The Epidermal Growth Factor Receptor Inhibitor Gefitinib Prevents the Progression of Pancreatic Lesions to Carcinoma in a Conditional LSL-Kras\textsuperscript{G12D/+} Transgenic Mouse Model

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Abstract
Pancreatic ductal adenocarcinoma (PDAC) is the most common pancreatic malignancy with a dismal prognosis. Developing novel strategies to prevent or delay pancreatic cancer is currently of intense interest. The chemopreventive efficacy of gefitinib, an epidermal growth factor receptor (EGFR) inhibitor, was evaluated against the progression of pancreatic intraepithelial neoplasms (PanIN) to PDAC in conditional LSL-Kras\textsuperscript{G12D/+} transgenic mice. LSL-Kras\textsuperscript{G12D/+} and p48\textsuperscript{Cre/+} mice were bred, and offspring of activated Kras\textsuperscript{G12D/+} were generated. Six-week-old male Kras\textsuperscript{G12D/+} (20 per group) and C57BL/6 wild-type (12 per group) mice were fed (AIN-76A) diets containing 0, 100, and 200 ppm of gefitinib for 35 weeks. At termination, pancreases were evaluated histopathologically for PanINs and PDAC, and various biomarkers were measured by immunohistochemistry, immunofluorescence, immunoblotting, and/or reverse transcription-PCR. Dietary gefitinib at 100 and 200 ppm significantly suppressed PDAC incidence by 77% and 100%, respectively ($P < 0.0001$) when compared with control diet. Importantly, a significant inhibition of carcinoma and a dose-dependent suppression of PanINs [PanIN-1, 37-62% ($P < 0.002$); PanIN-2, 38-41 ($P < 0.001$); and PanIN-3, 7-34% ($P < 0.0141$)] were observed in mice treated with gefitinib. Furthermore, mice treated with 100 and 200 ppm of gefitinib exhibited 67.6% to 77.3% of the pancreas to be free from ductal lesions. Also, gefitinib reduced EGFR, proliferating cell nuclear antigen, cyclin D1, C\textsubscript{2}GNT, RhoA, \textbeta-catelin, p38, phospho-extracellular signal–regulated kinase, caveolin-1, and mucin and increased cyclin B1 in the pancreatic lesions/PDAC. In summary, these results show that gefitinib can prevent the progression of pancreatic cancer precursor lesions to PDAC in a preclinical model. The present study highlights the promise of chemoprevention and the potential usefulness of EGFR inhibitors in individuals at high risk for pancreatic cancer. Cancer Prev Res; 3(11); 1417–26. ©2010 AACR.

Introduction
Pancreatic carcinoma is a devastating malignancy with a dismal prognosis, characterized by low responsiveness to conventional chemotherapies, and proving fatal for nearly all patients diagnosed with the disease. It is responsible for approximately 260,000 deaths per year worldwide, with most of the deaths occurring in developed countries (150,000 annually; ref. 1), and is the fourth leading cause of cancer-related deaths in the United States. Despite advances in the field of molecular genetics in human pancreatic cancers, the identification of various putative molecular targets associated with the development of targeted therapies has not yet translated to improved overall patient survival (2). The current standard of care for advanced pancreatic ductal adenocarcinoma (PDAC) is infusional gemcitabine, a deoxycytidine analogue and inhibitor of nucleic acid synthesis, which prolongs survival by only a few weeks and provides symptomatic improvement in a minority of patients (3). PDAC is generally believed to arise predominantly through the progression of pancreatic intraepithelial neoplasia (PanIN), ranging from low-grade PanINs (termed PanIN-1A and PanIN-1B) to high-grade PanINs (termed PanIN-2 and PanIN-3) to ductal adenocarcinoma (4). The preclinical study of PanINs has recently been made possible by the generation of genetically modified animal models, which recapitulate human PanINs at the genetic and histomorphologic levels (5). PDAC is characterized by a high frequency of Kras mutations at early stages.
and the accumulation over time of multiple additional genetic abnormalities (5).

To understand the biology of pancreatic cancer precursor lesions and to discover early detection markers, recent research interest in this field has adopted models and approaches to identify risk factors for the development and inhibition of pancreatic cancers. The conditional Kras \(^{G12D/+}\) model, first described by Hingorani et al. (6), is considered a very valuable tool to study PanIN biology. Mice harboring a conditional Kras mutant allele (LSL-Kras\(^{G12D/+}\)) in combination with a pancreas-specific Cre recombinase transgene (p48\(^{Cre/+}\)) develop a full range of premalignant lesions in the pancreas, termed pancreatic intraepithelial neoplasia (PanIN), before succumbing to invasive PDAC and other tumors at late ages (6–9). These mice are an excellent model of PanIN development and are useful for studying tumor progression. Importantly, these mice also serve as a valuable model to evaluate and identify the potential chemopreventive agents that can significantly suppress the progression of PanINs to PDAC.

Overexpression of epidermal growth factor and epidermal growth factor receptor (EGFR) has been observed in various malignancies, including carcinomas of the pancreas (10–12), stomach (13), and liver (14), as well as tumors of the brain (15), and is involved in tumor proliferation, survival, metastasis, and induction of angiogenesis. In addition, signaling through EGFR promotes tumor neovascularization and induces resistance to cytotoxic chemotherapy (16). Based on these multiple effects on cancer, the EGFR tyrosine kinase has been recognized as an attractive molecular target for selective treatment of solid tumors with increased EGFR expression levels. Stimulation of EGFR results in activation of multiple intracellular signaling cascades that increase cellular proliferation and induction of apoptosis. The ATP competitive kinase inhibitor gefitinib (Iressa, ZD1839) was the first EGFR-directed small-molecule drug that received approval for the treatment of non–small-cell lung cancer (18). Gefitinib is an orally active and selective EGFR tyrosine kinase inhibitor that blocks signal transduction pathways responsible for the proliferation and survival of cancer cells and other host-dependent processes that promote cancer growth. In clinical and preclinical animal models, gefitinib has been shown to be an effective therapeutic agent toward cancers of the lung, breast, colon, prostate, head and neck, and other organ sites when administered as a single agent or in combination with other chemotherapeutic agents (19–31). The potential beneficial effects of EGFR inhibitors such as gefitinib on the survival of pancreatic cancer patients have been limited (32, 33). However, the potential usefulness in the chemoprevention setting has not been established for EGFR inhibitors and/or other molecularly targeted agents. Thus, this study is the first to investigate the chemopreventive effects of gefitinib against the progression of PanIN to PDAC and the expression of important biomarkers of progression using the condition- al LSL-Kras\(^{G12D/+}\) mouse model.

Materials and Methods

Animals, diets, and care

All animal experiments were done in accordance with the institutional guidelines of the American Council of Animal Care. Breeder pairs of LSL-Kras\(^{G12D/+}\) and p48\(^{Cre/+}\) in the C57BL/6 genetic background were obtained from Dr. Howard Crawford at the University of New York, Stony Brook, NY. Required quantities of activated Kras\(^{G12D/+}\) mice were generated as described below. Animals were housed in ventilated cages under standardized conditions (21°C, 60% humidity, 12-h light/12-dark cycle, 20 air changes per hour) in the University of Oklahoma Health Sciences Center rodent barrier facility. Semi-purified modified AIN-76A diet ingredients were purchased from Bioserv, Inc. The selective EGFR inhibitor gefitinib was procured from the National Cancer Institute chemoprevention drug repository. Gefitinib (100 and 200 ppm) was premixed with small quantities of casein and then blended into the diet using a Hobart mixer. Both control and experimental diets were prepared weekly and stored in a cold room. Agent content in the experimental diets was determined periodically in multiple samples taken from the top, middle, and bottom portions of individual diet preparations to verify uniform distribution. Mice were allowed ad libitum access to the respective diets and to automated tap water purified by reverse osmosis.

Breeding and genotyping analysis

LSL-Kras\(^{G12D/+}\) and p48\(^{Cre/+}\) mice were maintained in a C57BL/6 heterozygous genetic background. LSL-Kras\(^{G12D/+}\) and p48\(^{Cre/+}\) mice were bred, and offspring of activated male Kras\(^{G12D/+}\) were generated at required quantities. The genetic background of each pup was confirmed by tail DNA extraction and PCR as described elsewhere (5). Briefly, genomic DNA was extracted from snap-frozen tail tissue samples using the mini-prep kit (Invitrogen). PCR was done for Kras and Cre using the following conditions: denaturation at 95°C for 5 minutes, followed by 35 cycles at 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute. The oligonucleotide primer sequences used were as follows: Kras, 5′-CCTTTACAAGCGCAAGCCAG-3′ (sense) and 5′-AGCTAGCCACATGCCTTGAAG-3′ (antisense); Cre, 5′-ACCGTCAGTACGTGAGATGG-3′ (sense) and 5′-ACCTGAGATGTTGGCGATATCGA-3′ (antisense). PCR products were separated on a 2% agarose gel. Successful recombination yields were 550- and 210-bp products.

Bioassay: chemopreventive efficacy of gefitinib

Genotyped male Kras\(^{G12D/+}\) transgenic mice were used in the efficacy study. The experimental protocol is summarized in Fig. 1A. Five-week-old mice were selected and randomized so that the average body weights in each group were equal \((n = 20\) p48\(^{Cre/+}\)-LSL-Kras\(^{G12D/+}\) mice per group and \(n = 12\) C57BL/6 wild-type mice per group) and were fed with AIN-76A diet for 1 week. At 6 weeks of age, mice
were fed with control or experimental diets containing 0, 100, or 200 ppm of gefitinib in the diet until termination of the study. Mice were routinely checked for signs of weight loss, toxicity, or any abnormalities. The food intake and body weight of each animal were measured once weekly for the first 6 weeks and then once a month until termination. After 35 weeks (~9 months) on experimental diets, all mice were euthanized by CO2 asphyxiation and necropsied; pancreases were collected, weighed, and snap frozen in liquid nitrogen for further analysis. Pancreases (head to tail) that required histopathologic and immunohistochemical evaluations to identify PanIN lesions and PDAC for evaluation of various molecular markers were fixed in 10% neutral-buffered formalin.

**Histologic evaluation**

Formalin-fixed, paraffin-embedded tissues were sectioned (4 μm) and stained with H&E. Twenty sections of each pancreas were histologically evaluated by a pathologist blinded to the experimental groups. PanIN lesions and carcinoma were classified according to histopathologic criteria as recommended elsewhere (4). To quantify the progression of PanIN lesions, the total number of ductal lesions and their grade were determined. Pancreatic ducts of the entire fixed specimen (head, body, and tail of the pancreatic sections) were analyzed for each animal. The relative proportion of each PanIN lesion to the overall number of analyzed ducts was recorded for each animal. Similarly, pancreatic carcinoma and normal-appearing pancreatic tissue were evaluated for all the animals.

**Immunohistochemistry and immunofluorescence histochemistry**

The effects of gefitinib on the expression of proliferating cell nuclear antigen (PCNA), β-catenin, EGFR, and caveolin-1 (Cav-1) were evaluated by immunohistochemistry and/or immunofluorescence histochemistry. Briefly, for immunohistochemistry of PCNA, β-catenin, EGFR, and Cav-1, paraffin sections were deparaffinized in xylene, rehydrated through graded ethanol solutions, and washed in PBS. Antigen retrieval was carried out by heating the sections in 0.01 mol/L citrate buffer (pH 6) for 30 minutes in a boiling water bath. Endogenous peroxidase activity was quenched by incubation in 3% H2O2 in PBS for 5 minutes. Nonspecific binding sites were blocked using Protein Block for 20 minutes. Sections were then incubated overnight at 4°C with 1:300 dilutions of monoclonal antibodies against PCNA, β-catenin, EGFR, and Cav-1 (Abcam/Santa Cruz Biotechnology). After several washes with PBS, the slides were incubated with appropriate secondary antibody for 2 hours and then washed and incubated with avidin-biotin complex reagent (Zymed Laboratories). After rinsing with PBS, the slides were incubated with the chromogen 3,3′-diaminobenzidine for 3 minutes, then rinsed and counterstained with hematoxylin. Nonimmune rabbit immunoglobulins for primary antibodies were used as negative controls. For immunofluorescence histochemistry, after overnight incubation with primary antibody, the slides were rinsed three times with PBS, each for 5 minutes, and then incubated with secondary antibody tagged with FITC/TRITC in the dark for 1 hour. Slides were then washed three times with PBS, each for 5 minutes, and incubated
with 0.5 μg/mL DAPI in the dark for 5 minutes. Slides were rinsed with PBS and observed for fluorescence under FITC/TRITC filters using an Olympus IX701, and digital computer images were recorded with an Olympus DP70 camera.

Alcian blue staining for mucinous PanINs

The effect of gefitinib on mucin secretion in the pancreas was evaluated by Alcian blue staining. Briefly, paraffin sections were deparaffinized in xylene, rehydrated through graded ethanol solutions as described earlier, stained with Alcian blue for 5 minutes, and then washed with PBS for 5 minutes. Sections were counterstained with aqueous neutral red, washed with distilled water, and mounted with a coverslip. Mucin staining (blue in color) was imaged using an Olympus IX 70 microscope as described earlier.

Reverse transcription-PCR for p38, C2GNT, cyclin D1, and cyclin B1 mRNA expression

Total RNA from pancreas samples was extracted using the Totally RNA Kit (Ambion) as per manufacturer's instructions. Equal quantities of DNA-free RNA were used in reverse transcription reactions for making cDNA using SuperScript reverse transcriptase (Invitrogen). PCR reactions were done for p38, C2GNT, cyclin D1, and cyclin B1 using the following conditions. For p38, denaturation was done at 94°C for 3 minutes, followed by 35 cycles at 94°C for 30 seconds, 60°C for 20 seconds, and 72°C for 45 seconds. The oligonucleotide primer sequences used for p38 were 5′-TCCCTAGGGTGTCCCTGCA-3′ (sense) and 5′-TTATGTGACCGCCCTCCCTGCA-3′ (antisense). For C2GNT, denaturation was done at 94°C for 2 minutes, followed by 30 cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute and 15 seconds. The oligonucleotide primer sequences used for C2GNT were 5′-AGAGGAAAGACGGCCACCCCTGCA-3′ (sense) and 5′-CGCTGAGGCTGCGCCAAAAG-3′ (antisense). For cyclin B1, denaturation was done at 94°C for 2 minutes, followed by 35 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45 seconds. The oligonucleotide primer sequences used for cyclin B1 gene were 5′-ACTTTCACTGAGCACCTC-3′ (sense) and 5′-GCAAGTGGTGTCAATC-3′ (antisense). For cyclin D1, denaturation was done at 94°C for 3 minutes, followed by 35 cycles at 94°C for 30 seconds, 60°C for 20 seconds, and 72°C for 45 seconds. The oligonucleotide primer sequences used for cyclin D1 gene were 5′-ATGGAAAGACCCGCTCCTGTC-3′ (sense) and 5′-ACCTCCAGCATCTCAGGTC-3′ (antisense). PCR was done using Taq polymerase, 10 mmol/L deoxynucleotide triphosphate, and buffers from Invitrogen. The PCR products were visualized and photographed under UV illumination.

Western blot analysis of protein expression

Pancreases harvested from mice fed with or without gefitinib were homogenized and lysed in ice-cold lysis buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L DTT, and protease inhibitor cocktail]. After a brief vortexing, the lysates were separated by centrifugation at 12,000 × g for 15 minutes at 4°C, and protein concentrations were measured with the Bio-Rad protein assay reagent. An aliquot (50 μg protein/lane) of the total protein was separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. After blocking with 5% milk powder, membranes were probed for expression of RhoA, phosphoextracellular signal-regulated kinase (pERK), PCNA, and β-catenin in hybridizing solution (1:500, in TBS-Tween 20 solution) using the respective primary antibodies (Santa Cruz Biotechnology) and then probed with horseradish peroxidase-conjugated secondary antibodies. Detection was done following the SuperSignal West Pico Chemiluminescence procedure (Pierce). The bands were captured on Ewen Parker, Blue sensitive X-ray films.

Statistical analysis

The data are presented as mean ± SE. Differences in body weights were analyzed by ANOVA. Statistical differences between the control and treated groups were evaluated using unpaired t test with Welch’s correction. Differences between groups are considered significant at P < 0.05.

Results

General observations

Kras<sup>G12D/+</sup> mice fed control and experimental diets had steady body weight gains. At the end of the experiment, wild-type mice had slightly higher body weight gains (P > 0.05) in comparison with Kras<sup>G12D/+</sup> mice. No significant body weight changes were observed within the treatment groups and the control group during the course of the study (Fig. 2A). None of the animals fed the experimental diets exhibited any observable toxicity or any gross changes attributable to liver, kidney, or lung toxicity, with notable difference in pancreatic weights as described below.

Dietary administration of gefitinib significantly inhibits PDAC and delays the progression of PanIN lesions to PDAC in Kras<sup>G12D/+</sup> mice

Kras<sup>G12D/+</sup> mice spontaneously develop pancreatic cancer arising through the progression of PanINs, ranging from low-grade PanINs (1A and 1B) to high-grade PanINs (PanIN-2 and PanIN-3). C57BL/6 wild-type mice fed with control diet or experimental diets containing gefitinib showed no evidence of PanIN lesions or carcinoma (data not shown). The efficacy end point used in this study was inhibition of PanINs and PDAC. At the termination of the experiment, pancreases were collected and weighed. The pancreases from C57BL/6 wild-type mice fed control or experimental diets weighed about 0.24 g (0.21-0.26 g) and showed no significant difference
However, the pancreases of Kras<sup>G12D/+</sup> mice fed control diet weighed 0.95 g (0.72-1.4 g), almost 4.1-fold higher than the weight of wild-type mouse pancreas, whereas the pancreases of Kras<sup>G12D/+</sup> mice fed with gefitinib diet showed a >50% decrease in weight (P < 0.002; Fig. 2B). Figure 2C summarizes the chemopreventive efficacy of gefitinib against PDAC incidence in Kras<sup>G12D/+</sup> mice that were fed control diet with a 65% incidence (percentage of mice with PDAC). Whereas mice fed with 100 ppm of gefitinib showed only a 15% incidence (percentage with PDAC), whereas mice fed with 200 ppm gefitinib had no evidence of carcinoma by histologic analysis. Also, control diet–fed Kras<sup>G12D/+</sup> mice developed, on average, about 253 PanIN-1, 159 PanIN-2, and 173 PanIN-3 lesions, whereas dietary administration of 100 and 200 ppm of gefitinib for 35 weeks showed significant inhibition of PanIN-1, PanIN-2, and PanIN-3 lesions [PanIN-1, 37.2-61.6% (P < 0.02-0.002); PanIN-2, 38.4-41.0% (P < 0.0016-0.0035); and PanIN-3, 34.7-0% (P < 0.014-0.663); Fig. 2D]. Although a dose-dependent decrease in the incidence of PanIN-1 lesions was observed, such dose-response effects were not observed for the PanIN-2 and PanIN-3 lesions (Fig. 2D). Furthermore, mice fed with 100 and 200 ppm of gefitinib exhibited respectively 67% and 77% normal-appearing (free from PanINs and PDAC) pancreatic tissues in comparison with the control group (Fig. 2E).

**Inhibition of PCNA, β-catenin, EGFR, and Cav-1 expression in PanINs and pancreatic carcinoma by gefitinib**

Figure 3A summarizes the effects of gefitinib on tumor cell proliferation as measured by PCNA overexpression. Qualitative microscopic examination of PCNA-stained sections showed a substantial decrease in PCNA-positive cells in the pancreas of gefitinib-treated mice compared with untreated controls. As shown in Fig. 3, we observed overexpression of β-catenin, EGFR, and Cav-1 in the PanIN lesions and PDAC of Kras<sup>G12D/+</sup> mice in comparison with the normal pancreases of C57BL/6 wild-type mice. As shown in Fig. 2B, minimal to no expression of nuclear accumulation of β-catenin was observed in the pancreatic ductal lesions of Kras<sup>G12D/+</sup> mice fed with gefitinib diets when compared with Kras<sup>G12D/+</sup> mice fed with control diet. Similarly, the expression levels of EGFR and Cav-1 were significantly suppressed by gefitinib treatment. Also, administration of diets with 100 and 200 ppm of gefitinib resulted in a significant decrease in EGFR and Cav-1 protein expression levels in pancreatic lesions as compared with the pancreases of mice fed control diet. Furthermore,
immunofluorescence histochemistry analysis revealed that EGFR and Cav-1 expression was primarily localized to the cell membrane (Fig. 4A and C). Importantly, membrane expression of EGFR and Cav-1 was significantly reduced in mice fed 200 ppm of gefitinib as shown in Fig. 4B and D.

**Inhibition of PCNA, β-catenin, RhoA, pERK, p38, cyclin D1, and cyclin B1 overexpression**

Western blot analysis showed that pancreatic tissues from gefitinib-fed mice exhibited significantly reduced expression of PCNA and β-catenin compared with control diet–fed mice (Fig. 5A). These results further confirm the immunohistologic observations described earlier. In addition, RhoA and pERK, which are known to be upregulated due to the Kras mutations in various malignancies, are downregulated in the pancreases of mice fed gefitinib compared with those fed with control diet (Fig. 5A). Similarly, a significant downregulation of p38 mRNA expression was observed in the pancreatic tumor tissues of gefitinib-fed mice compared with control diet–fed mice. Also, a significant decrease of cyclin D1 and an increase...
in cyclin B1 mRNA were observed in gefitinib-fed mice (Fig. 5B).

Inhibition of mucin production and C2GNT expression in pancreatic lesions

Alcian blue staining showed very high mucin expression levels in PanIN lesions of 10-month-old KrasG12D/+ mice, whereas such expression was not detected in normal pancreas (Fig. 3E). This was further confirmed by reverse transcription-PCR analysis of C2GNT levels (Fig. 5C) in pancreatic tissues. A significant decrease in the number of mucinous cysts/glands was observed in gefitinib-treated mice (Figs. 2F and 3E, 69-79%, P < 0.04-0.02). Similarly, this was consistent with a significant decrease in mucin expression in gefitinib-fed mice (Fig. 5B).

Fig. 4. Effect of gefitinib on EGFR and Cav-1 expression in PDAC; localization is shown by the immunofluorescence histochemical method as described in the text. A, EGFR expression in the PDAC of KrasG12D/+ mice fed with control diet. As shown in the merged images (right), EGFR expression is localized to the cell membrane, and mouse pancreatic lesion treated with gefitinib (100 ppm) shows significantly decreased levels of EGFR (B). C and D, Cav-1 expression in PDAC of mice fed with control diet was mostly localized to the membrane (C), whereas in gefitinib-treated mice, PDAC had limited expression (D).
and C2GNT expression levels with gefitinib treatment in the pancreas of Kras^{G12D/+} mice in comparison with the pancreas of control diet–fed mice (Fig. 5B).

**Discussion**

Substantial efforts in the molecular genetics of human PDAC have delineated a number of common genetic alterations. Among the most common alterations in PDAC are activating mutations in the Kras proto-oncogene, and such mutations are found in almost 95% of human pancreatic cancer cases (34). Although other common alterations involve inactivation of tumor suppressor genes [p16^{INK4a} (90% of cases), p53 (~75% of cases), SMAD4 (55%), and BRCA2 (10%; ref. 35)], these alterations accumulate with increasing frequency as PanINs progress to PDAC (36). Results from our efficacy study clearly suggest that an early intervention with an EGFR inhibitor, gefitinib, significantly suppresses the PanIN lesions and their progression to PDAC in this pancreatic cancer model. These results have important implications for the chemoprevention of human pancreatic cancer.

The role of EGFR in pancreatic tumor promotion is well established (37, 38). In pancreatic cancer patients, EGFR overexpression is detected in approximately 90% of clinical specimens (37, 38). Thus, targeting EGFR with small-molecule inhibitors has led to the development of clinically successful agents such as gefitinib and erlotinib. Studies have shown that EGFR inhibitors are promising for the treatment of pancreatic cancer when they are combined with gemcitabine (39). As in the case of many chemotherapeutic agents, the effectiveness of EGFR inhibitors in improving survival in advanced stage PDAC patients has been modest (40). However, there are several lines of evidence showing that gefitinib inhibits EGFR in various cancers in different mouse models (41–44). Because dysregulation of EGFR signaling may occur during the progression of PanINs, application of EGFR inhibitors may be more effective in suppressing PDAC, and the present study clearly supports this concept. A strong dose-responsive protective effect of gefitinib against the Kras-driven progression of PanIN to PDAC was observed in the present study. This is the first study to validate the pronounced chemopreventive beneficial effects of EGFR inhibitors in a transgenic mouse model of pancreatic cancer. To our knowledge, there is only one report on chemoprevention of PanIN lesions. This study used a selective cyclooxygenase-2 inhibitor, nimesulide, to inhibit PanIN lesions in Kras-transgenic mice; however, the number of mice used in this study was only six (45).

The development of pancreatic lesions and their progression to PDAC are complex processes. It is well known that the endogenous mouse models show very few and less extensive acinar-ductal metaplasia (ADM) compared with the acinar promoter models, which are characterized by prominent and extensive ADM (4, 46). In the present study, we have used an endogenous Kras mouse model and have evaluated the pancreases after more than 10 months of age, and we did not find a statistically significant number of ADM. However, there were very few and infrequent ADM and mucinous metaplastic lesions seen in untreated p48^{Cre}/LSL-Kras^{G12D/+} mouse pancreas. Moreover, our histopathologic observations suggest that mucinous ducts were rare, suggesting that there was no clear evidence of acini becoming metaplastic ducts. Almost all the ducts present seemed to come from normal periacinar ducts.

To understand the possible mechanism(s) of the progression of PanIN to PDAC and the inhibitory effects of gefitinib, we analyzed a number of key molecular markers associated with the EGFR pathway and pancreatic tumor growth (47–50). Higher expression levels of EGFR were associated with Cav-1 expression and positively correlated with histologic grade and advanced tumor stage. Clinical studies have shown that Cav-1 expression is associated with pancreatic tumor progression and poor prognosis for patient survival (50). In the present study, overexpression of Cav-1 was corroborated with proliferation markers and several of the downstream signal molecules, such as RhoA, pERK, and the mitogen-activated protein kinase (MAPK) signaling molecule p38. The high expression levels of the proliferation markers PCNA, β-catenin, and cyclin D1 observed in Kras^{G12D/+} mouse pancreatic lesions were significantly inhibited by dietary gefitinib in correlation with EGFR and Cav-1 expression. EGFR and Cav-1 coexpression levels are known to regulate a number of

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**Fig. 5.** A, effect of gefitinib on the protein expression levels of β-catenin, pERK, RhoA, and PCNA. Protein expression was analyzed by Western blot analysis as described in the text. B, effect of gefitinib on the mRNA expression levels of cyclin D1, p38, cyclin B1, and C2GNT by reverse transcription-PCR. C, expression levels of C2GNT mRNA in the normal pancreas of wild-type mice and PDAC of Kras^{G12D/+} mice.
signaling pathways including phosphatidylinositol 3-kinase-AKT and MAPK pathways that contribute to the progression and development of PDAC (48). Our results support previous observations that oncogenic Kras enhances the progression of pancreatic ductal cells to a malignant phenotype through activation of MAPK and RhoA. Also, a dose-dependent decrease in cyclin D1 and an increase in cyclin B1 were observed in the pancreas of gefitinib-fed Kras \(^{G12D/+}\) mice. These results suggest that the cells are arrested in G2-M phase, which is similar to the inhibition of pancreatic cancer cell growth by the farnesyl transferase inhibitor L-744,832 (31).

There is a strong relationship between the risk of pancreatic carcinoma and the presence of combinations of Kras gene mutation, papillary growth, and expression of mucins (32). In the present study, gefitinib treatment not only decreased the number of PanIN lesions but also significantly decreased the expression of mucin and a mucin-producing enzyme in these lesions.

In summary, this study showed for the first time that blockade of the EGFR signaling pathway in the pancreatic cancer mouse model by dietary gefitinib exerts significant chemopreventive efficacy in the inhibition of PanIN formation and their progression to PDAC. Inhibition of PanINs and PDAC by gefitinib is associated with significant suppression of tumor cell proliferation; mucin bio-


10. Pryczynicz A, Guzin K, Kemona A, Ewska JC. Expression of EGF and RhoA, MAPK, and Wnt. These results and the similarity of this pancreatic mouse model to human premalignant and malignant pancreatic lesions highlight the potential application of EGFR pathway inhibitors in preventing and delaying the progression of pancreatic cancer in the clinical setting.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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