Erlotinib prolongs survival in pancreatic cancer by blocking gemcitabine-induced MAPK signals

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Running title: Erlotinib inhibits gemcitabine-induced MAPK signaling in pancreatic cancer

Key words: pancreatic ductal adenocarcinoma, erlotinib, gemcitabine, genetically-engineered mouse model, EGFR·MAPK signaling

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The authors have declared that no conflict of interest exists.

This manuscript contains 4997 words (excluding references), 8 figures and no tables.
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 epidermal growth factor receptor (EGFR) inhibitor, erlotinib with gemcitabine has been the only molecular targeted drug tested so far which has been superior to gemcitabine alone. The mechanism underlying this effective combinational regimen remains unknown. Here we demonstrate 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 MAPK 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.
Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the leading causes of cancer death in Japan and worldwide, with a 5-yr survival rate of less than 5% for all stages combined\(^1\),\(^2\),\(^3\). 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, EGFR inhibitor erlotinib in combination with gemcitabine was the only regimen using molecular target agents that showed prolonged survival compared to 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 to 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. With regard to PDAC, previous studies reported that the overall rate of EGFR expression was 30-70% and no obvious impact of EGFR expression on outcome was observed. Activating EGFR mutations have rarely been reported in human PDAC. Moreover, since a majority of PDAC patients carry downstream KRAS mutations, 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. 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.

We have already established pancreas-specific TGF-beta receptor II (Tgfbr2) knockout mice in the context of Kras activation (KrasG12D+Tgfbr2KO). The clinical and histopathological manifestations of the mice recapitulated human PDAC. This model histologically demonstrates 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\textsuperscript{13}. With regard to TGF-β signaling, \textit{SMAD4} gene mutation or deletion is frequently observed in human PDAC patients\textsuperscript{20}, however, mice containing \textit{Smad4} knockout with the \textit{Kras} activation in the pancreas was reported to demonstrate cystic tumors of the pancreas, distinct precancer lesions from PanIN, intraductal papillary mucinous neoplasm, or mucious cystic neoplasm\textsuperscript{21-23}. Therefore, our \textit{Kras}\textsuperscript{G12D+Tgfbr2KO} 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 \textit{Kras}\textsuperscript{G12D+Tgfbr2KO} model and propose mechanisms explaining why PDAC with extremely frequent \textit{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. A MEK inhibitor, PD0325901 was purchased from WAKO.

Mouse colonies and treatment with reagents

\( Tgfbr2^{\text{flk/flo}} \), \( Ptf1a^{\text{cre/+}} \) and \( LSL\cdot Kras^{G12D/+} \) were described previously. The three lines were intercrossed to generate \( Ptf1a^{\text{cre/+}}; LSL\cdot Kras^{G12D/+}; Tgfbr2^{\text{flk/flo}} \) (\( Kras^{G12D+Tgfbr2\text{KO}} \)) on > 95% C57BL/6 background. 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.

The \( Kras^{G12D+Tgfbr2\text{KO}} \) mice were treated with vehicle, gemcitabine, and gemcitabine + erlotinib (n=9-11, each) for the survival experiment. For the histological and biochemical analyses, mice (n=3-4 each group) were treated and euthanized at 7 weeks of age, then 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+Tgfbr2KO} 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 μM) for 48 h 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 h 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 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 RT-PCR.**

Details are described in Supplementary Methods.

**ELISA**

The cells were treated with vehicle or gemcitabine (10 nM) for 24 h in serum-containing media and cell culture media were centrifuged at 2000 rpm for 10 min 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 15000 G for 15 min 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 min. The cells were then analyzed for cell cycle status using the Guava EasyCyte Plus (Guava Technologies). Annexin assay (Guava annexin kit; Millipore) was performed according to the manufacture’s protocol.
Phospho-RTK antibody array

Mouse phospho-receptor-type tyrosine kinase (RTK) antibody array (R&D Systems) analysis was performed according to the manufacturer’s instructions. 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 immunohistochemistry

Mouse tissue was harvested and processed as described previously. The slides with hematoxylin & eosin staining were subjected to histological analysis. Immunohistochemistry was performed as described previously. Details are described in Supplementary Methods.

Statistical Analysis

Except when indicated, quantitative data were shown as mean ± SD and the two-sided Student’s t test was used for statistical analysis, with \( P < 0.05 \) taken as significant. The IC50 and the combination index (CI) indicating the interaction of the drugs
were calculated by CalcuSyn software. Logrank test was used to determine the survival significance.
Results

Erlotinib inhibits the \text{Kras}^{G12D+Tgfb2KO} \text{PDAC progression and prolongs survival of the PDAC mice in combination with gemcitabine.}

We first evaluated the survival of the \text{Kras}^{G12D+Tgfb2KO} \text{PDAC mice by treating with gemcitabine, erlotinib, and gemcitabine + erlotinib (Figure 1A). Median survival times were 52.5, 69, 61, and 74 days for control, gemcitabine alone, erlotinib alone, and gemcitabine + erlotinib group, respectively (Figure 1B). Logrank test comparing the two groups, a standard chemoreagent gemcitabine alone significantly extended the survival compared to the control (p = 0.046). Further, adding erlotinib to gemcitabine prolonged the survival significantly compared to gemcitabine alone (p = 0.0095). We also evaluated survival efficacy of erlotinib alone, which revealed that erlotinib alone extended the survival compared to the control (p = 0.0005) and gemcitabine + erlotinib further prolonged the survival compared to 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 (Figure 1C, 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$^{G12D+Tgfbr2^{K0}}$ PDAC tissues were basically well-differentiated ductal adenocarcinoma with rich stromal components and also contained poorly-differentiated and invasive ductal adenocarcinoma (Figure 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 (Figure 1F). The tumor area on the microscope was almost consistent with that judged macroscopically. There were no apparent pathological differences such as grade of malignancy between tumor tissues with or without gemcitabine, whereas gemcitabine + erlotinib-treated PDACs were less frequent poorly-differentiated PDAC (Figure 1E).

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

Next 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 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 (Figure 2A). A flow cytometry analysis showed that erlotinib induced G1 arrest in mouse PDAC cells (K375) (Figure 2B). Immunoblot analysis showed that erlotinib affected endogenous intracellular signaling and inhibited phosphorylation of EGFR, MEK and ERK. Erlotinib also inhibited phosphorylation of STAT3 and AKT at higher concentrations (Figure 2C, 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, AKT in all the cells irrespective of KRAS mutation status and erlotinib clearly inhibited the phosphorylation (Figure 2D, E). STAT3 phosphorylation was not induced by EGF treatment (Figure 2D, 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 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 demonstrated that the control group showed a frequent and strong staining in the nuclei, while gemcitabine treatment dramatically reduced the nuclear staining and adding erlotinib further diminished the staining (Figure 3A).

Immunoblot analysis of mouse PDAC tissue lysates showed that gemcitabine activated phosphorylation of Erk (Figure 3B). Further, erlotinib in combination with gemcitabine inhibited the phosphorylation. In the gemcitabine alone-treated group, the mice with larger pancreatic tumors (457, 988) demonstrated 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 demonstrated that adding erlotinib to gemcitabine synergistically inhibited the growth of mouse and human PDAC
cells (K375 and BxPC-3) (Figure 4A, B, Table S1). In mouse PDAC cells we detected that gemcitabine alone activated phosphorylation of Erk and Mek, which was inhibited by adding erlotinib (Figure 4C). The gemcitabine-induced Erk phosphorylation was observed in a time-dependent manner for 0 to 24 h (Figure 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 (Figure 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 or without fibroblasts (data not shown).

Accordingly, gemcitabine activated Egfr and 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 didn’t affect the expression of Egfr ligands (Figure 5A). In the human PDAC cells (Capan-1, CFPAC-1), Amphiregulin, TGF-α, and EGF were all significantly elevated after gemcitabine treatment (Figure 5B). Next, we performed ELISA assays for EGFR ligands. In murine PDAC lysates, Amphiregulin and Egf were significantly elevated in gemcitabine-treated PDACs compared to vehicle-treated ones (Figure 5C). In the human PDAC cells (BxPC-3), Amphiregulin and TGF-α were significantly elevated after gemcitabine treatment. TGF-α was also significantly elevated after gemcitabine treatment in Capan-1 and CFPAC-1 (Figure 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 ligands upregulation might be associated with anti-apoptotic response of the PDAC cells against gemcitabine (Figure 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 receptor-type tyrosine kinases (RTKs) other than EGFR were activated in response to gemcitabine by using a phospho-RTK antibody array, which contained 39 RTKs. We compared four 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 (Figure 6A, B). The array also showed Erbb4 induction by gemcitabine, which was also inhibited by adding erlotinib (Figure 6A, 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 (Figure 6C). The Erbb2 expression pattern was also confirmed by immunohistochemistry (Figure 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 (Figure 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 (Figure 7B). Quantitative RT-PCR demonstrated 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 (Figure 7C). Immunoblotting showed that gemcitabine + erlotinib clearly decreased the protein level of Erbb2 (Figure 7D), suggesting that erlotinib in combination with gemcitabine might induce degradation of Erbb2 protein, but 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) (Figure S2). Adding PD0325901 to gemcitabine also reduced the expression of EGFR ligands compared to gemcitabine alone (Figure 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 demonstrating that selective inhibitors of BRAF and MEK can induce EGFR (Figure S2). The expression of EGFR ligands were also reduced after incubation with PD325901 alone (Figure S3).

We also observed that gemcitabine + PD0325901 significantly inhibited the growth of PDAC cells in vitro compared to gemcitabine alone and similarly to the gemcitabine + erlotinib, which suggested that the effect of erlotinib was mainly through the inhibition of MAPK signaling (Figure 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 two drugs. We showed a signal diagram in Figure 8.

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

We evaluated whether gemcitabine-induced EGFR/ERBB2 induction was related to \textit{KRAS} status or gemcitabine sensitivity. We observed gemcitabine induced the activation of EGFR/ERBB2 dose-dependently in PDAC cell lines irrespective of \textit{KRAS} status and gemcitabine sensitivity (Figure S5A, C). 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 \textit{KRAS} status (Figure S5B).
Discussion

Our Kras^{G12D+Tgfbr2KO} PDAC recapitulates well human PDAC in its clinical and histopathological 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 PDAC. 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 demonstrate 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%)\textsuperscript{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\textsuperscript{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 time course. 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. Previous reports also described that overexpression of ErbB3 is related to tumorigenesis and progression of pancreatic cancer and to sensitivity of erlotinib. 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 similar but somewhat different mechanisms are involved in certain type of PDAC. We further observed that the gemcitabine-induced EGFR ligands 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. By contrast,
Erlotinib inhibited many downstream pathways of EGFR other than MAPK signaling, which could be the difference between these two drugs (Figure 8).

ErbB2 is a well-known prognostic factor and therapeutic target in breast and gastric cancers. Overexpression and amplification of ErbB2 is observed in 20-30% of breast cancer\textsuperscript{37,38}, and in 7-34% of gastric cancer\textsuperscript{40-42}. Trastuzumab, a monoclonal antibody against ErbB2, has a survival benefit in ErbB2-positive breast cancer\textsuperscript{39} and gastric cancer\textsuperscript{43}. In PDAC, ErbB2 overexpression is observed (10-82%), but does not correlate with poor prognosis\textsuperscript{44-46}. Although the antitumor effect of trastuzumab was documented in patients with high ErbB2 expression\textsuperscript{47}, survival effects of ErbB2 inhibitor in PDAC was not significant in clinical trials\textsuperscript{48}. The true clinical advantage of ErbB2-targeted therapy in PDAC therefore remains unclear. We observed that the Kras\textsuperscript{G12D+Tgfbr2KO} PDAC showed a better survival response to the gemcitabine plus erlotinib compared to the result of human clinical trial\textsuperscript{4}. The suggested mechanism involved ErbB2. In addition, murine PDAC with high Egfr expression occupied the entire pancreas following gemcitabine-alone treatment, while PDAC with low Egfr expression showed frequent normal pancreas (Figures 1, 3, 6). Taken together, it might be possible that certain subpopulation of PDAC patients, e.g., with disrupted TGF-\beta signaling and high EGFR expression, can especially have the survival benefit from EGFR/ErbB2-targeted therapy.
Since the $\text{Kras}^{G12D+}\text{Tgfbr2KO}$ 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 didn’t 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 erlotinib’s major effect is on EGFR-MAPK signaling in PDAC cells rather than the stromal cells.

In conclusion, the $\text{Kras}^{G12D+}\text{Tgfbr2KO}$ 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 $\text{KRAS}$ 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 $\text{Kras}$-mutant PDAC. PDAC 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.
Acknowledgements

We thank Christopher V.E. Wright (Vanderbilt University) 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.I.

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”.
References


Figure legends

**Figure 1**: Erlotinib inhibits the Kras<sup>G12D</sup>+Tgfbr2<sup>KO</sup> PDAC progression and extends survival of the PDAC mice in combination with gemcitabine
(A) Treatment schedule. (B) Kaplan-Meyer curve. Logrank test demonstrated 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>KO</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>KO</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 (upper) and 200 μm (middle and lower); 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 Kras<sup>G12D</sup>+Tgfbr2<sup>KO</sup> 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 h. *: p < 0.05 v.s. without erlotinib, respectively. (B) Erlotinib induced G1 arrest in mouse PDAC cells (K375) in the flow cytometry. *: p < 0.05. (C, D, 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.

**Figure 3**: Gemcitabine activates the phosphorylation of Egfr and Erk, which is inhibited by adding erlotinib in the Kras<sup>G12D</sup>+Tgfbr2<sup>KO</sup> 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, 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).

**Figure 4**: Gemcitabine activates the phosphorylation of ERK in PDAC cells, which is inhibited by adding erlotinib in vitro
(A, B) Cell viability of Kras^{G12D+Tgfbr2^{KO}} mouse PDAC cells (K375) (A) and human PDAC cells (BxPC-3) (B) treated with vehicle, 10 nM gemcitabine alone or 10 nM gemcitabine + 1 μM erlotinib for the indicated hours. *: p < 0.05 v.s. 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 nM gemcitabine. (E) Immunoblot analysis of mouse fibroblast (K643f) treated with 10 nM gemcitabine with or without 1 μM erlotinib. Gem: gemcitabine; Gem+Erl: gemcitabine + erlotinib.

**Figure 5: Gemcitabine induces the expression of EGFR ligands in PDAC cells**
(A, B) Quantitative RT-PCR of EGFR ligands (Amphiregulin, TGF-α and EGF) after incubation with 10 nM 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 to vehicle-treated ones. (D) ELISA assays for TGF-α in human pancreatic cancer cells. *: p < 0.05; Gem: gemcitabine; Gem+Erl: gemcitabine + erlotinib.

**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; Gem+Erl: gemcitabine + erlotinib group.

**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). The lower panel shows a shorter exposure of the blots. In the upper panel, the blot of input was excised and a longer-exposure is shown. IP: immunoprecipitation; IB: immunoblotting.
(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 nM gemcitabine and 1 μM erlotinib for 24 h, with 10 ng/ml EGF stimulation for the last 4 h in vitro. Gem: gemcitabine; Gem+Erl: gemcitabine + erlotinib.

**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 ligands 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 ligands expression and gemcitabine-induced MAPK activation.
Figure 2

A

% viable cells

0 10 50

erlotinib concentration (µM)

K375 K399

B

Control erlotinib

G0/G1 40.9
G2/M 13.4
S 20.0
subG1 1.2

G0/G1 53.8
G2/M 9.0
S 15.6
subG1 2.7

DNA histogram

C

erlotinib(µM) - 0.1 1 10 - 0.1 1 10

K375 K399

Erk p-Erk Mek p-Mek Stat3 p-Stat3 Akt p-Akt

D

K375 K399

EGF(10nM) erlotinib(µM) - + + + - + + +

Egfr p-Egfr Erk p-Erk Stat3 p-Stat3 β-actin

E

BxPC-3 Capan-1 CFPAC-1

EGF(10nM) erlotinib(µM) - + + + - + + + - + + + - + + +

Egfr p-Egfr Erk p-Erk Stat3 p-Stat3 β-actin
Figure 3

A

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Figure 5

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K643f

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K643f

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Figure 8
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.

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