Therapeutics, Targets, and Chemical Biology

Cancer Research

Erlotinib Prolongs Survival in Pancreatic Cancer by Blocking Gemcitabine-Induced MAPK Signals

Koji Miyabayashi[1], Hideaki Ijichi[1], Dai Mohri[1], Motohisa Tada[4], Keisuke Yamamoto[1], Yoshinari Asaoka[1], Tsuneo Ikenoue[3], Keisuke Tateishi[1], Yousuke Nakai[1], Hiroyuki Isayama[1], Yasuyuki Morishita[2], Masao Omata[5], Harold L. Moses[6], and Kazuhiko Koike[1]

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most deadly cancers worldwide. Although many regimens have been used for PDAC treatment, the combination of the EGF receptor (EGFR) inhibitor erlotinib with gemcitabine has been the only molecular-targeted drug tested so far that has been superior to gemcitabine alone. The mechanism underlying this effective combinational regimen remains unknown. Here, we show that the combination is superior to gemcitabine alone in blocking progression and prolonging survival in a murine model of PDAC (Kras activation with Tgfbr2 knockout). We found that gemcitabine induced mitogen-activated protein kinase signaling, which was dramatically inhibited by erlotinib even in the Kras-activated PDAC cells in the mouse model. Mechanistic investigations suggested that gemcitabine induces EGFR ligand expression and ERBB2 activation by increasing heterodimer formation with EGFR, thereby maintaining high levels of ERBB2 protein in PDAC cells. Overall, our findings suggest a significant role of ERBB in PDAC treatment. *Cancer Res; 73(7); 1–14. ©2013 AACR.*

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the leading causes of cancer death in Japan and worldwide, with a 5-year survival rate of less than 5% for all stages combined (1–3). Most patients are already unresectable when diagnosed, and even after successful resection, the cancers frequently relapse. In addition, PDAC is highly resistant to conventional chemotherapy regimens. Although molecular-targeted drugs have been extensively evaluated in a number of clinical trials, EGF receptor (EGFR) inhibitor erlotinib in combination with gemcitabine was the only regimen using molecular target agents that showed prolonged survival compared with gemcitabine alone (4). The impact of clinical benefit previously reported appears relatively small; however, considering that almost no regimens have shown any statistically significant benefits compared with gemcitabine in PDAC, this is one of the important options in this field. Moreover, erlotinib has just been approved by the government in Japan as a formal regimen for the treatment of PDAC. Therefore, understanding the detailed mechanisms whereby erlotinib shows an efficacy on PDAC in combination with gemcitabine is gaining more significance.

The predictive factors for treatment with EGFR inhibitor have been established in some cancers. In metastatic colorectal cancer, EGFR inhibition benefits only the *KRAS* wild-type patients (5, 6). In non–small cell lung cancer (NSCLC), patients with activating *EGFR* mutation have shown better response and survival (7, 8). With regard to PDAC, previous studies reported that the overall rate of EGFR expression was 30% to 70% (9, 10), and no obvious impact of EGFR expression on outcome was observed (4, 10). Activating *EGFR* mutations have rarely been reported in human PDAC. Moreover, as a majority of patients with PDAC carry downstream *KRAS* mutations (11, 12), it is difficult to explain why upstream EGFR inhibition has a beneficial effect on the PDAC.

Recently, by using pancreas-specific conditional activation or knockout of clinically relevant PDAC-related genes and signaling pathways, genetically engineered murine PDAC progression models have been described (13–17). Previous studies reported that the genetically engineered models can recapitulate clinical tumor microenvironment better than xenograft tumor models and also can recapitulate the survival effect of clinical trials of human patients (18, 19).

We have already established pancreas-specific TGF-β receptor II (*Tgfbr2*) knockout mice in the context of *Kras* activation (Kras^{G12D}+Tgfbr2^{KO}; ref. 13). The clinical and histopathologic manifestations of the mice recapitulated human PDAC.

Authors' Affiliations: Departments of [1]Gastroetnterology and [2]Molecular Pathology, Graduate School of Medicine, [3]Division of Clinical Genome Research, Institute of Medical Science, The University of Tokyo, Tokyo; [4]Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Chiba; [5]Yamanashi Prefectural Hospital Organization, Yamanashi, Japan; and [6]Vanderbilt-Ingram Comprehensive Cancer Center, Nashville, Tennessee

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Hideaki Ijichi, Department of Gastroetnterology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Phone: 81-3-3815-5411, ext. 33070; Fax: 81-3-3814-0021; E-mail: hideijichi-gi@umin.ac.jp

doi: 10.1158/0008-5472.CAN-12-1453

This model histologically show differentiated ductal adenocarcinoma with abundant stromal components including desmoplastic reaction, but not sarcomatoid or undifferentiated tumors, which are rare in human pancreatic cancer and were reported in other genetically engineered models (13). With regard to TGF-β signaling, *SMAD4* gene mutation or deletion is frequently observed in human patients with PDAC (20); however, mice containing *Smad4* knockout with the *Kras* activation in the pancreas were reported to show cystic tumors of the pancreas, distinct precancer lesions from pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm, or mucious cystic neoplasm (21–23). Therefore, our Kras^{G12D}+Tgfbr2^{KO} might be the closest approximation of the human PDAC in terms of histology that can be expected to recapitulate response to the therapy.

In the present study, we investigated the mode of action of gemcitabine and erlotinib *in vivo* using the Kras^{G12D}+Tgfbr2^{KO} model and propose mechanisms explaining why PDAC with extremely frequent *KRAS* mutation benefits from the EGFR inhibitor in combination with gemcitabine.

Materials and Methods

Reagents

Gemcitabine was purchased from Eli Lilly Japan. Erlotinib was purchased from Chugai Pharmaceutical. An MEK inhibitor, PD0325901, was purchased from WAKO.

Mouse colonies and treatment with reagents

Tgfbr2$^{flox/flox}$ (24), *Ptf1a$^{cre/+}$* (25), and *LSL-Kras$^{G12D/+}$* (26) were described previously. The 3 lines were intercrossed to generate *Ptf1a$^{cre/+}$;LSL-Kras$^{G12D/+}$;Tgfbr2$^{flox/flox}$* (Kras^{G12D}+Tgfbr2^{KO}) on >95% C57BL/6 background(13). All of the experimental protocols were approved by the ethics committee for animal experimentation and conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Graduate School of Medicine, the University of Tokyo (Tokyo, Japan).

The Kras^{G12D}+Tgfbr2^{KO} mice were treated with vehicle, gemcitabine, and gemcitabine + erlotinib ($n = 9–11$, each) for the survival experiment. For the histologic and biochemical analyses, mice ($n = 3–4$ each group) were treated and euthanized at 7 weeks of age, the pancreas was excised, the long and short diameter of tumors was measured, and fixed in 4% paraformaldehyde in PBS or frozen. Details are described in Supplementary Methods.

Cell lines

The cell lines were purchased from the American Type Culture Collection or the RIKEN Cell Bank and passaged in our laboratory for fewer than 6 months after resuscitation. Mouse pancreatic cancer cell lines (K375, K399) were established from Kras^{G12D}+Tgfbr2^{KO} mice, and mouse pancreatic fibroblast (K643f) was established from the *Kras* alone-activated mice as described previously (13, 27).

Cell growth assays

The cell lines were treated with erlotinib, gemcitabine (0–10 μmol/L) for 48 hours in serum-containing media. MTT solution (Sigma) was added to each well to a final concentration of 0.1 mg/mL, and plates were incubated for 1 hour at 37°C. Then, the formazan crystals were dissolved with EtOH and absorbance was read at 570 nm.

Western blotting, immunoprecipitation

Mouse pancreatic tumors and the cells were homogenized with radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors. In immunoprecipitation, the cells were lysed with 1% Nonidet P-40 buffer and subjected to immunoprecipitation analysis. Details are described in Supplementary Methods.

Quantitative reverse transcriptase PCR

Details are described in Supplementary Methods.

ELISA

The cells were treated with vehicle or gemcitabine (10 nmol/L) for 24 hours in serum-containing media and cell culture media were centrifuged at 2,000 rpm for 10 minutes at 4°C, and the supernatants were subjected to the ELISA (Ray Biotech, Inc). Pancreatic tumors from the treated mice were homogenized with lysis buffer (Raybiotech, Inc) and centrifuged at 15,000 × *g* for 15 minutes at 4°C and the supernatants were also subjected to the ELISA.

Flow cytometry

The cells suspended in PBS were incubated in propidium iodide solution (Dojindo Laboratories, 50 μg/mL in PBS) for 30 minutes. The cells were then analyzed for cell-cycle status using the Guava EasyCyte Plus (Guava Technologies). Annexin assay (Guava annexin kit; Millipore) was conducted according to the manufacture's protocol.

Phospho-receptor tyrosine kinase antibody array

Mouse phospho-receptor tyrosine kinase (RTK) antibody array (R&D Systems) analysis was conducted according to the manufacturer's instructions. A total of 250 μg of lysates from the treated PDAC tissues at 7 weeks of age were subjected to analysis. The densitometric data after subtraction of background density were normalized by those of positive controls on each membrane and compared between the treatment groups.

Histology and immunohistochemsitry

Mouse tissues was harvested and processed as described previously (13). The slides with hematoxylin & eosin (H&E) staining were subjected to histologic analysis. Immunohistochemistry was conducted as described previously (28). Details are described in Supplementary Methods.

Statistical analysis

Except when indicated, quantitative data were shown as mean ± SD, and the 2-sided Student t test was used for statistical analysis, with $P < 0.05$ taken as significant. The IC$_{50}$ and the combination index (CI), indicating that the interaction of the drugs was calculated by CalcuSyn software. Log-rank test was used to determine the survival significance.

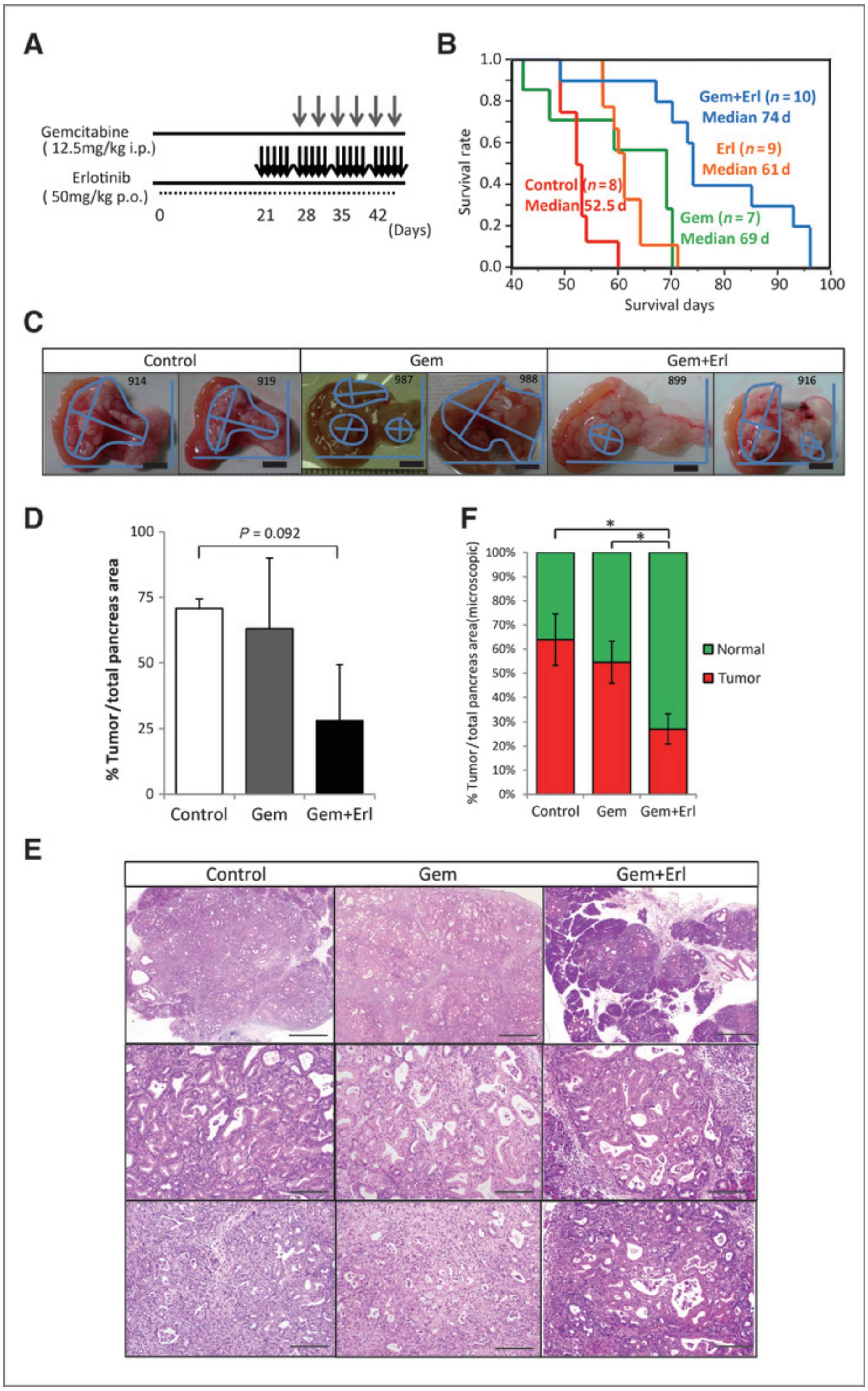


Figure 1. Erlotinib inhibits the Kras^{G12D} + Tgfbr2^{KO} PDAC progression and extends survival of the PDAC mice in combination with gemcitabine. A, treatment schedule. B, Kaplan–Meier curve. Log-rank test showed a statistical difference between the gemcitabine + erlotinib and gemcitabine, or erlotinib alone group ($P = 0.0095$ and 0.0006, respectively), as well as between the gemcitabine or erlotinib alone and control group ($P = 0.046$ and 0.0005, respectively). C, macroscopic appearance of the Kras^{G12D} + Tgfbr2^{KO} pancreas at 7 weeks of age. Tumors are outlined with blue line. Bars, 5 mm. D, the proportion of tumor area to total pancreas tissue. E, H&E staining of the Kras^{G12D} + Tgfbr2^{KO} PDAC tissues. Representative figures of each treatment group are shown. F, quantification of the remaining normal pancreas area (green) and tumor area (red) in the PDAC tissues calculated under the microscope. *, $P < 0.05$. Bars, 1 mm (top) and 200 μm (middle and bottom). Gem, gemcitabine alone group; Gem + Erl, gemcitabine + erlotinib group.

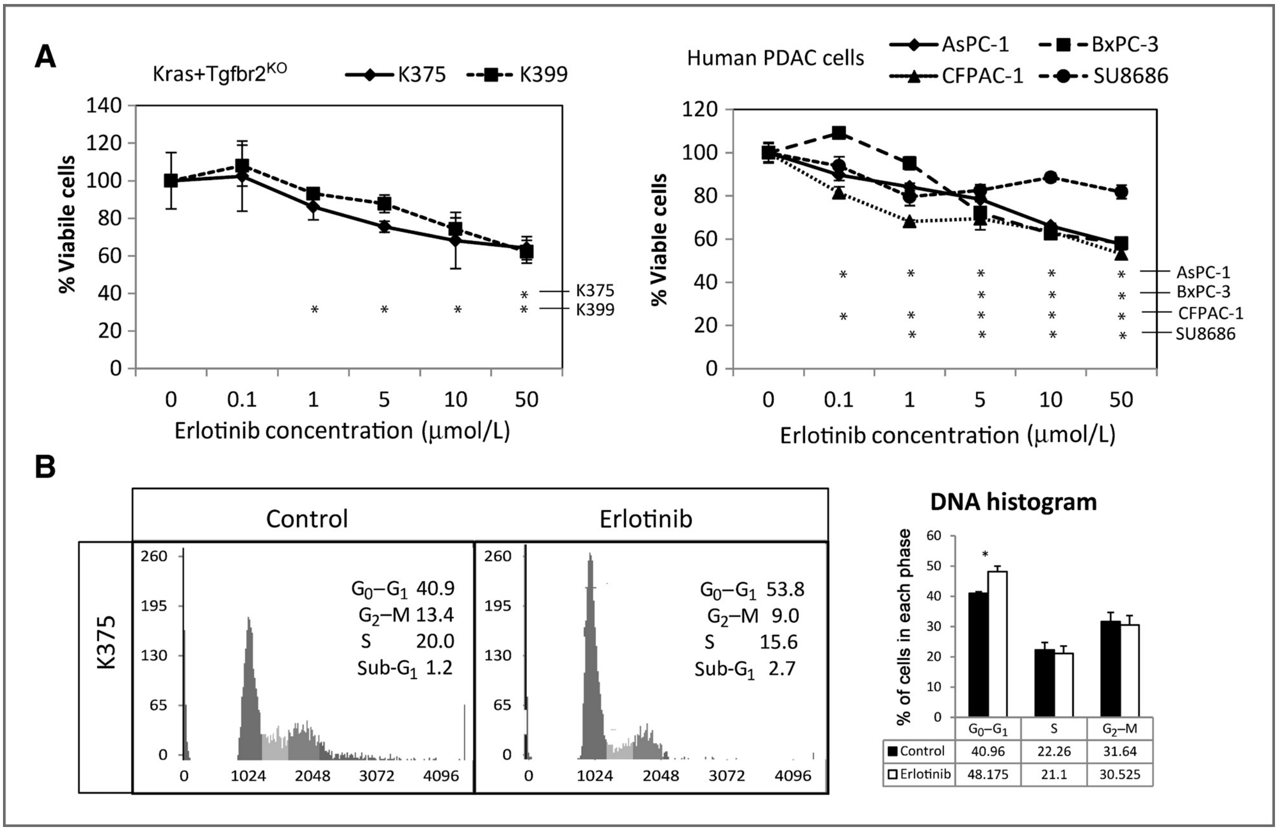


Figure 2. Erlotinib inhibits the growth and intracellular signaling of PDAC cells *in vitro*. A, cell viability of $\mathrm{Kras}^{\mathrm{G12D}}$+$\mathrm{Tgfbr2}^{\mathrm{KO}}$ mouse PDAC cells (K375, K399) and human PDAC cell lines (AsPC-1, BxPC-3, Capan-1, CFPAC-1) treated with the indicated concentrations of erlotinib for 72 hours. *, $P < 0.05$ versus without erlotinib, respectively. B, erlotinib induced G_1 arrest in mouse PDAC cells (K375) in the flow cytometry. *, $P < 0.05$.

Results

Erlotinib inhibits the $\mathrm{Kras}^{\mathrm{G12D}}$ + $\mathrm{Tgfbr2}^{\mathrm{KO}}$ PDAC progression and prolongs survival of the PDAC mice in combination with gemcitabine

We first evaluated the survival of the $\mathrm{Kras}^{\mathrm{G12D}}$+$\mathrm{Tgfbr2}^{\mathrm{KO}}$ PDAC mice by treating with gemcitabine, erlotinib, and gemcitabine + erlotinib (Fig. 1A). Median survival times were 52.5, 69, 61, and 74 days for control, gemcitabine alone, erlotinib alone, and gemcitabine + erlotinib group, respectively (Fig. 1B). Log-rank test comparing the 2 groups, a standard chemoreagent gemcitabine alone significantly extended the survival compared with the control ($P = 0.046$). Furthermore, adding erlotinib to gemcitabine prolonged the survival significantly compared with gemcitabine alone ($P = 0.0095$). We also evaluated survival efficacy of erlotinib alone, which revealed that erlotinib alone extended the survival compared with the control ($P = 0.0005$) and gemcitabine + erlotinib further prolonged the survival compared with the erlotinib alone ($P = 0.0006$).

When dissected at 7 weeks of age, vehicle-treated PDACs were so large that they occupied the entire pancreas, whereas gemcitabine + erlotinib-treated PDACs showed focal nodules in the pancreas. Gemcitabine-treated PDACs were intermediate, some occupied the entire pancreas and some were focal. The proportion of tumor area to total pancreas tissue of gemcitabine + erlotinib–treated PDACs seemed smaller than that of vehicle-treated PDACs (Fig. 1C and D), although the difference did not reach a statistical significance ($P = 0.092$). There was no metastasis at 7 weeks of age in this mouse model.

H&E staining showed that the $\mathrm{Kras}^{\mathrm{G12D}}$ + $\mathrm{Tgfbr2}^{\mathrm{KO}}$ PDAC tissues were basically well-differentiated ductal adenocarcinoma with rich stromal components and also contained poorly differentiated and invasive ductal adenocarcinoma (Fig. 1E). The quantification of remaining normal pancreas area confirmed a statistical difference between the gemcitabine + erlotinib group and the control group, as well as the gemcitabine + erlotinib group and the gemcitabine group (Fig. 1F). The tumor area on the microscope was almost consistent with that judged macroscopically. There were no apparent pathologic differences such as grade of malignancy between tumor tissues with or without gemcitabine, whereas gemcitabine + erlotinib–treated PDACs were less frequent poorly differentiated PDACs (Fig. 1E).

Erlotinib inhibits the growth and intracellular signaling of PDAC cells *in vitro*

We examined the effect of erlotinib on the growth of PDAC cells established from this mouse model (K375, K399) and human pancreatic cancer cell lines (BxPC-3, Capan-1,

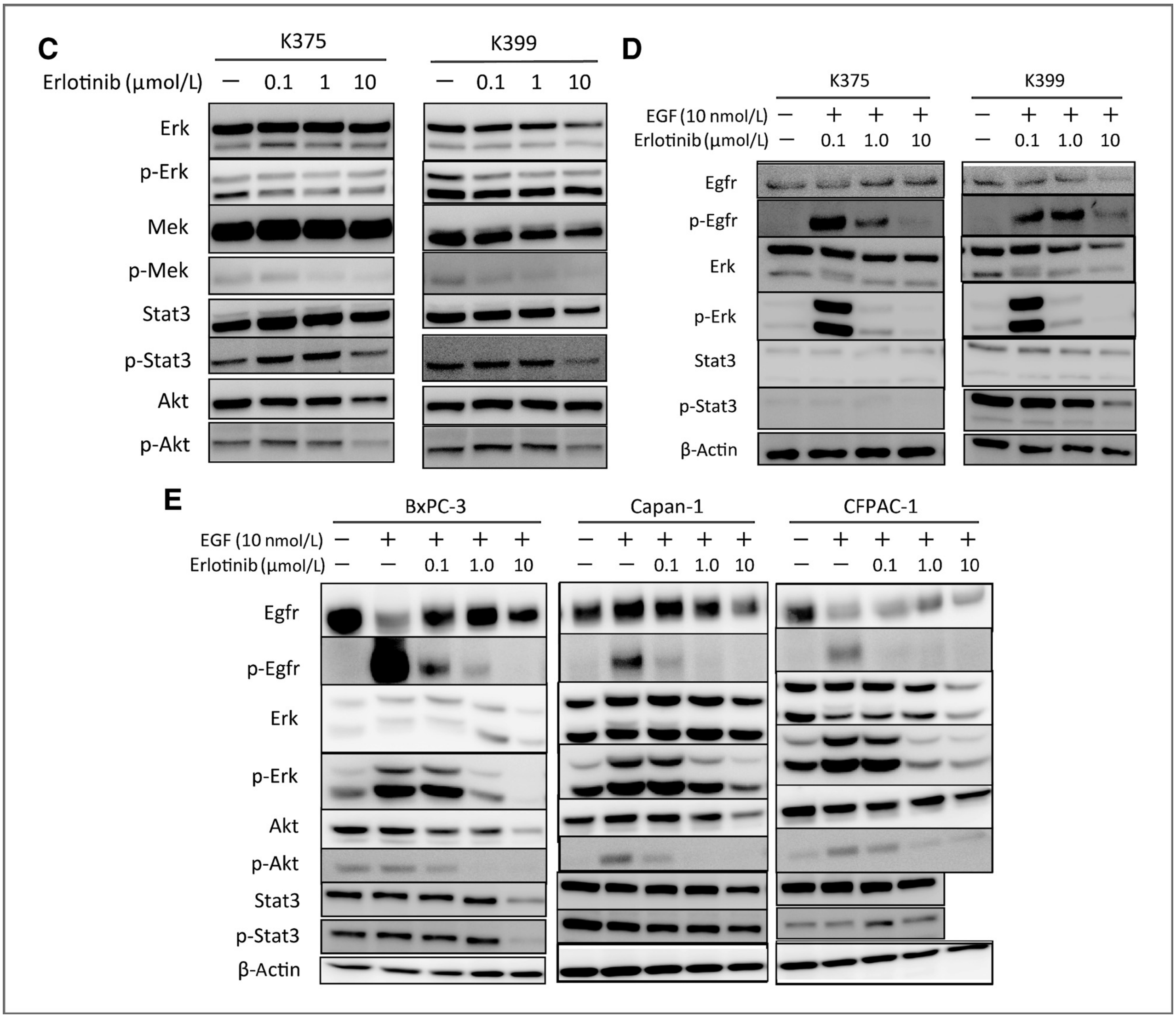


Figure 2. (*Continued*) C–E, Western blot analysis of signal transduction in mouse PDAC cells (K375, K399), with EGF (D) or without EGF (C) and in human PDAC cell lines (BxPC-3, Capan-1, CFPAC-1; E) treated by erlotinib at the indicated concentrations.

and CFPAC-1). Erlotinib inhibited the growth of all PDAC cells, irrespective of their human or mouse origin, although most of them except for BxPC-3 contained constitutively active *KRAS* mutation (Fig. 2A). A flow cytometric analysis showed that erlotinib induced G_1 arrest in mouse PDAC cells (K375; Fig. 2B). Immunoblot analysis showed that erlotinib affected endogenous intracellular signaling and inhibited phosphorylation of EGFR, MEK, and extracellular signal–regulated kinase (ERK). Erlotinib also inhibited phosphorylation of STAT3 and AKT at higher concentrations (Fig. 2C and D). We next treated the PDAC cells with EGF. Although the K375, K399, Capan-1, and CFPAC-1 contained the *KRAS* mutation, we observed that EGF treatment dramatically induced phosphorylation of EGFR, ERK, and AKT in all the cells irrespective of *KRAS* mutation status and erlotinib clearly inhibited the phosphorylation (Fig. 2D and E). STAT3 phosphorylation was not induced by EGF treatment (Fig. 2D and E).

Gemcitabine activates the phosphorylation of Egfr and Erk, which is inhibited by adding erlotinib in the Kras^{G12D} + Tgfbr2^{KO} PDAC *in vivo*

We sacrificed the mice at 7 weeks of age and evaluated the effect of gemcitabine and erlotinib on signal transduction.

Immunohistochemistry showed that Egfr and Erk were strongly phosphorylated in the control group and gemcitabine treatment and increased the phosphorylation of Egfr and Erk. Erlotinib in combination with gemcitabine inhibited the activation of Egfr and Erk. Proliferating cell nuclear antigen (PCNA) staining showed that the control group showed a frequent and strong staining in the nuclei, whereas gemcitabine treatment dramatically reduced the nuclear

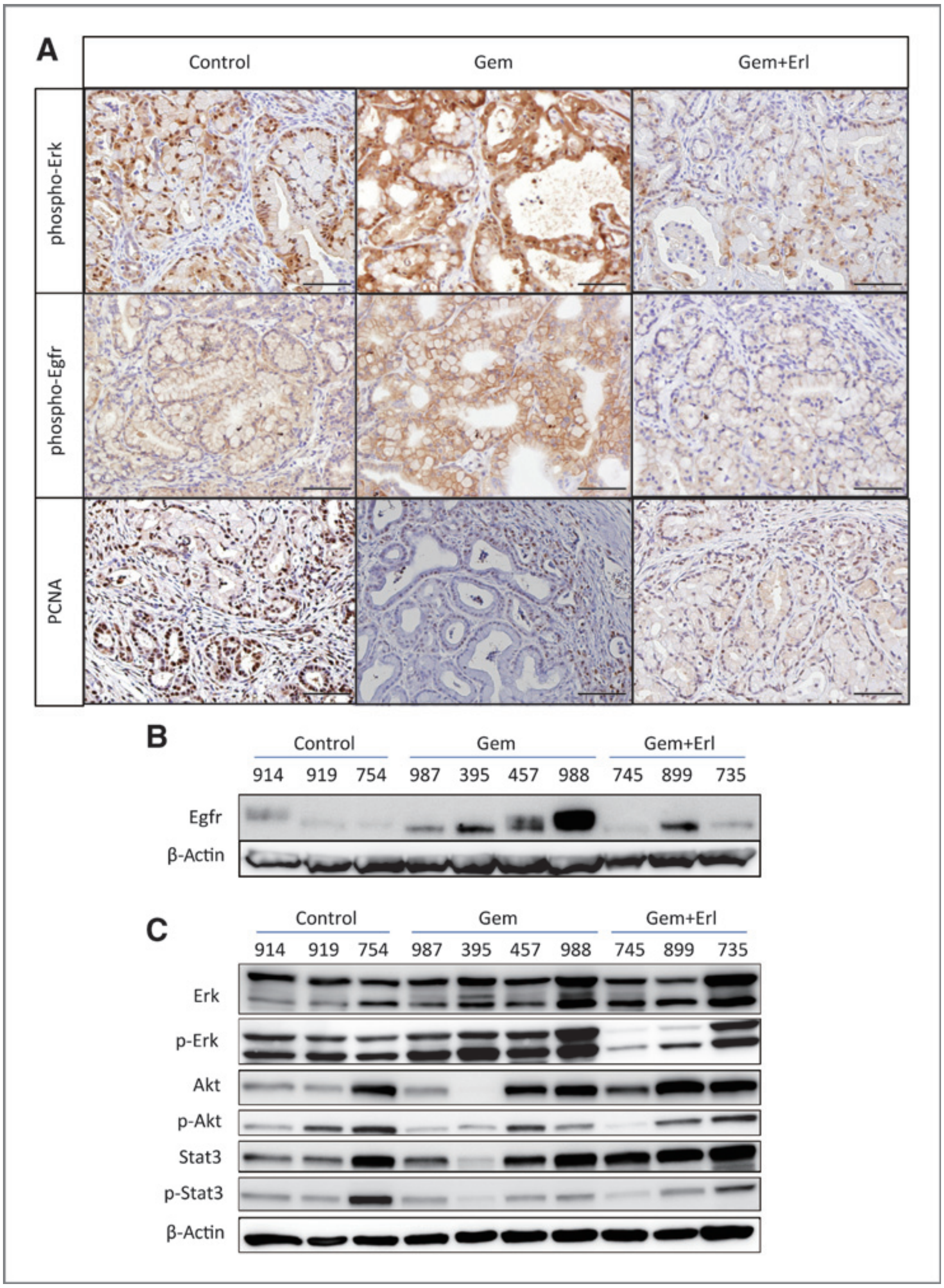


Figure 3. Gemcitabine activates the phosphorylation of Egfr and Erk, which is inhibited by adding erlotinib in the $Kras^{G12D}$ + $Tgfbr2^{KO}$ PDAC *in vivo*. A, immunohistochemistry of the murine pancreatic tumors at 7 weeks of age with anti-phospho-Egfr, phospho-Erk, and PCNA. Bars, 200 μm. Gem, gemcitabine alone group; Gem + Erl, gemcitabine + erlotinib group. B and C, immunoblot analysis of total Egfr (B) and intracellular transduction (C) of the murine pancreatic tumors at 7 weeks of age: vehicle-treated (#914, #919, #754), gemcitabine-treated (#987, #395, #457, #988), and gemcitabine + erlotinib-treated (#745, #899, #735).

staining and adding erlotinib further diminished the staining (Fig. 3A).

Immunoblot analysis of mouse PDAC tissue lysates showed that gemcitabine activated phosphorylation of Erk (Fig. 3B). Furthermore, erlotinib in combination with gemcitabine inhibited the phosphorylation. In the gemcitabine alone–treated group, the mice with larger pancreatic tumors (457, 988) showed strong expression of Egfr and strong phosphorylation of Erk. Erlotinib also diminished the total protein level of Egfr.

There were no apparent differences in the inflammatory cell infiltration (neutrophils and macrophages) by treatment with gemcitabine or in combination with erlotinib (data not shown).

Gemcitabine activates the phosphorylation of ERK in PDAC cells, which is inhibited by adding erlotinib *in vitro*

We examined whether gemcitabine and erlotinib affected the intracellular signaling of PDAC cells *in vitro*. First, we examined the cell viability and showed that adding erlotinib to gemcitabine synergistically inhibited the growth of mouse and human PDAC cells (K375 and BxPC-3; Fig. 4A and B, Supplementary Table S1). In mouse PDAC cells, we detected that gemcitabine alone activated phosphorylation of Erk and Mek, which was inhibited by adding erlotinib (Fig. 4C). The gemcitabine-induced Erk phosphorylation was observed in a time-dependent manner for 0 to 24 hours (Fig. 4D).

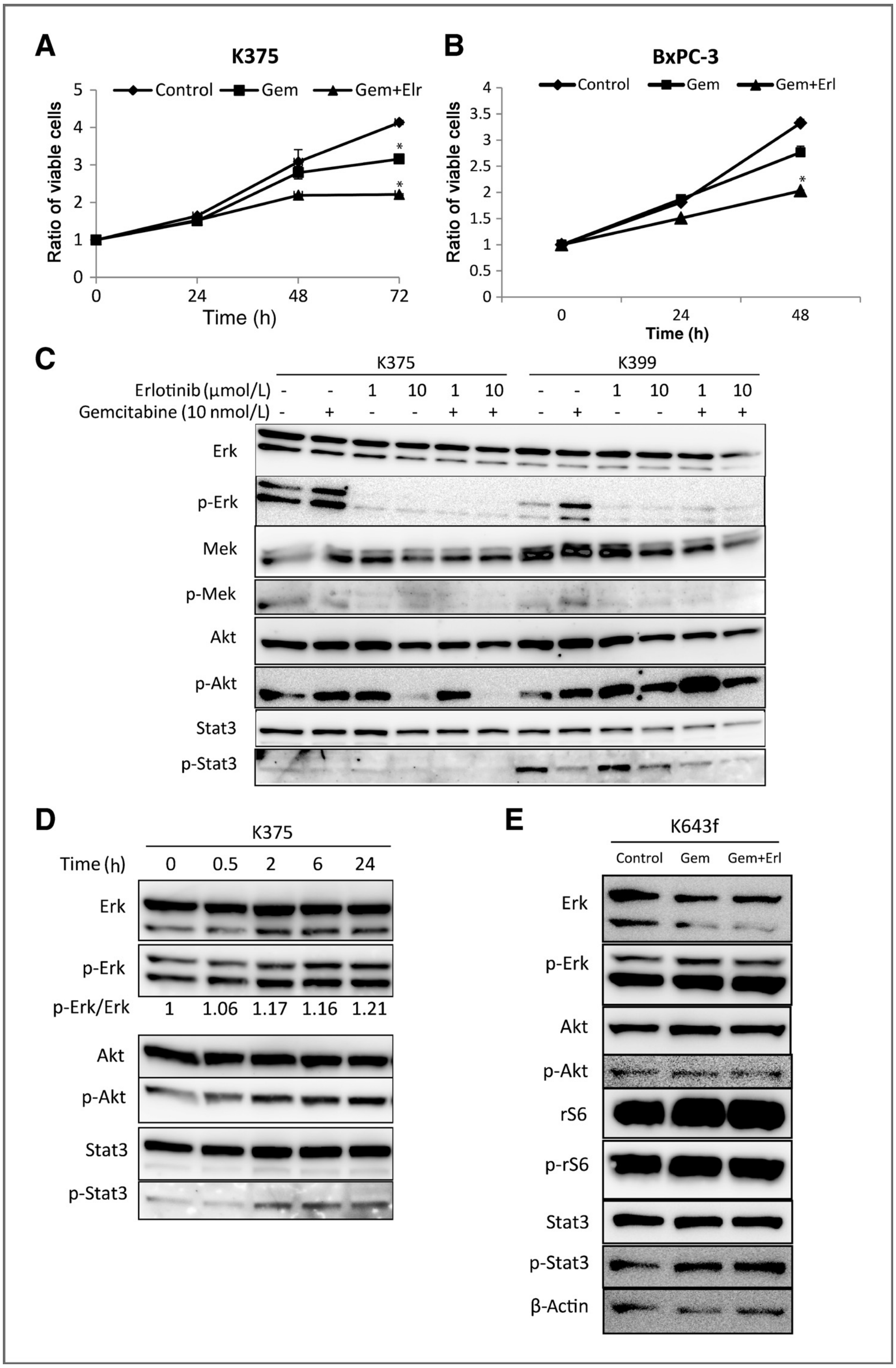


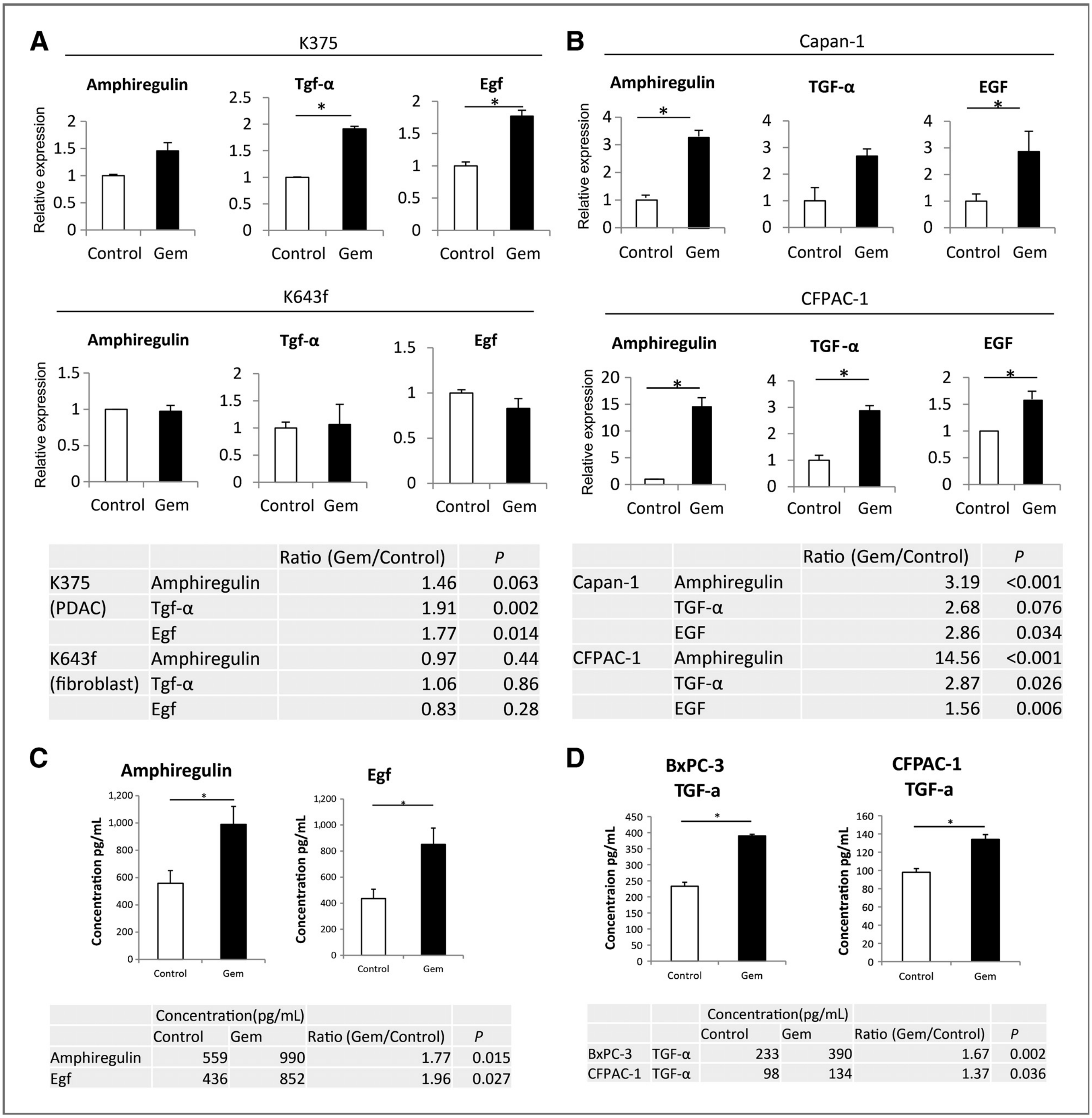
Figure 4. Gemcitabine activates the phosphorylation of ERK in PDAC cells, which is inhibited by adding erlotinib *in vitro*. A and B, cell viability of Kras^{G12D} + Tgfbr2^{KO} mouse PDAC cells (K375; A) and human PDAC cells (BxPC-3; B) treated with vehicle, 10 nmol/L gemcitabine alone, or 10 nmol/L gemcitabine + 1 μmol/L erlotinib for the indicated hours. *, $P < 0.05$ versus control. C, immunoblot analysis of mouse PDAC cells (K375, K399) treated with gemcitabine and erlotinib at the indicated doses. D, Western blot analysis for the time-dependent signal transduction in mouse PDAC cell (K375) after treatment with 10 nmol/L gemcitabine. E, immunoblot analysis of mouse fibroblast (K643f) treated with 10 nmol/L gemcitabine with or without 1 μmol/L erlotinib. Gem, gemcitabine; Gem + Erl, gemcitabine + erlotinib.

We also examined the intracellular signaling of mouse pancreatic fibroblasts (K643f) treated with vehicle, gemcitabine, or gemcitabine + erlotinib *in vitro*, but no obvious change was found in the mouse pancreatic fibroblasts (Fig. 4E). To evaluate a possible contribution of tumor–stromal interactions on the Erk phosphorylation induced by gemcitabine, we next admixed mouse PDAC cells and mouse fibroblasts at 4:1 ratio, but there were no differences in the gemcitabine-induced Erk phosphorylation between with and without fibroblasts (data not shown).

Accordingly, gemcitabine activated Egfr and mitogen-activated protein kinase (MAPK) signaling in the PDAC cells and adding erlotinib inhibited the activation *in vivo* and *in vitro* irrespective of the *Kras* mutation status. Gemcitabine and erlotinib seemed to regulate mainly the PDAC cells not affecting obviously the stromal fibroblasts in the PDAC tissue.

Gemcitabine induces the expression of EGFR ligands in PDAC cells

To examine the mechanism of gemcitabine-induced activation of EGFR/ERK, we evaluated the effect of gemcitabine on the expression of EGFR ligands *in vitro*. Relative RNA levels of *amphiregulin*, *TGF-α*, and *EGF* after incubation with gemcitabine were determined by real-time quantitative PCR. In the murine PDAC cells K375, *Tgf-α* and *Egf* were significantly elevated and *amphiregulin* also seemed to be elevated after gemcitabine treatment, whereas in the murine fibroblast, K643f gemcitabine did not affect the expression of Egfr ligands (Fig. 5A). In the human PDAC cells (Capan-1, CFPAC-1), *amphiregulin*, *TGF-α*, and *EGF* were all significantly elevated after gemcitabine treatment (Fig. 5B). Next, we conducted ELISA for EGFR ligands. In murine PDAC lysates, amphiregulin and Egf were



		Ratio (Gem/Control)	*P*
K375 (PDAC)	Amphiregulin	1.46	0.063
	Tgf-α	1.91	0.002
	Egf	1.77	0.014
K643f (fibroblast)	Amphiregulin	0.97	0.44
	Tgf-α	1.06	0.86
	Egf	0.83	0.28

		Ratio (Gem/Control)	*P*
Capan-1	Amphiregulin	3.19	<0.001
	TGF-α	2.68	0.076
	EGF	2.86	0.034
CFPAC-1	Amphiregulin	14.56	<0.001
	TGF-α	2.87	0.026
	EGF	1.56	0.006

	Concentration(pg/mL)			
	Control	Gem	Ratio (Gem/Control)	*P*
Amphiregulin	559	990	1.77	0.015
Egf	436	852	1.96	0.027

		Concentration(pg/mL)			
		Control	Gem	Ratio (Gem/Control)	*P*
BxPC-3	TGF-α	233	390	1.67	0.002
CFPAC-1	TGF-α	98	134	1.37	0.036

Figure 5. Gemcitabine induces the expression of EGFR ligands in PDAC cells. A and B, quantitative RT-PCR of EGFR ligands (*amphiregulin, TGF-α*, and *EGF*) after incubation with 10 nmol/L gemcitabine in mouse PDAC cell (K375) and mouse fibroblast (K643f; A) and human PDAC cells (Capan-1, CFPAC-1; B). C, ELISA assays for amphiregulin and Egf in gemcitabine-treated PDACs compared with vehicle-treated ones. D, ELISA assays for TGF-α in human pancreatic cancer cells. *, $P < 0.05$; Gem, gemcitabine; Gem + Erl, gemcitabine + erlotinib.

significantly elevated in gemcitabine-treated PDACs compared with vehicle-treated ones (Fig. 5C). In the human PDAC cells (BxPC-3), amphiregulin and TGF-α were significantly elevated after gemcitabine treatment. TGF-αwas also significantly elevated after gemcitabine treatment in Capan-1 and CFPAC-1 (Fig. 5D and data not shown). Thus, the EGFR ligands upregulation can explain the gemcitabine-induced EGFR/ERK activation.

We observed that gemcitabine induced the PDAC cell apoptosis and adding EGF reduced the apoptosis in flow cytometry, which suggested that the gemcitabine-induced EGFR ligand upregulation might be associated with antiapoptotic response of the PDAC cells against gemcitabine (Supplementary Fig. S1). There might be release of EGFR ligands from dying cells by chemotherapy; however, we observed the upregulation of the ligands at mRNA levels, which indicated that the response was derived from live PDAC cells.

Gemcitabine induces activation of Erbb2 in the Kras^{G12D} + Tgfbr2^{KO} PDAC mouse model

We assessed whether RTKs other than EGFR were activated in response to gemcitabine by using a phospho-RTK antibody array, which contained 39 RTKs. We compared 4 groups of mouse pancreatic tissue lysates; vehicle-treated, gemcitabine-treated with low Egfr expression, gemcitabine-treated with high Egfr expression, and gemcitabine + erlotinib–treated. The Egfr phosphorylation in the gemcitabine-treated group with high Egfr expression was inhibited in combination with erlotinib. Most notably, phospho-Erbb2 was more strongly induced than phospho-Egfr in gemcitabine-treated group and was almost completely inhibited in combination with erlotinib (Fig. 6A and B). The array also showed Erbb4 induction by gemcitabine, which was also inhibited by adding erlotinib (Fig. 6A and B).

We evaluated this result by immunoblot analysis. The expression and phosphorylation of Erbb2 were found to be increased in the gemcitabine-treated mice and adding erlotinib inhibited the induction (Fig. 6C). The Erbb2 expression pattern was also confirmed by immunohistochemistry (Fig. 6D).

Gemcitabine induces Erbb2 protein level and a heterodimer formation with Egfr in the PDAC cells *in vitro*, which is diminished by adding erlotinib

We observed that gemcitabine treatment increased the total protein level and phosphorylation of Erbb2, which was inhibited by adding erlotinib in the PDAC cells *in vitro* (Fig. 7A). Next we examined an effect of gemcitabine and erlotinib on heterodimer formation of Egfr with Erbb2 in the PDAC cells. Immunoprecipitation assay revealed that the heterodimer formation of Egfr with Erbb2 was enhanced by gemcitabine treatment and inhibited in combination with erlotinib (Fig. 7B). Quantitative reverse transcriptase PCR (RT-PCR) showed that gemcitabine treatment induced Egfr and Erbb2 expression in the PDAC cells (K375) at transcriptional level, which was further induced by adding erlotinib *in vitro* (Fig. 7C). Immunoblotting showed that gemcitabine + erlotinib clearly decreased the protein level of Erbb2 (Fig. 7D), suggesting that erlotinib in combination with gemcitabine might induce degradation of Erbb2 protein but does not inhibit *Erbb2* transcription in the PDAC cells. Thus, we propose that gemcitabine activated Erbb2 by increasing the total protein level and also heterodimerization with Egfr. Both were inhibited by adding erlotinib.

Gemcitabine-induced EGFR/ErbB2-MAPK signal activation is also dependent on active MAPK signaling

To assess whether the effect of gemcitabine on EGFR/ErbB2 activation is secondary to MAPK signal activation, we evaluated the effect of MEK inhibition. We observed that adding MEK inhibitor (PD0325901) reduced the gemcitabine-induced activation of EGFR and ErbB2 in the PDAC cells (K375; Supplementary Fig. S2). Adding PD0325901 to gemcitabine also reduced the expression of EGFR ligands compared with gemcitabine alone (Supplementary Fig. S3). These results indicated that the gemcitabine-induced EGFR ligands upregulation and EGFR/ErbB2 activation require intact MAPK signaling and these are secondary effects of MAPK signal activation. On the other hand, PD325901 without gemcitabine rather activated the phosphorylation of EGFR, which was consistent with several recent reports showing that selective inhibitors of BRAF and MEK can induce EGFR (refs. 29, 30; Supplementary Fig.S2). The expression of EGFR ligands were also reduced after incubation with PD325901 alone (Supplementary Fig. S3).

We also observed that gemcitabine + PD0325901 significantly inhibited the growth of PDAC cells *in vitro* compared with gemcitabine alone and similarly to the gemcitabine + erlotinib, which suggested that the effect of erlotinib was mainly through the inhibition of MAPK signaling (Supplementary Fig. S4). While MEK inhibition itself might induce certain feedback loop of other signal transduction (as shown in the PD325901 alone-induced EGFR/ErbB2 activation), erlotinib inhibited many downstream pathways of EGFR other than MAPK signaling, which could be the difference between these 2 drugs. We showed a signal diagram in Fig. 8.

Gemcitabine-induced EGFR/ErbB2-MAPK signal activation is a common phenomenon in PDAC and lung cancer cells irrespective of *KRAS* mutation status and gemcitabine sensitivity

We evaluated whether gemcitabine-induced EGFR/ERBB2 induction was related to *KRAS* status or gemcitabine sensitivity. We observed that gemcitabine induced the activation of EGFR/ERBB2 dose dependently in PDAC cell lines irrespective of *KRAS* status and gemcitabine sensitivity (Supplementary Fig.S5A and S5C). Besides, we also examined human lung cancer cells because gemcitabine is commonly used in the treatment and found that gemcitabine also induced EGFR/ERBB2 activation irrespective of *KRAS* status (Supplementary Fig. S5B).

Discussion

Our Kras^{G12D} + Tgfbr2^{KO} PDAC recapitulates human PDACs well in its clinical and histopathologic manifestations. In addition, gemcitabine, a standard chemotherapeutic for PDAC,

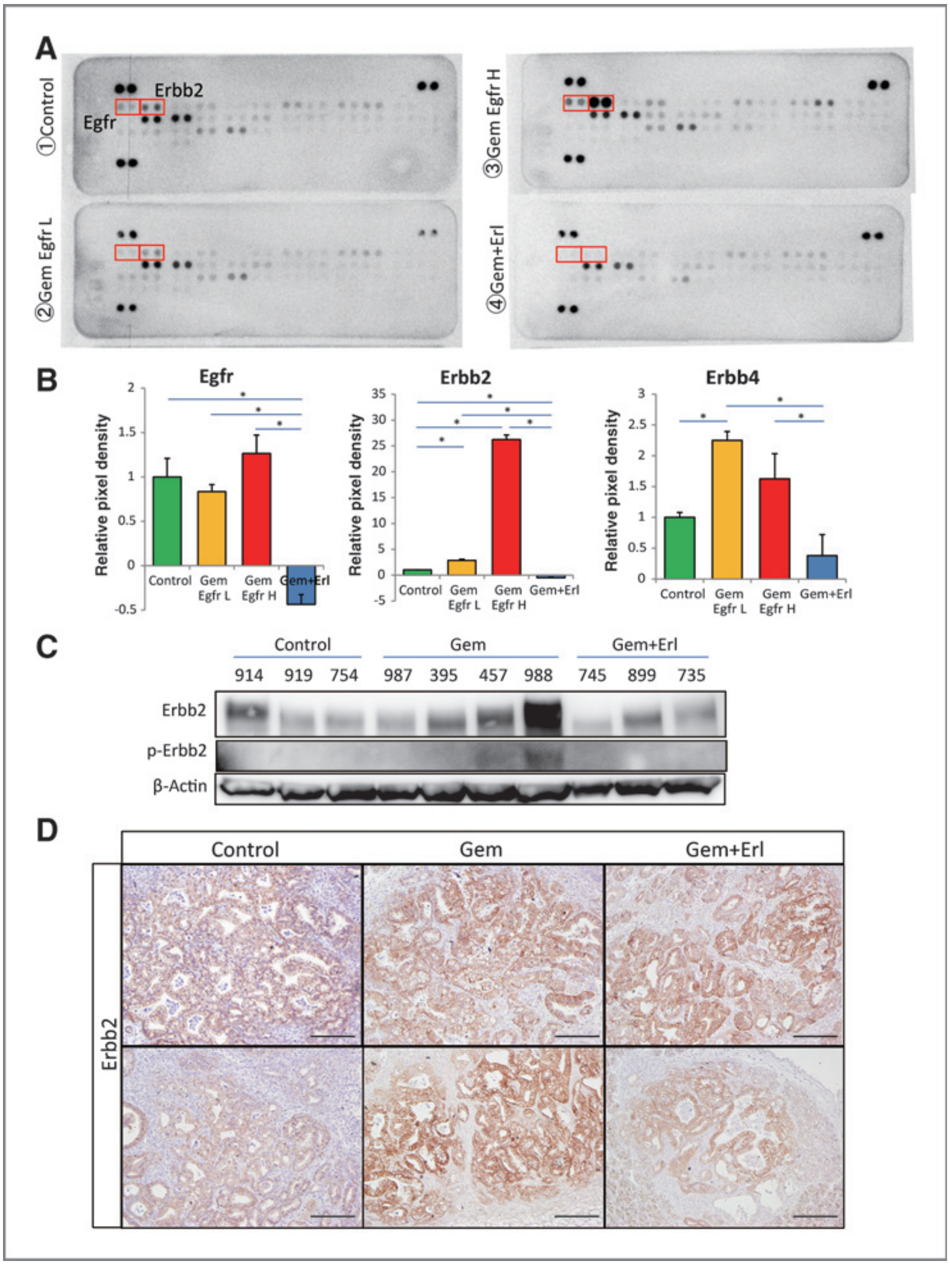


Figure 6. Gemcitabine induces activation of Erbb2 in the Kras^{G12D} + Tgfbr2^{KO} PDAC mouse model. A, phospho-RTK antibody array of 4 groups (vehicle-treated, gemcitabine-treated with low Egfr expression, gemcitabine-treated with high Egfr expression, and gemcitabine + erlotinib-treated). B, quantification of the RTK array data by densitometry. All the densitometric data after subtraction of background density were normalized by those of the positive controls and shown. *, $P < 0.05$. Gem Egfr L, gemcitabine-treated with low Egfr expression; Gem Egfr H, gemcitabine-treated with high Egfr expression; Gem + Erl, gemcitabine + erlotinib-treated. C, immunoblotting of total and phospho-Erbb2 using the PDAC lysates. D, representative figures of immunohistochemistry of Erbb2 expression using the PDAC tissues. Bars, 100 μm. Gem, gemcitabine alone group.

extended survival of the mice significantly, which suggested that this model might be suitable for evaluating treatment regimens for PDACs. The survival period was further dramatically extended by adding erlotinib to gemcitabine. This model is useful for evaluating not only the survival impact but also the mode of action of therapeutic regimens for PDAC.

In this study, we show one of the mechanisms by which the EGFR inhibitor, erlotinib, inhibits PDAC with extremely frequent *KRAS* mutation. Although EGFR overexpression has been reported as a common feature of PDAC (30%–70%; refs. 9, 10), activating *EGFR* mutations have rarely been reported, and *EGFR* gene copy number and *KRAS* mutational status were not found to be predictive markers of a survival benefit from EGFR inhibitor (9). The human PDAC and lung cancer cells used in this study had no *EGFR* mutations. We showed that gemcitabine induced activation of EGFR and ErbB2 as well as downstream MAPK signal activation, which was completely inhibited by adding erlotinib even in the *KRAS*-mutant PDAC cells. This phenomenon was commonly seen in the PDAC cells irrespective of gemcitabine sensitivity or *KRAS* mutation status and was also common in lung cancer cells.

We observed that EGFR ligand upregulation and EGFR/ErbB2 heterodimer formation were involved in gemcitabine-induced MAPK signal activation in PDAC. The phenomenon that gemcitabine induced EGFR-MAPK signaling activation has been reported in previous studies; however, EGFR phosphorylation might rapidly cause EGFR degradation and the signal activation might vary depending on the cell context and

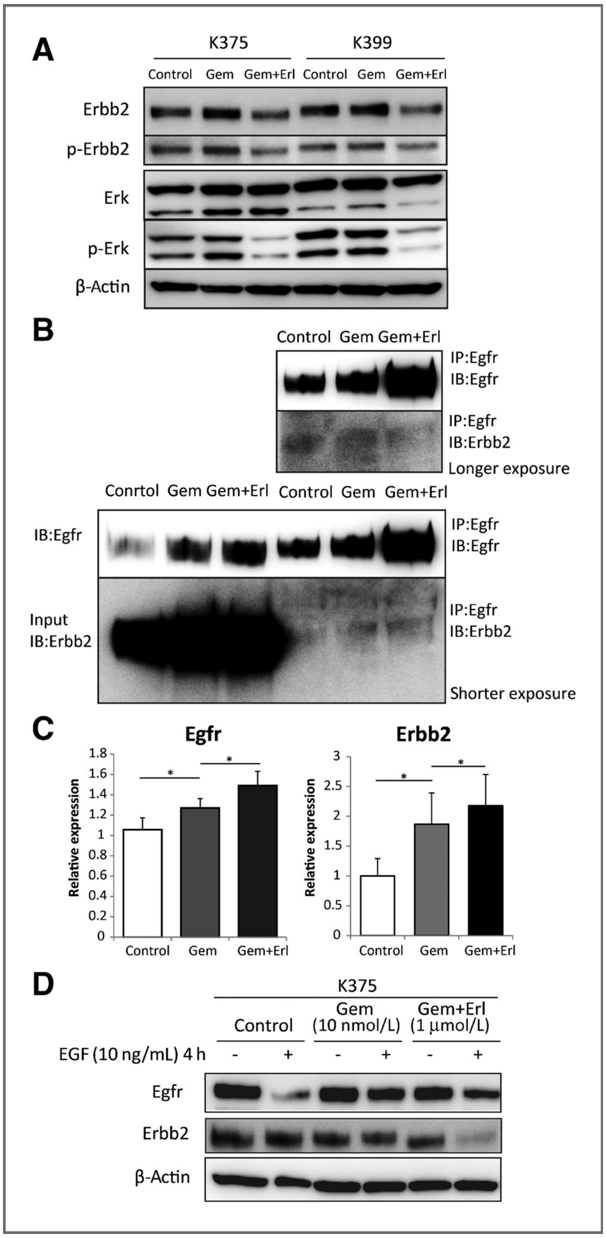


Figure 7. Gemcitabine induces Erbb2 protein level and a heterodimer formation with Egfr in the PDAC cells *in vitro*, which is diminished by adding erlotinib. A, immunoblot analysis of mouse PDAC cells (K375, K399) treated with vehicle, gemcitabine, or gemcitabine + erlotinib *in vitro*. B, immunoprecipitation assay for the heterodimer formation of Egfr with Erbb2 using the lysates from mouse PDAC cells (K375). Bottom, a shorter exposure of the blots. Top, the blot of input was excised and a longer exposure is shown. IB, immunoblotting; IP, immunoprecipitation. C, quantitative RT-PCR of *Egfr* and *Erbb2* in mouse PDAC cells (K375) treated with vehicle, gemcitabine, or gemcitabine + erlotinib *in vitro*. *, $P < 0.05$. D, immunoblot analysis of Egfr and Erbb2 protein level in mouse PDAC cells (K375) treated with 10 nmol/L gemcitabine and 1 µmol/L erlotinib for 24 hours, with 10 ng/mL EGF stimulation for the last 4 hours *in vitro*. Gem, gemcitabine; Gem + Erl, gemcitabine + erlotinib.

time course (31–33). Recently, it was reported that gemcitabine enhanced the heterodimer formation of EGFR with ErbB3 and secretion of amphiregulin, resulting in MAPK signal activation in human PDAC cells *in vitro* (34). Previous reports also described that overexpression of ErbB3 is related to tumorigenesis and progression of pancreatic cancer and to sensitivity of erlotinib (34–36). Our results revealed that gemcitabine treatment upregulated not only amphiregulin but also TGF-α and EGF and induced a heterodimer formation of EGFR with ErbB2 in the PDAC cells. These results indicate that similar but somewhat different mechanisms are involved in certain type of PDAC. We further observed that the gemcitabine-induced EGFR ligand expression and EGFR/ErbB2-MAPK signaling was a secondary effect of active MAPK signaling, which suggested that there might be certain signaling loop amplifying EGFR/ErbB2-MAPK signaling. Although MEK inhibition suppressed the expression of EGFR ligands, it activated EGFR/ErbB2. In contrast, erlotinib inhibited many downstream pathways of EGFR other than MAPK signaling, which could be the difference between these 2 drugs (Fig. 8).

ErbB2 is a well-known prognostic factor and therapeutic target in breast and gastric cancers. Overexpression and amplification of ErbB2 is observed in 20% to 30% of breast cancer (37, 38) and in 7% to 34% of gastric cancer (39–41). Trastuzumab, a monoclonal antibody against ErbB2, has a survival benefit in ErbB2-positive breast cancer (42) and gastric cancer (43). In PDAC, ErbB2 overexpression is observed (10%–82%) but does not correlate with poor prognosis (44–46). Although the antitumor effect of trastuzumab was documented in patients with high ErbB2 expression (47), survival effects of ErbB2 inhibitor in PDAC were not significant in clinical trials (48). The true clinical advantage of ErbB2-targeted therapy in PDAC therefore remains unclear. We observed that the Kras^{G12D}+Tgfbr2^{KO} PDAC showed a better survival response to the gemcitabine plus erlotinib compared with the result of human clinical trial (4). The suggested mechanism involved ErbB2. In addition, murine PDAC with high Egfr expression occupied the entire pancreas following gemcitabine alone treatment, whereas PDAC with low Egfr expression showed frequent normal pancreas (Figs. 1, 3, and 6). Taken together, it might be possible that certain subpopulation of patients with PDAC, for example, with disrupted TGF-b signaling and high EGFR expression, can especially have the survival benefit from EGFR/ErbB2-targeted therapy.

Because the Kras^{G12D} + Tgfbr2^{KO} PDAC contained abundant stromal components similar to human PDAC, we also tried to clarify the effects of erlotinib on the tumor microenvironment, such as fibroblasts, neutrophils, and macrophages. Gemcitabine and erlotinib did not affect the intracellular signaling of pancreatic fibroblasts *in vitro*, and no prominent differences were detected in immunohistochemistry of neutrophil and macrophage in the treated PDAC tissues. Thus, we concluded that the major effect of erlotinib is on EGFR-MAPK signaling in PDAC cells rather than the stromal cells.

In conclusion, the Kras^{G12D}+Tgfbr2^{KO} PDAC recapitulates chemosensitivity of human PDAC and was useful in the investigation of efficacy and mode of action of therapeutic agents, which might provide important insights into the predictive markers, beneficial drug combinations, and also beneficial patient subpopulation. We found the underlying mechanisms explaining why PDAC with highly frequent *KRAS*

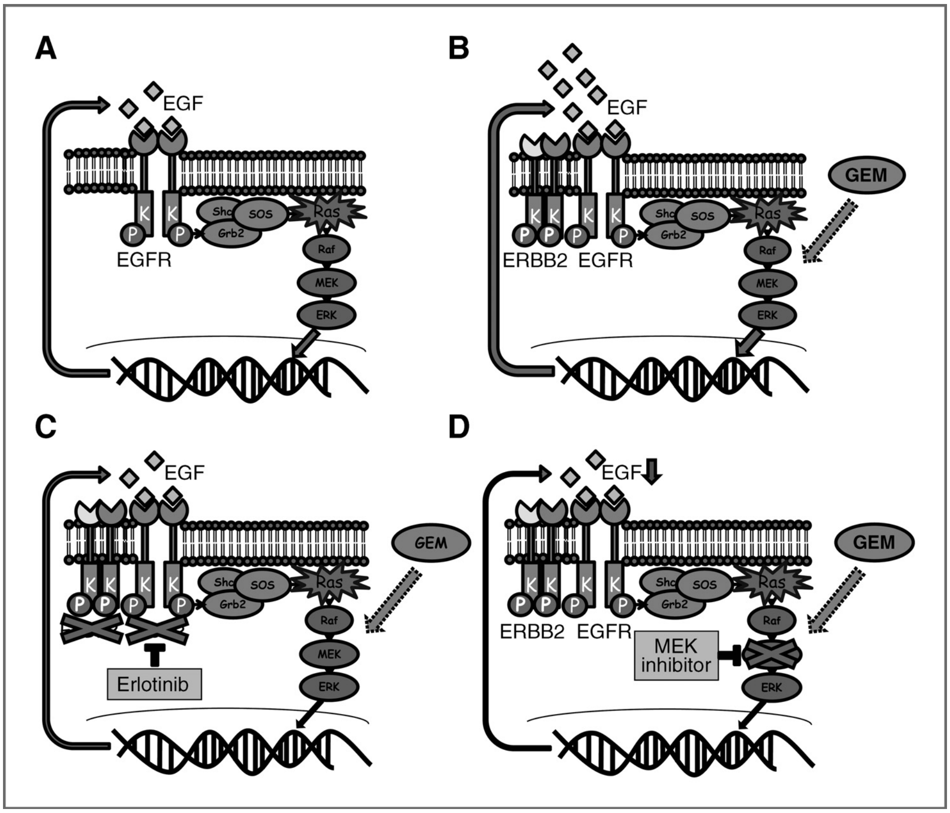


Figure 8. Signaling diagram. A, EGFR-MAPK signaling without gemcitabine. (It is already activated by mutant *Kras*.) B, gemcitabine further activates EGFR-MAPK signaling, by increasing EGFR ligand expression, ERBB2 protein expression, and EGFR–ERBB2 heterodimer formation. The increase of EGFR ligands is dependent on MAPK activation. C, erlotinib inhibited the gemcitabine-induced EGFR-MAPK signaling. D, MEK inhibitor reduced EGFR ligand expression and gemcitabine-induced MAPK activation.

mutation benefits from erlotinib in combination with gemcitabine *in vitro* and *in vivo*. Gemcitabine induced EGFR-MAPK signal activation, which was dramatically diminished by adding erlotinib even in the *Kras*-mutant PDACs. PDACs with high EGFR and ErbB2 expression as well as disrupted TGF-β signaling might be the beneficial subpopulation for this combination therapy. Further translational research using genetically engineered mouse models such as the one used in this study might accelerate our understanding and development effective therapies to overcome the most obstinate cancer, PDAC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: K. Miyabayashi, H. Ijichi
Development of methodology: K. Miyabayashi, H. Ijichi, M. Tada
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Miyabayashi, H. Ijichi, D. Mohri, K. Yamamoto, Y. Nakai, H. Isayama
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Miyabayashi, H. Ijichi, Y. Asaoka, T. Ikenoue, H.L. Moses
Writing, review, and/or revision of the manuscript: K. Miyabayashi, H. Ijichi, K. Tateishi, H.L. Moses, K. Koike
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Tada, K. Tateishi, Y. Morishita
Study supervision: M. Tada, M. Omata, K. Koike

Acknowledgments

The authors thank Christopher V.E. Wright (Vanderbilt University, Nashville, TN) and Tyler Jacks (Massachusetts Institute of Technology) for providing the mice and Mitsuko Tsubouchi for technical assistance.

Grant Support

This study was supported by Kakenhi, grants of Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT) and grants from Japanese Society of Gastroenterology, Foundation for Promotion of Cancer Research in Japan and Foundation for Advancement of International Science to H. Ijichi. This work was also supported by Japan Society for the Promotion of Science (JSPS) Core-to-Core Program "Cooperative International Framework in TGF-β Family Signaling".

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 20, 2012; revised January 2, 2013; accepted January 13, 2013; published OnlineFirst February 1, 2013.

References

1. Warshaw AL, Fernández-del Castillo C. Pancreatic carcinoma. N Engl J Med 1992;326:455–65.
2. Hezel AF, Kimmelman AC, Stanger BZ, Bardeesy N, Depinho RA. Genetics and biology of pancreatic ductal adenocarcinoma. Genes Dev 2006;20:1218–49.
3. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, et al. Cancer statistics, 2006. CA Cancer J Clin 2006;56:106–30.
4. Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the

National Cancer Institute of Canada Clinical Trials Group. J Clin Oncol 2007;25:1960–6.

5. Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol 2008;26: 1626–34.
6. Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 2008;359:1757–65.
7. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39.
8. Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med 2009;361:947–57.
9. da Cunha Santos G, Dhani N, Tu D, Chin K, Ludkovski O, Kamel-Reid S, et al. Molecular predictors of outcome in a phase 3 study of gemcitabine and erlotinib therapy in patients with advanced pancreatic cancer: National Cancer Institute of Canada Clinical Trials Group Study PA.3. Cancer 2010;116:5599–607.
10. Garcea G, Neal CP, Pattenden CJ, Steward WP, Berry DP. Molecular prognostic markers in pancreatic cancer: a systematic review. Eur J Cancer 2005;41:2213–236.
11. Almoguera C, Shibata D, Forrester K, Martin J, Arnheim N, Perucho M. Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. Cell 1988;53:549–54.
12. Tada M, Omata M, Ohto M. Clinical application of ras gene mutation for diagnosis of pancreatic adenocarcinoma. Gastroenterology 1991;100: 233–8.
13. Ijichi H, Chytil A, Gorska AE, Aakre ME, Fujitani Y, Fujitani S, et al. Aggressive pancreatic ductal adenocarcinoma in mice caused by pancreas-specific blockade of transforming growth factor-beta signaling in cooperation with active Kras expression. Genes Dev 2006;20:3147–60.
14. Aguirre AJ, Bardeesy N, Sinha M, Lopez L, Tuveson DA, Horner J, et al. Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma. Genes Dev 2003;17: 3112–26.
15. Hingorani SR, Petricoin EF, Maitra A, Rajapakse V, King C, Jacobetz MA, et al. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer Cell 2003;4: 437–50.
16. Hingorani SR, Wang L, Multani AS, Combs C, Deramaudt TB, Hruban RH, et al. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. Cancer Cell 2005;7:469–83.
17. Bardeesy N, Aguirre AJ, Chu GC, Cheng KH, Lopez LV, Hezel AF, et al. Both p16(Ink4a) and the p19(Arf)-p53 pathway constrain progression of pancreatic adenocarcinoma in the mouse. Proc Natl Acad Sci U S A 2006;103:5947–52.
18. Olive KP, Jacobetz MA, Davidson CJ, Gopinathan A, McIntyre D, Honess D, et al. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. Science 2009;324:1457–61.
19. Singh M, Lima A, Molina R, Hamilton P, Clermont AC, Devasthali V, et al. Assessing therapeutic responses in Kras mutant cancers using genetically engineered mouse models. Nat Biotechnol 2010;28:585–93.
20. Hahn SA, Schutte M, Hoque AT, Moskaluk CA, da Costa LT, Rozenblum E, et al. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science 1996;271:350–3.
21. Bardeesy N, Cheng KH, Berger JH, Chu GC, Pahler J, Olson P, et al. Smad4 is dispensable for normal pancreas development yet critical in progression and tumor biology of pancreas cancer. Genes Dev 2006;20:3130–46.
22. Izeradjene K, Combs C, Best M, Gopinathan A, Wagner A, Grady WM, et al. Kras(G12D) and Smad4/Dpc4 haploinsufficiency cooperate to induce mucinous cystic neoplasms and invasive adenocarcinoma of the pancreas. Cancer Cell 2007;11:229–43.
23. Kojima K, Vickers SM, Adsay NV, Jhala NC, Kim HG, Schoeb TR, et al. Inactivation of Smad4 accelerates Kras(G12D)-mediated pancreatic neoplasia. Cancer Res 2007;67:8121–30.
24. Chytil A, Magnuson MA, Wright CV, Moses HL. Conditional inactivation of the TGF-beta type II receptor using Cre:Lox. Genesis 2002;32:73–5.
25. Kawaguchi Y, Cooper B, Gannon M, Ray M, MacDonald RJ, Wright CV. The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. Nat Genet 2002;32:128–34.
26. Jackson EL, Willis N, Mercer K, Bronson RT, Crowley D, Montoya R, et al. Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. Genes Dev 2001;15:3243–8.
27. Ijichi H, Chytil A, Gorska AE, Aakre ME, Bierie B, Tada M, et al. Inhibiting Cxcr2 disrupts tumor-stromal interactions and improves survival in a mouse model of pancreatic ductal adenocarcinoma. J Clin Invest 2011;121:4106–17.
28. Ohta M, Seto M, Ijichi H, Miyabayashi K, Kudo Y, Mohri D, et al. Decreased expression of the RAS-GTPase activating protein RASAL1 is associated with colorectal tumor progression. Gastroenterology. 2009;136:206–16.
29. Turke AB, Song Y, Costa C, Cook R, Arteaga CL, Asara JM, et al. MEK inhibition leads to PI3K/AKT activation by relieving a negative feedback on ERBB receptors. Cancer Res 2012;72:3228–37.
30. Prahallad A, Sun C, Huang S, Di Nicolantonio F, Salazar R, Zecchin D, et al. Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. Nature 2012;483:100–03.
31. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 2007;316:1039–43.
32. Feng FY, Varambally S, Tomlins SA, Chun PY, Lopez CA, Li X, et al. Role of epidermal growth factor receptor degradation in gemcitabine-mediated cytotoxicity. Oncogene 2007;26:3431–9.
33. Correale P, Marra M, Remondo C, Migali C, Misso G, Arcuri FP, et al. Cytotoxic drugs up-regulate epidermal growth factor receptor (EGFR) expression in colon cancer cells and enhance their susceptibility to EGFR-targeted antibody-dependent cell-mediated-cytotoxicity (ADCC). Eur J Cancer 2010;46:1703–11.
34. Yotsumoto F, Fukami T, Yagi H, Funakoshi A, Yoshizato T, Kuroki M, et al. Amphiregulin regulates the activation of ERK and Akt through epidermal growth factor receptor and HER3 signals involved in the progression of pancreatic cancer. Cancer Sci 2010;101:2351–60.
35. Frolov A, Schuller K, Tzeng CW, Cannon EE, Ku BC, Howard JH, et al. ErbB3 expression and dimerization with EGFR influence pancreatic cancer cell sensitivity to erlotinib. Cancer Biol Ther 2007;6:548–54.
36. Liles JS, Arnoletti JP, Tzeng CW, Howard JH, Kossenkov AV, Kulesza P, et al. ErbB3 expression promotes tumorigenesis in pancreatic adenocarcinoma. Cancer Biol Ther 2010;10:555–63.
37. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med 2005;353:1659–72.
38. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 2001;344:783–92.
39. Gravalos C, Jimeno A. HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target. Ann Oncol 2008;19:1523–9.
40. Hofmann M, Stoss O, Shi D, Büttner R, van de Vijver M, Kim W, et al. Assessment of a HER2 scoring system for gastric cancer: results from a validation study. Histopathology 2008;52:797–805.
41. Tanner M, Hollmén M, Junttila TT, Kapanen AI, Tommola S, Soini Y, et al. Amplification of HER-2 in gastric carcinoma: association with Topoisomerase IIalpha gene amplification, intestinal type, poor prognosis and sensitivity to trastuzumab. Ann Oncol 2005;16:273–8.
42. Smith I, Procter M, Gelber RD, Guillaume S, Feyereislova A, Dowsett M, et al. 2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial. Lancet 2007;369:29–36.
43. Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive

advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. Lancet 2010;376: 687–97.

44. Safran H, Miner T, Resnick M, Dipetrillo T, McNulty B, Evans D, et al. Lapatinib/gemcitabine and lapatinib/gemcitabine/oxaliplatin: a phase I study for advanced pancreaticobiliary cancer. Am J Clin Oncol 2008;31:140–4.

45. Novotný J, Petruzelka L, Vedralová J, Kleibl Z, Matous B, Juda L. Prognostic significance of c-erbB-2 gene expression in pancreatic cancer patients. Neoplasma 2001;48:188–91.

46. Kimura K, Sawada T, Komatsu M, Inoue M, Muguruma K, Nishihara T, et al. Antitumor effect of trastuzumab for pancreatic cancer with high HER-2 expression and enhancement of effect by combined therapy with gemcitabine. Clin Cancer Res 2006;12:4925–32.

47. Safran H, Iannitti D, Ramanathan R, Schwartz JD, Steinhoff M, Nauman C, et al. Herceptin and gemcitabine for metastatic pancreatic cancers that overexpress HER-2/neu. Cancer Invest 2004;22:706–12.

48. Safran H, Miner T, Bahary N, Whiting S, Lopez C, Sun W, et al. Lapatinib and gemcitabine for metastatic pancreatic cancer: A phase II study. Am J Clin Oncol 2011;34:50–2.

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

American Association for Cancer Research

Erlotinib Prolongs Survival in Pancreatic Cancer by Blocking Gemcitabine-Induced MAPK Signals

Koji Miyabayashi, Hideaki Ijichi, Dai Mohri, et al.

Cancer Res Published OnlineFirst February 1, 2013.