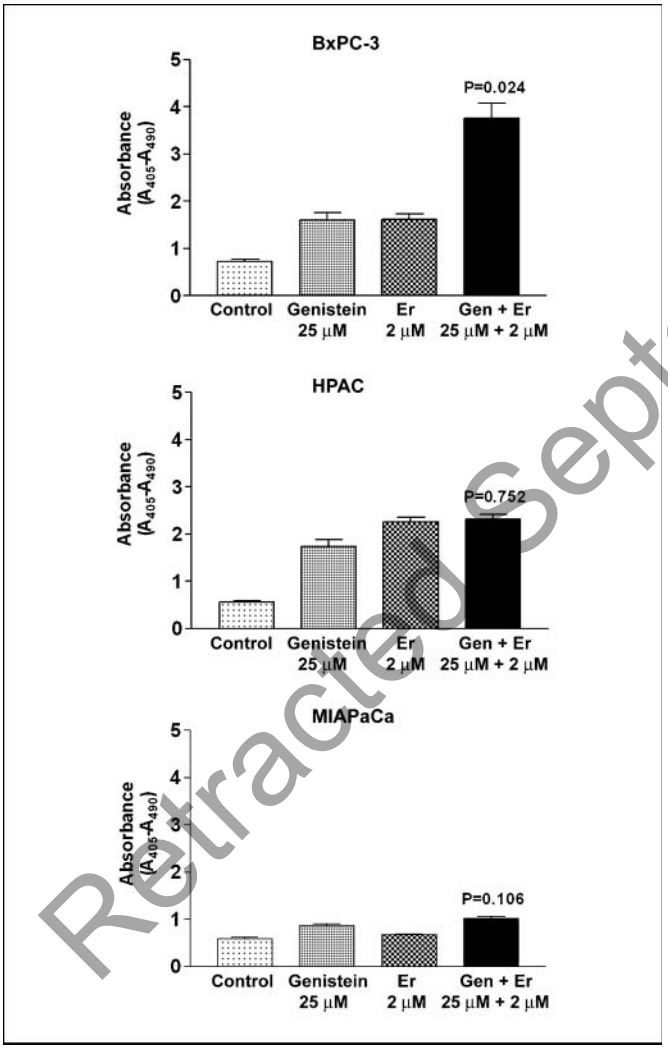

Figure 4. Induction of apoptosis in human pancreatic cancer cell lines treated with genistein, erlotinib, and the combination was evaluated by the ELISA assay. Cells were treated with 25 μmol/L genistein, 2 μmol/L erlotinib, or the combination as described in Material and Method section. There was a significant potentiation of apoptosis observed in BxPC-3 cells treated with genistein and erlotinib compared with cells treated with either agent alone. No potentiation of apoptosis was observed in either HPAC or MIAPaCa cell lines.

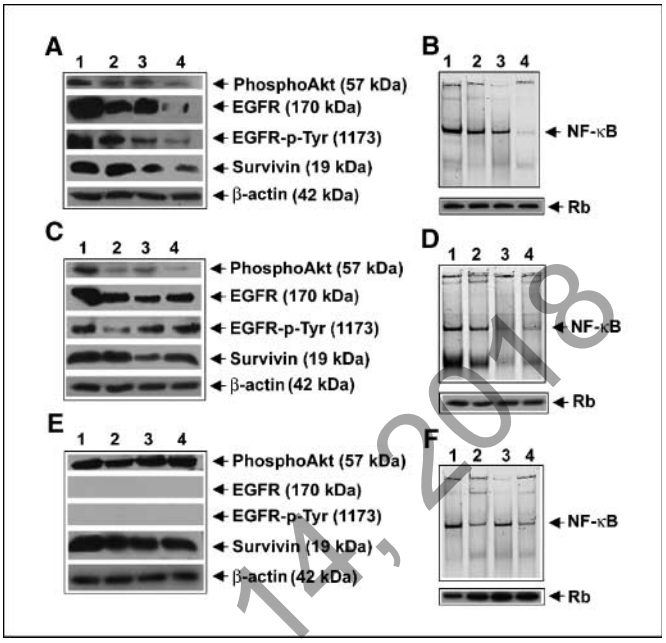

Figure 5. The expression of phosphorylated AKT, EGFR, phosphorylated EGFR, survivin, and NF-κB activation in BxPC-3, HPAC, and MIAPaCa human pancreatic cell lines. *Lane 1,* untreated cells; *lane 2,* 25 μmol/L genistein; *lane 3,* 2 μmol/L erlotinib; *lane 4,* the combination. β-Actin and retinoblastoma antibodies were used as internal control for cytoplasmic and nuclear proteins, respectively. *A,* Western blot analysis of BxPC-3 cells. *B,* NF-κB activation in BxPC-3 cells. *C,* Western blot analysis of HPAC cells. *D,* NF-κB activation in HPAC cells. *E,* Western blot analysis of MIAPaCa cells. *F,* NF-κB activation in MIAPaCa cells. Significant down-regulation of phosphorylated AKT, EGFR, phosphorylated EGFR, survivin protein, and NF-κB activation was observed in BxPC-3 cells treated with the combination of genistein and erlotinib compared with cells treated with either drug alone. No significant down-regulation of any protein or NF-κB activation was observed in MIAPaCa cell lines.

Neither the expression of EGFR nor the baseline activation of Akt/NF-κB predicted the effects of the genistein erlotinib combination. This is supported by the results of recently conducted clinical trials indicating that tumoral EGFR expression does not predict the outcome of therapy with EGFR inhibitors (10). The potentiation of erlotinib and genistein is most probably limited to cell lines with dysregulation of both EGFR and PI3K. Given the heterogeneous dysregulation of the EGFR pathway in pancreatic cancer, no one combination of targeted agents is appropriate for all patients. Therefore, one aim of future trials in pancreatic cancer should be to define the appropriate combination of targeted therapies based on the specific dysregulation of the EGFR pathway.

The combination of gemcitabine and erlotinib was a recently Food and Drug Administration–approved regimen for the treatment of advanced pancreatic cancer. In this study, we evaluated

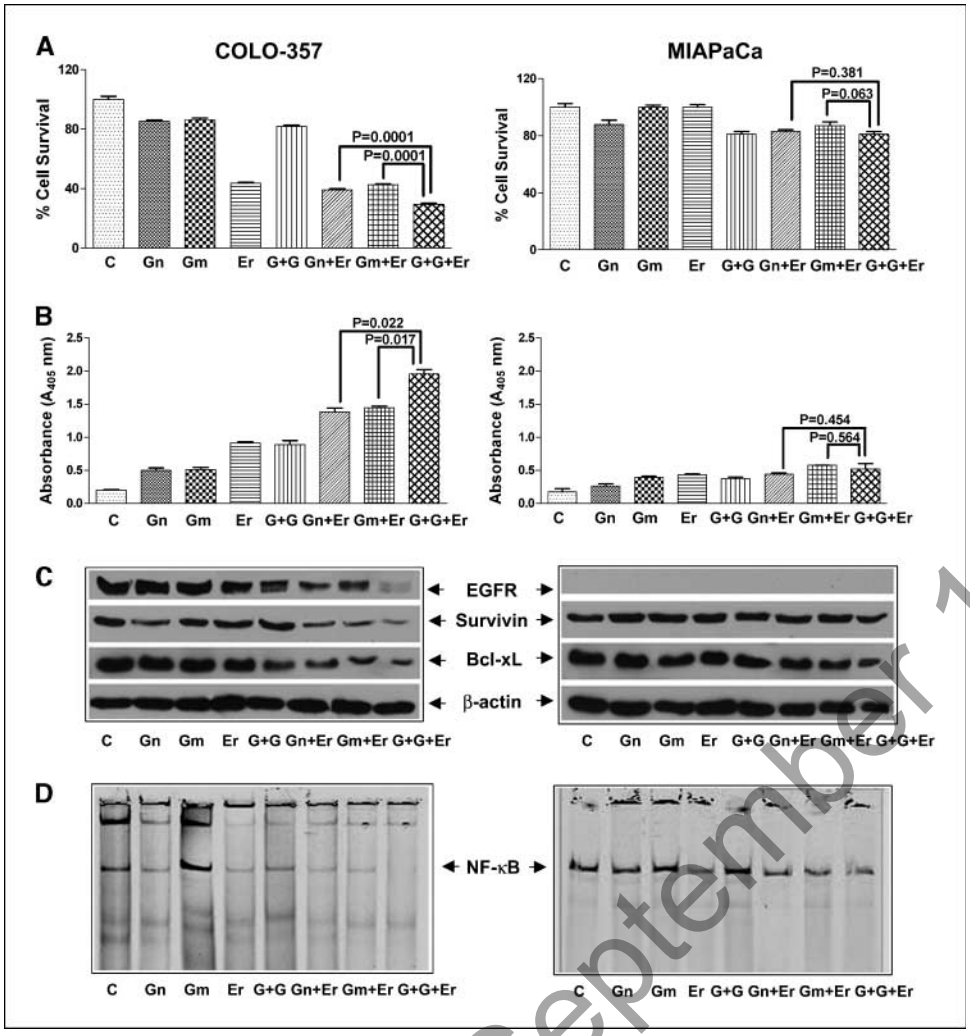

Figure 6. Growth inhibition (*A*), induction of apoptosis (*B*), Western blot (*C*), and NF-κB activation (*D*) of human pancreatic cancer cell lines COLO-357 and MIAPaCa. The cells treated with 20 μmol/L genistein, 10 nmol/L gemcitabine, 1 μmol/L erlotinib, and the dual and triple combination was evaluated by the MTT assay and the Cell Death Detection by histone DNA ELISA method. There was a significant reduction in growth in COLO-357 cells treated with triple combination (*G+G+Er*) compared with cells treated with dual combination or single agent alone. No potentiation in growth inhibition was observed in MIAPaCa cells (*A*). A significant potentiation of apoptosis was observed in the cells treated with triple combination (*G+G+Er*) compared with cells treated with dual combination or single agent alone. No induction of apoptosis was observed in MIAPaCa cells (*B*). Significant down-regulation of EGFR, Bcl-xL, survivin protein (*C*), and NF-κB activation (*D*) was observed in COLO-357 cells treated with the combination of gemcitabine, genistein, and erlotinib compared with cells treated with either drug alone. No significant down-regulation of any protein or NF-κB activation was observed in MIAPaCa cell lines.

the potentiation of gemcitabine and erlotinib by genistein. Although both erlotinib and genistein potentiated the growth inhibition and apoptosis induced by gemcitabine in COLO-357, the potentiation was significantly more when gemcitabine was combined with both erlotinib and genistein. This further supports that the modulation of chemoresistance through the inhibition of the EGFR pathway requires targeting the pathway at multiple levels.

Because NF-κB has a central role in the regulation of apoptotic pathways in pancreatic cancer, we evaluated the effect of erlotinib and genistein on expression of the NF-κB downstream molecules survivin and Bcl-xL as a possible mechanism for potentiation of gemcitabine-induced apoptosis. The cellular effects of survivin include inhibition of caspase-3 and caspase-7 (34). Wang et al. (35) have shown that activation of the EGFR leads to over-expression of survivin through the PI3K signaling pathway. Our results suggest that genistein potentiates the down-regulation of survivin by erlotinib in the BxPC-3 but not in HPAC or MIAPaCa cell lines. The inhibition of the Akt/NF-κB pathway down-regulates Bcl-2 and Bcl-xL and up-regulates the proapoptotic Bax (19, 36, 37). Similarly, in the COLO-357 cell line, inhibition of NF-κB activation by genistein was associated with increased apoptotic effect of erlotinib and gemcitabine as well as down-regulation of EGFR, survivin, and Bcl-xL expression. Therefore, the erlotinib and genistein combination can lower threshold for the induction of apoptosis through the inhibition of NF-κB and can sensitize pancreatic cancer cells to proapoptotic effects associated with cytotoxic agents, such as gemcitabine.

In conclusion we have shown the potentiation of erlotinib and gemcitabine by genistein in certain pancreatic cancer cell lines through the inhibition of the EGFR-independent activation of Akt/NF-κB pathway. The erlotinib and genistein combination can lower the apoptotic threshold of pancreatic cancer cell lines and therefore could reverse chemoresistance. The observed effects were achieved using concentration of genistein comparable with levels observed in the plasma of patients receiving genistein from supplements (38). Therefore, combining genistein with erlotinib and gemcitabine could represent a rational and novel approach for further preclinical and clinical trials.

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