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

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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-targeted drugs 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 (KrasG12D þ Tgfbr2KO; ref. 13). The clinical and histopathologic manifestations of the mice recapitulated human PDAC.
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-$\beta$ 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 mucinous 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$^{lox/lox}$ (24), Ptf1a$^{rec-}$ (25), and LSL-Kras$^{G12D/+}$ (26) were described previously. The 3 lines were intercrossed to generate Ptf1a$^{mot-}$ LSL-Kras$^{G12D/+}$; Tgfbr2$^{lox/lox}$ (Kras$^{G12D}$ + Tgfbr2$^{KO}$) on $>95\%$ C57BL/6 background (15). 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 $\mu$mol/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 EIOH 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 $\times$ 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 $\mu$g/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 manufacturer’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 $\mu$g 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 immunohistochemistry

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 $\pm$ 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<sup>G12D</sup> + Tgfbr2<sup>−/−</sup> 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<sup>G12D</sup> + Tgfbr2<sup>−/−</sup> 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<sup>G12D</sup> + Tgfbr2<sup>−/−</sup> 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.
Results

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

We first evaluated the survival of the Kras^{G12D} + Tgfbr2^{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 ($P = 0.0046$). Furthermore, adding gemcitabine to erlotinib prolonged the survival significantly compared with gemcitabine alone ($P = 0.0005$). 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 Kras^{G12D} + Tgfbr2^{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, CFPAC-1) (Fig. 2A). Erlotinib induced G1 arrest in mouse PDAC cells (K375) (Fig. 2B). 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 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 G1 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 KrasG12D + Tgfbr2KO 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...
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).
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 Egrf 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

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.
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-β was 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<sup>G12D</sup> + Tgfbr2<sup>KO</sup> 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 Egr expression, gemcitabine-treated with high Egr expression, and gemcitabine + erlotinib-treated. The Egr phosphorylation in the gemcitabine-treated group with high Egr expression was inhibited in combination with erlotinib. Most notably, phospho-ErbB2 was more strongly induced than phospho-Egr 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 activation 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<sup>G12D</sup> + Tgfbr2<sup>KO</sup> PDAC recapitulates human PDACs well in its clinical and histopathologic manifestations. In addition, gemcitabine, a standard chemotherapeutic for PDAC,
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
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 KrasG12D–Tgfbr2KO PDAC showed a better survival response to the gemicitabine 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-β signaling and high EGFR expression, can especially have the survival benefit from EGFR/ErbB2-targeted therapy.

Because the KrasG12D + Tgfbr2KO 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 KrasG12D–Tgfbr2KO 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...
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.

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