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Amphiregulin regulates the activation of ERK and Akt through epidermal growth factor receptor and HER3 signals involved in the progression of pancreatic cancer

Fusanori Yotsumoto,[1,2] Tatsuya Fukami,[2,3] Hiroshi Yagi,[4] Akihiro Funakoshi,[5] Toshiyuki Yoshizato,[3] Masahide Kuroki[1,2] and Shingo Miyamoto[2,3,6]

[1]Department of Biochemistry, School of Medicine, [2]Center for Advanced Molecular Medicine, [3]Department of Obstetrics & Gynecology, School of Medicine, Fukuoka University, Fukuoka, Japan; [4]Cell Growth Regulation Section, Oral and Pharyngeal Cancer Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland, USA; [5]Department of Gastroenterology, National Kyushu Cancer Center, Fukuoka, Japan

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Pancreatic cancer is one of the most lethal malignancies. Epidermal growth factor receptor (EGFR), HER3, Akt, and amphiregulin have been recognized as targets for pancreatic cancer therapy. Although gemcitabine + erlotinib has been the recommended chemotherapy for pancreatic cancer, the prognosis is extremely poor. The development of molecularly targeted therapies has been required for patients with pancreatic cancer. To assess the validation of amphiregulin as a target for pancreatic cancer therapy, we examined its expression in pancreatic cancer using real-time PCR analyses and ELISA. We also measured the apoptotic cell rate using TUNEL assays. In addition, alterations in signaling pathways were detected by immunoblotting analyses. Treatment with gemcitabine, which reduced the cell viability and augmented the cell apoptotic rate, activated and subsequently attenuated ERK and EGFR signals. However, gemcitabine, paclitaxel, or cisplatin treatment enhanced the Akt activation, heterodimer formation of EGFR with HER3, and secretion of amphiregulin, indicating that the presence of gemcitabine promoted the activity of targeted molecules including amphiregulin, Akt, and HER3 for pancreatic cancer therapy. Combined treatment with an inhibitor for amphiregulin and gemcitabine, paclitaxel, or cisplatin induced synergistic antitumor effects, accompanied by the suppression of Akt and ERK activation. Blockade of amphiregulin suppressed the activities of EGFR, HER3, and Akt and the expression of amphiregulin itself. According to this evidence, combination chemotherapy of conventional anticancer drugs plus an inhibitor for amphiregulin would allow us to provide more favorable clinical outcomes for patients with pancreatic cancer. (*Cancer Sci* 2010; 101: 2351–2360)

The prognosis of pancreatic cancer, one of the most devastating forms of cancer, is extremely poor, mainly because 80–85% of pancreatic cancer patients are not diagnosed until they reach an unresectable status.[1,2] Although the chemotherapeutic regimen of gemcitabine + erlotinib, a potent inhibitor of the epidermal growth factor receptor (EGFR) tyrosine kinase, has been regarded as the standard chemotherapy for advanced pancreatic cancer,[3,4] the efficacy of this regimen seems to have become debatable.[5] Therefore, the development of molecularly targeted therapies for pancreatic cancer has been required to ameliorate the clinical prognosis in pancreatic cancer.

Epidermal growth factor receptor has been proposed as a promising target for pancreatic cancer therapies. In immunohistological analyses, pancreatic cancer patients with phosphorylated Akt had a poor prognosis compared with those with unphosphorylated Akt.[6] Accordingly, there is increasing evidence that Akt signaling plays pivotal roles in the mechanisms for resistance to gemcitabine.[7] Overexpression of HER3 has also been shown to be a prognostic factor in patients with pancreatic cancer.[8] The heterodimer formation mediated by HER3 was reported to be involved in the acquisition of aggressive behavior by pancreatic cancer cells through phosphatidylinositol 3-kinase (PI3K)/Akt signaling.[9] Previously, we reported that amphiregulin was validated as an attractive target for pancreatic cancer therapy using *in vitro* analyses.[10] Epidermal growth factor receptor, HER3, Akt, and amphiregulin, which are all members of the HER family, are considered to be putative targets for the development of molecularly targeted therapies for patients with pancreatic cancer.

Amphiregulin, originally isolated from MCF-7 breast cancer cells,[11] was secreted through ectodomain shedding mainly through the actions of a disintegrin and metalloproteinase (ADAM)17.[12] Amphiregulin knockout mice show impaired proliferative responses after partial liver resection[13,14] and female mice show impaired mammary gland development and/or functions.[15,16] Amphiregulin transgenic mice display small intralobular ducts and centroacinar cell proliferation, whereas transforming growth factor (TGF)-α transgenic mice show tubular complex formation with a strong fibrogenic response.[17,18] These characteristics indicate enhanced expression of amphiregulin, thereby suggesting that amphiregulin may be involved in the proliferation of pancreatic duct cells. In addition, the presence of amphiregulin in cancer cells was associated with an increased frequency of local lymph node involvement.[19] According to this evidence, it is plausible that amphiregulin may play a pivotal role in the acquisition of a malignant phenotype in pancreatic cancer.

In the present study, in order to reconfirm the validation of amphiregulin as a target for pancreatic cancer therapy, we examined its antitumor effects as well as the alterations in signals after treatment with an inhibitory agent against amphiregulin compared with inhibitory agents against other HER family members.

Materials and Methods

Reagents and antibodies. Cross-reacting material 197 (CRM197) was a kind gift from Professor Eisuke Mekada (Department of Cell Biology, Osaka University, Osaka, Japan). Gemcitabine was purchased from Enzo Life Sciences International (Plymouth Meeting, PA, USA). Erlotinib, an EGFR tyrosine kinase inhibitor, was kindly provided by F. Hoffmann–La Roche (Basel, Switzerland). Cetuximab, a chimeric (mouse/

[6]To whom all correspondence should be addressed.
E-mail: smiya@cis.fukuoka-u.ac.jp

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human) monoclonal antibody against EGFR, was kindly provided by Merck KGaA (Darmstadt, Germany). Recombinant human amphiregulin, neuregulin, neutralizing antibodies against amphiregulin, TGF-α and neuregulin, and control IgG were purchased from R&D Systems (Minneapolis, MN, USA). Polyclonal antibodies against EGFR, HER2, and ERK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibodies against HER3 and anti-phospho-ERK, and anti-phosphotyrosine antibodies were purchased from Millipore–Upstate Biotechnology (Lake Placid, NY, USA). Polyclonal anti-Akt and monoclonal anti-phospho-Akt (Ser473) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). A monoclonal anti-β-actin antibody was purchased from Sigma (St. Louis, MO, USA). 5-Fluorouracil (5-FU), cisplatin, and paclitaxel were obtained from Calbiochem (San Diego, CA, USA).

Cell lines and tissue samples. The following cell lines were obtained commercially: KLM-1 cells from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan); MIA-PaCa-2 cells from the Japanese Collection of Research Bioresources (Osaka, Japan); and PANC-1, AsPC-1, CAPAN1, and CFPAC-1 cells from the American Type Culture Collection (Manassas, VA, USA). All cells were maintained in RPMI-1640 medium supplemented with 10% FBS (ICN Biomedicals, Irvine, CA, USA), 100 U/mL penicillin G, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere of 5% CO_2 at 37°C. All six patients examined in this study had undergone surgery at the National Kyushu Cancer Center (Fukuoka, Japan) and provided written informed consent to participate in this study. The study was approved by the Institutional Review Board of National Kyushu Cancer Center.

Real-time quantitative PCR. RNA extraction, cDNA synthesis, and Real-time quantitative PCR were carried out as previously described.[10]

Soluble HB-EGF, EGF, amphiregulin, and TGF-α in cell culture media (CM). The levels of heparin-binding epidermal growth factor-like growth factor (HB-EGF), amphiregulin, TGF-α, and epidermal growth factor (EGF) in CM of cells incubated for 48 h were determined using a commercially available sandwich ELISA (DuoSet kit; R&D Systems) according to the manufacturer's instructions and as previously described.[20] When the levels were less than the detection limits, the amounts of HB-EGF, EGF, TGF-α, and amphiregulin were recorded as 31, 39, 78, and 156 pg/mL, respectively. All the samples were assayed in triplicate. Each mean value was considered to be representative of the corresponding CM.

Immunoprecipitation and immunoblotting analyses. To evaluate the alterations in phosphorylation of EGFR, HER3, Akt, or ERK before the occurrence of significant cell apoptosis, cells

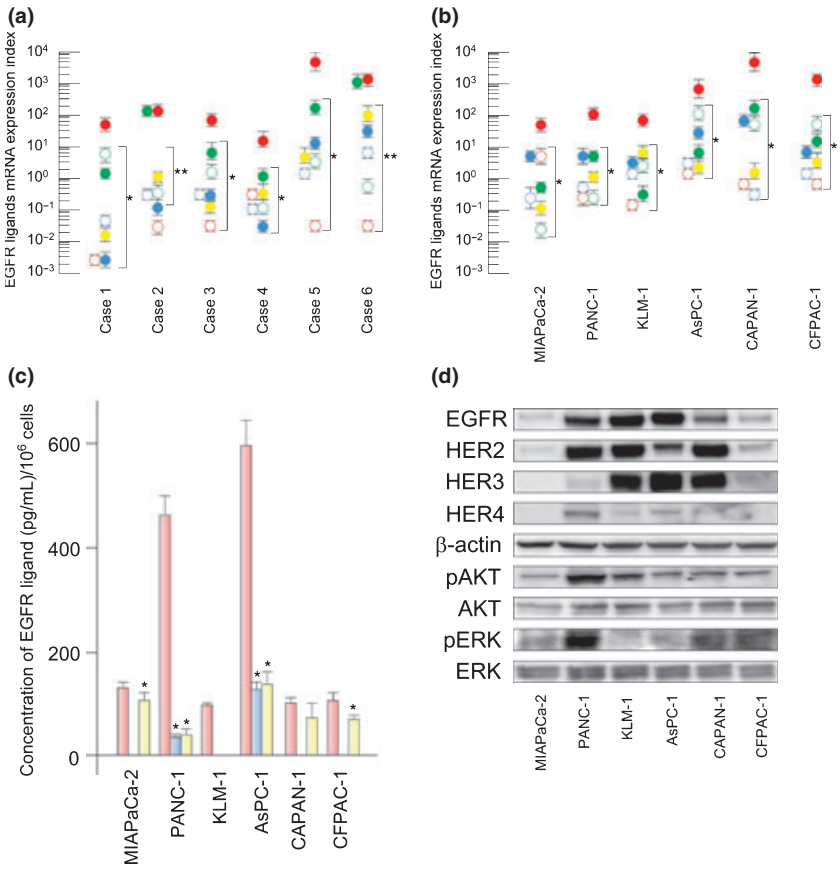

Fig. 1. Cell characteristics in pancreatic cancer. Differences in the expressions of epidermal growth factor receptor (EGFR) ligands in pancreatic cancer patients (a) and pancreatic cancer cell lines (b). Each value represents the mean and SD of the mRNA expression index for an EGFR ligand (n = 4). Closed blue circles HB-EGF; closed green circles, epiregulin; closed red circles, amphiregulin; closed yellow circles, transforming growth factor (TGF)-α; open blue circles, betacellulin; open green circles, epigen; open red circles, EGF. *P < 0.05 *versus* each value of the other six EGFR ligands; **P < 0.05 *versus* each value of the other five EGFR ligands. (c) Amounts of EGFR ligands in culture media from cancer cells incubated for 48 h. The concentrations of HB-EGF, amphiregulin, TGF-α, and EGF are presented as the concentrations per 1×10^6 cells. Blue bars, TGF-α; red bars, amphiregulin; yellow bars, HB-EGF; EGF could not be determined. Each value represents the mean and SD (n = 3). *P < 0.05 *versus* each amount of HB-EGF, TGF-α, or EGF. (d) Differences in the protein expressions of EGFR, HER2, HER3, HER4, Akt, ERK, and β-actin in pancreatic cell lines.

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were harvested for 24 h during incubation with an anticancer drug such as gemcitabine, paclitaxel, cisplatin, or 5-FU then extracted with radio immunoprecipitation assay (RIPA) buffer as previously described.[21] To analyze the alterations in heterodimer formation of EGFR with HER3 induced by treatment with an anticancer agent, cells were incubated with RPMI-1640 alone for 12 h then incubated with gemcitabine, paclitaxel, cisplatin, or 5-FU for an additional 12 h. After treatment, the cells were extracted with RIPA buffer and subjected to immunoprecipitation analyses.

To address the alterations in heterodimer formation of EGFR with HER3 induced by a molecularly targeted agent, cells were incubated with RPMI-1640 alone for 12 h then incubated with an inhibitory antibody against amphiregulin, neuregulin, or HER3 for an additional 12 h. Finally, the cells were treated with recombinant amphiregulin or neuregulin for 15 min. After the treatment, the cells were extracted with RIPA buffer and subjected to immunoprecipitation analyses. Cells were washed twice with ice-cold PBS containing 1 mM sodium orthovanadate. For total cell lysate (TCL)s and immunoprecipitation, the cells were lysed with 0.5 mL RIPA buffer. After removal of the cell debris by centrifugation at 15 000g for 30 min at 4°C, the supernatants were collected. The samples for TCLs were boiled for 5 min at 95°C in an equal volume of 2× Laemmli sample buffer. The samples for immunoprecipitation were incubated with 5 μg anti-EGFR or anti-HER3 antibody overnight at 4°C with slow agitation. On the following day, 15 μL protein G–Sepharose was added for 1 h at 4°C with slow agitation. The immunocomplexes were collected by centrifugation at 15 000g for 15 min at 4°C, washed twice with RIPA buffer, resuspended in 50 μL of 2× Laemmli sample buffer, and boiled for 5 min at 95°C. The extracts and immunoprecipitants were subjected to SDS-PAGE and immunoblotting analysis.[21] The expression levels of proteins detected by immunoblotting were quantified by densitometric analysis as previously described.[20]

Cell viability and cell apoptosis assays. To assess cell viability and cell apoptosis, cells were seeded in polylysine-coated 6-cm dishes (50–60% confluence) then incubated with RPMI-1640 plus 10% FCS in the presence of an anticancer agent, namely gemcitabine, paclitaxel, cisplatin, or 5-FU, for 48 h. For treatment with a molecularly targeted agent, such as CRM197, cetuximab, erlotinib, or an inhibitory antibody against amphiregulin, TGF-α,

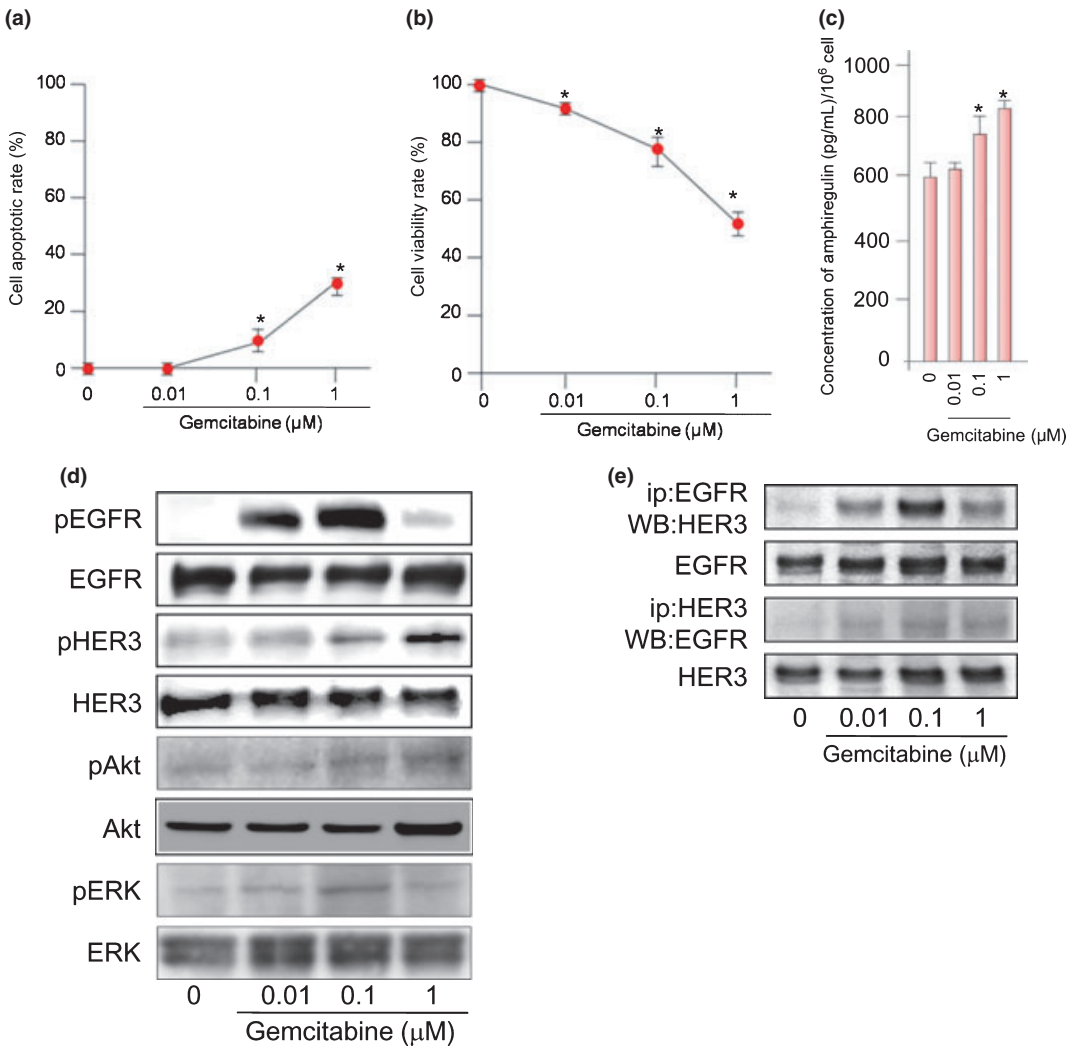

Fig. 2. Cell behavior after treatment with gemcitabine in AsPC-1 pancreatic cancer cells. Differences in the cell apoptotic rate (a), cell viability rate (b), and amount of amphiregulin in the culture medium (c) after treatment with various doses of gemcitabine for 48 h. Each value represents the mean and SD ($n = 4$). *$P < 0.05$ *versus* the value of the cell apoptotic rate, cell viability rate, or concentration of amphiregulin without gemcitabine treatment. (d) Alterations in the expressions of phosphorylated epidermal growth factor receptor (EGFR), HER3, Akt, and ERK after treatment with various doses of gemcitabine for 48 h. (e) Analysis of the heterodimer formation of EGFR with HER3 in the presence of various doses of gemcitabine. ip, immunoprecipitation; WB, western blotting.

neuregulin, or HER3, cells were incubated with RPMI-1640 alone for 48 h. For combined treatment with an anticancer agent and a molecularly targeted agent, the cells were incubated with RPMI-1640 plus 10% FCS for 48 h. The cells were counted using a hemocytometer after addition of Trypan blue exclusion dye to determine viability. TUNEL-positive cells were quantified as apoptotic cells by flow cytometric analysis as previously described.[10]

Three-dimensional culture. AsPC-1 cells were detached with trypsin–EDTA, washed three times with serum-free medium and suspended at a final concentration of 5×10^5 cells/3 mL. Aliquots (3 mL) were applied to the wells of 6-well plates pre-coated with 1.5 mL/well of growth factor-reduced Matrigel (Biocoat Cellware; Becton Dickinson, Franklin Lakes, NJ, USA). The cells were then cultured in medium containing 10% FBS. After 3 days, the plates were photographed. To count the numbers of cells using a hemocytometer, the cells were retrieved from colonies using a BD Cell Recovery Solution (Biocoat Cellware; Becton Dickinson). The cell viability was determined by Trypan blue exclusion.

Statistical analysis. Data for two experiments were analyzed using the Mann–Whitney *U*-test. Data for multiple experiments were analyzed using a Tukey HSD test. Values of $P < 0.05$ were considered statistically significant. The effects of drug–drug combinations were evaluated by a combination index (CI) value calculated on the basis of the following equation (termed the Loewe combination index): $CI = dx/Dx + dy/Dy$, where Dx and Dy are the doses of individual drugs required to exert the same effect as doses dx and dy used in combination. If the CI value is significantly below or above 1, the data are considered to be synergistic or antagonistic, respectively, whereas if the CI value is almost equal to 1, the data are considered to be additive.[22]

Results

Abundant expressions of amphiregulin in pancreatic cancer. To address the clinical significance of amphiregulin as a target for pancreatic cancer therapy, we examined the expressions of EGF family members in pancreatic cancer patients and pancreatic

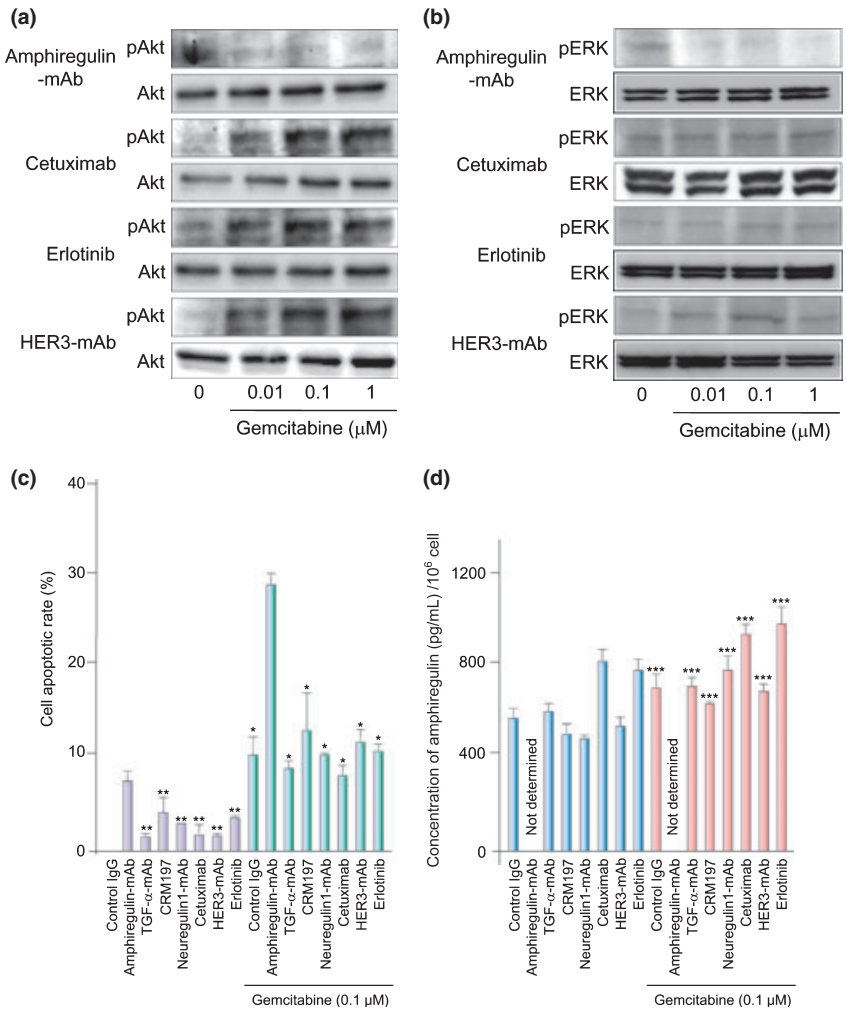

Fig. 3. Antitumor effects of combined treatment with gemcitabine and a variety of inhibitors in AsPC-1 pancreatic cancer cells. Alterations in the expression of phosphorylated Akt (a) and ERK (b) after combined treatment with gemcitabine and an inhibitory anti-amphiregulin antibody (10 μg/mL), cetuximab (10 μg/mL), erlotinib (1 μM), or an inhibitory anti-HER3 antibody (10 μg/mL) for 48 h. Differences in the cell apoptotic rates (c) and amounts of amphiregulin in the culture medium (d) after treatment with gemcitabine (0.1 μM) with or without each inhibitory antibody against amphiregulin (10 μg/mL), transforming growth factor (TGF)-α (10 μg/mL), neuregulin (10 μg/mL), or HER3 (10 μg/mL), cross-reacting material (CRM)197 (10 μg/mL), cetuximab (10 μg/mL), or erlotinib (1 μM). Each value represents the mean and SD ($n = 4$). *$P < 0.05$ *versus* the value of the cell apoptotic rate after treatment with the inhibitory anti-amphiregulin antibody with gemcitabine (0.1 μM); **$P < 0.05$ *versus* the value of the cell apoptotic rate after treatment with the inhibitory anti-amphiregulin antibody without gemcitabine; ***$P < 0.05$ *versus* the value of the corresponding amount of amphiregulin only in the treatment with each inhibitor (minus 0.1 μM gemcitabine).

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cancer cell lines. Amphiregulin was primarily expressed among the EGFR ligands in both the pancreatic cancer patients and pancreatic cancer cell lines (Fig. 1a,b). In addition, amphiregulin was prominently secreted into the culture media, compared with the amounts of HB-EGF, TGF-α, and EGF (Fig. 1c). Epidermal growth factor receptor was highly expressed in PANC-1, KLM-1, and AsPC-1 cells, and HER2 was predominantly expressed in PANC-1, KLM-1, and CAPAN-1 cells (Figs 1d,S1). Overexpression of HER3 was observed in KLM-1, AsPC-1, and CAPAN-1 cells, whereas significant expression of HER4 was not detected in any of the pancreatic cancer cells (Figs 1d,S1). Definite activation of ERK and Akt was found in all of these cells (Figs 1d,S1). Accordingly, AsPC-1 cells exhibited expression of the therapeutic target molecules for pancreatic cancer therapy, including overexpression of amphiregulin, EGFR, and HER3, and Akt activation.

Alterations in cell behavior and signaling induced by gemcitabine treatment. The *in vitro* antitumor effects, including the cell apoptotic and cell viability rates, were examined in AsPC-1 cells after treatment with gemcitabine. The apoptotic rate and cell viability rate of the cells increased and decreased, respectively, in a gemcitabine dose-dependent manner (Fig. 2a,b). As most of the cells became detached at gemcitabine concentrations above 1 μM, all subsequent analyses were carried out with gemcitabine concentrations of <1 μM. An increased amount of amphiregulin in the culture medium was found after gemcitabine treatment (Fig. 2c). The phosphorylation of EGFR and ERK was augmented by gemcitabine treatment (0–0.1 μM), whereas little phosphorylated EGFR and ERK was detected for treatment with 1 μM gemcitabine (Figs 2d,S2a). However, HER3 and Akt became increasingly phosphorylated in a gemcitabine dose-dependent manner (Figs 2d,S2a). The heterodimer formation of EGFR with HER3 was also enhanced in a gemcitabine dose-dependent manner, although a slight decrease in heterodimer formation of EGFR with HER3 was observed in the presence of 1 μM gemcitabine (Figs 2e,S2b). The ectodomain shedding of amphiregulin, which was induced by treatment with gemcitabine, was mainly regulated by ADAM17 (Fig. S3a). The introduction of a siRNA for ADAM17 augmented the cell apoptotic rate through blockade of amphiregulin cleavage (Fig. S3b). Taken together, these results suggest that treatment with gemcitabine attenuated the activation of ERK as well as EGFR independently of the increased amount of amphiregulin, and stimulated Akt activation through enhanced heterodimer formation of EGFR with HER3. These findings produced two issues requiring clarification. The first was whether inhibition of amphiregulin enhanced the antitumor effects of gemcitabine. The second was whether inhibition of

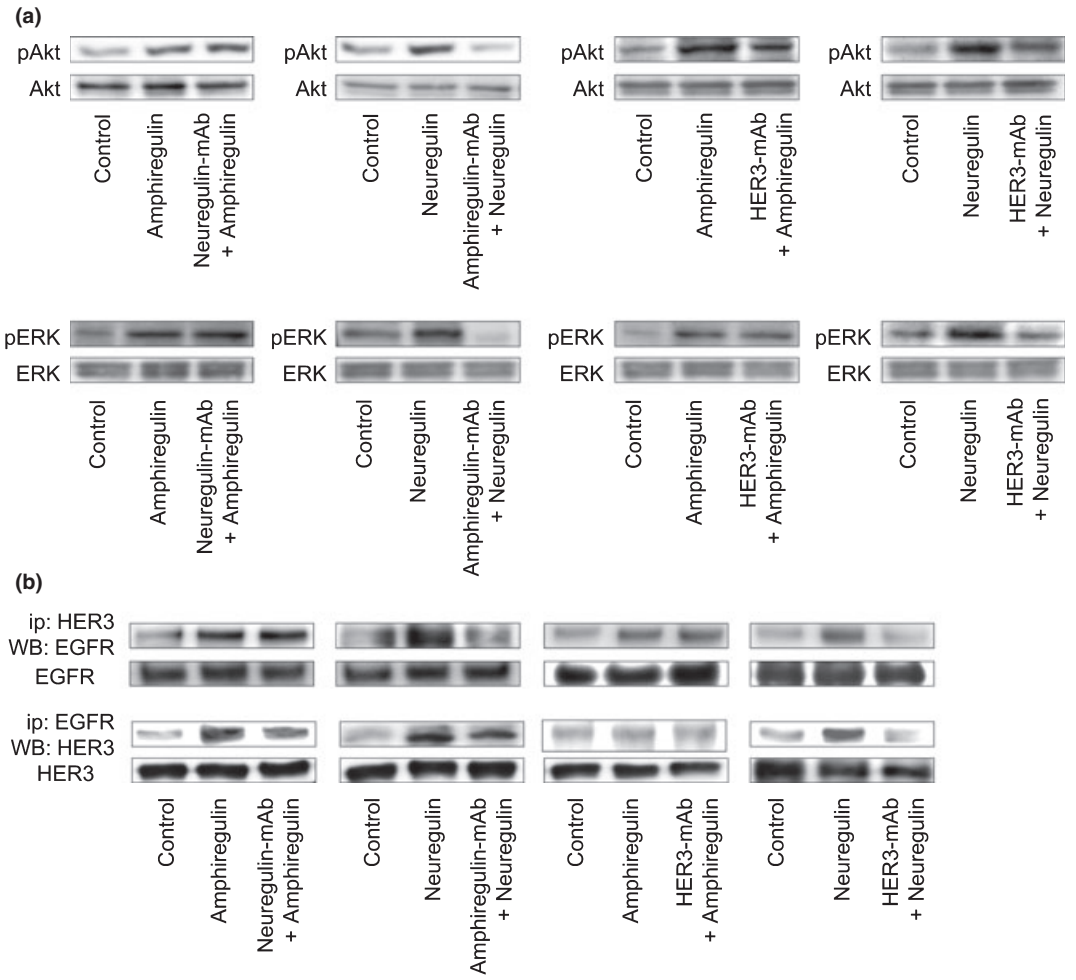

Fig. 4. Activation of Akt and ERK mediated by heterodimer formation of epidermal growth factor receptor (EGFR) with HER3 in AsPC-1 pancreatic cancer cells. (a) Phosphorylation of Akt (upper panels) and ERK (lower panels) stimulated by amphiregulin (50 ng/mL) or neuregulin (50 ng/mL) in the absence or presence of inhibitory anti-amphiregulin (10 μg/mL), anti-neuregulin (10 μg/mL), or anti-HER3 (10 μg/mL) antibodies. (b) Stimulation of heterodimer formation of EGFR with HER3 by amphiregulin (50 ng/mL) or neuregulin (50 ng/mL) in the absence or presence of inhibitory anti-amphiregulin (10 μg/mL), anti-neuregulin (10 μg/mL), or anti-HER3 (10 μg/mL) antibodies. ip, immunoprecipitation; WB, western blotting.

amphiregulin blocked the activation of Akt and ERK through heterodimer formation of EGFR with HER3.

Combined treatment with gemcitabine and molecularly targeted therapies in pancreatic cancer. To evaluate the *in vitro* antitumor effects mediated by combined treatment with gemcitabine and molecularly targeted therapies, we examined the alterations in the amount of amphiregulin, Akt signaling, ERK signaling, and cell apoptotic rate. Combined treatment with an inhibitory anti-amphiregulin antibody and gemcitabine blocked both Akt and ERK activation (Figs 2d,3a,b and S2a,S4a,b). Combined treatment with EGFR inhibitors, including cetuximab and erlotinib, or an inhibitory anti-HER3 antibody and gemcitabine, partly suppressed ERK activation, although these agents did not inhibit Akt activation (Figs 3a,b,S4a,b). The cell apoptotic rates were highest in the presence of an inhibitory anti-amphiregulin antibody with or without gemcitabine treatment, compared with those in the presence of inhibitory antibodies against TGF-α, neuregulin, EGFR and HER3, and CRM197 (Fig. 3c and Table S1). Each combined treatment with an inhibitor + gemcitabine promoted the cell apoptotic rate, compared with the corresponding rate without gemcitabine (Fig. 3c and Table S1). However, the amount of amphiregulin was significantly increased in the presence of each inhibitor with gemcitabine, compared with the corresponding amount without gemcitabine (Fig. 3d). The combined treatments with EGFR inhibitors + gemcitabine augmented the most abundant amount of amphiregulin, compared with the other inhibitors or other inhibitors + gemcitabine (Fig. 3d). In KLM-1 cells, gemcitabine augmented the number of apoptotic cells in a dose-dependent manner, accompanied by an increase in amphiregulin expression (Fig. S5a,b). In CAPAN-1 cells, only a slight increase in apoptotic cells was found after treatment with gemcitabine even at a high dosage. The increase in amphiregulin was also minimal for the high dose of gemcitabine (Fig. S5a,b). Incubation with gemcitabine and an inhibitory anti-amphiregulin antibody induced synergistic antitumor effects in AsPC-1 and KLM-1 cells, but

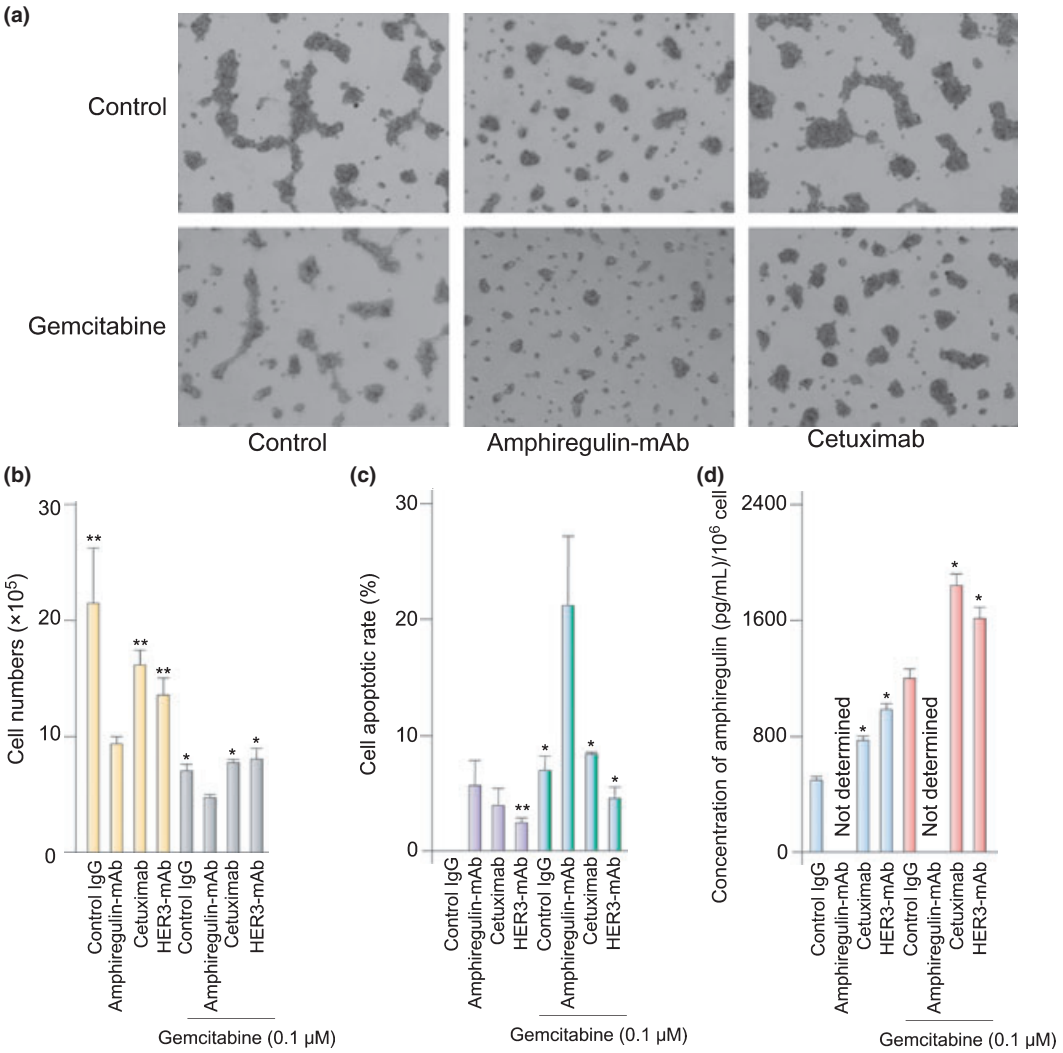

Fig. 5. Antitumor effects of combined treatment with gemcitabine and a variety of inhibitors in AsPC-1 cells using a Matrigel 3D culture system. (a) Appearances of growing cells by phase-contrast microscopy after treatment with control IgG (10 μg/mL), an inhibitory anti-amphiregulin antibody (10 μg/mL), or cetuximab (10 μg/mL) with or without gemcitabine (0.1 μM) for 48 h. Differences in the cell number (b), cell apoptotic rate (c), and concentration of amphiregulin in the culture medium (d) after treatment with control IgG (10 μg/mL), an inhibitory anti-amphiregulin antibody (10 μg/mL), an inhibitory anti-HER3 antibody (10 μg/mL), or cetuximab (10 μg/mL) with or without gemcitabine (0.1 μM) for 48 h. Each value represents the mean and SD ($n = 4$). *$P < 0.05$ *versus* the value for the cell number or apoptotic rate after treatment with an inhibitory anti-amphiregulin antibody with gemcitabine treatment (0.1 μM); **$P < 0.05$ *versus* the value for the cell number or apoptotic rate after treatment with an inhibitory anti-amphiregulin antibody without gemcitabine treatment.

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not in CAPAN-1 cells (Fig. S5c, Table S3). Regarding the treatment with gemcitabine, the enhancement of amphiregulin expression resulted in synergistic antitumor effects for the use of gemcitabine and the anti-amphiregulin antibody. In the presence or absence of gemcitabine, the introduction of an siRNA for amphiregulin dominantly induced an increase in the cell apoptotic rate and a decreased amount of amphiregulin in culture media from AsPC-1 cells, compared with the effects of siRNAs for TGF-α, HB-EGF, EGFR, and HER3 (Fig. S6). In addition, the amount of amphiregulin was significantly increased for the combined treatment with each siRNA and gemcitabine, compared with the corresponding amount for treatment with each siRNA without gemcitabine (Fig. S6). Gemcitabine is the most frequently used drug in the treatment of pancreatic cancer patients. Cisplatin, 5-FU, and paclitaxel are also available for the treatment of pancreatic cancer patients.[23,24] Therefore, we also tested the effects of these conventional cytotoxic anticancer agents in a dose-dependent manner on the amounts of amphiregulin, apoptotic cell rates, and activations of EGFR, HER3, Akt, and ERK in AsPC-1 cells. At doses above 0.01 μM paclitaxel or 0.1 μM cisplatin, marked increases in amphiregulin expression and cell apoptosis were observed, whereas no significant increases in amphiregulin expression or the cell apoptotic rate were observed in the presence of 5-FU (Fig. S7a,b). At 1 μM paclitaxel, most of the cells were detached from the plate, and the apoptotic cell rate and amount of amphiregulin were not measurable (Fig. S7a,b). The phosphorylation levels of EGFR, HER3, Akt, and ERK were enhanced by treatment with 0.01 μM paclitaxel or cisplatin, whereas the activations of EGFR, HER3, Akt, and ERK were barely detectable even at a high dose of 5-FU (Fig. S7c,d). Next, we analyzed the combined antitumor effects of an inhibitory anti-amphiregulin antibody and conventional chemotherapeutic agents. Synergistic *in vitro* antitumor effects were found for the combination of the anti-amphiregulin antibody with 0.01 μM paclitaxel or >0.1 μM cisplatin (Fig. S7e, Table S4). No synergistic antitumor effects were found for the combined treatment of 5-FU with the inhibitory anti-amphiregulin antibody (Fig. S7e, Table S4). The heterodimer formation of EGFR with HER3 was also enhanced in a paclitaxel or cisplatin dose-dependent manner, although slight decreases in heterodimer formation of EGFR with HER3 were observed in the presence of 1 μM paclitaxel or cisplatin (Fig. S7f,g), similar to the findings for gemcitabine. Treatment with 5-FU did not induce heterodimer formation of EGFR with HER3 (Fig. S7f,g). Taking this evidence together, inhibition of amphiregulin evoked synergistic antitumor effects in combination with gemcitabine, paclitaxel, or cisplatin.

Akt and ERK signal through heterodimer formation of EGFR with HER3. To investigate the signals mediated by HER3 in pancreatic cancer, we examined the Akt and ERK activation induced by amphiregulin or neuregulin in AsPC-1 cells. The addition of amphiregulin or neuregulin led to the phosphorylation and activation of both Akt and ERK (Figs 4a,S8a). The activation of Akt and ERK was blocked by treatment with an inhibitor for amphiregulin, but was not suppressed by treatment with an inhibitor for neuregulin or HER3 (Figs 4a,S8a). The activation of Akt and ERK mediated by amphiregulin or neuregulin was completely inhibited by treatment with inhibitory antibodies against amphiregulin or neuregulin (Figs 4a,S8a). An inhibitory anti-HER3 antibody partly abolished the phosphorylation of Akt but not the phosphorylation of ERK stimulated by neuregulin, although the activation of ERK induced by neuregulin was very weak (Figs 4a,S8a). Stimulation by amphiregulin or neuregulin promoted the heterodimer formation of EGFR with HER3 (Figs 4b,S8b). An inhibitory anti-amphiregulin antibody attenuated the heterodimer formation of EGFR with HER3 mediated by neuregulin, whereas an inhibitor for neuregulin or HER3 did not block the heterodimer formation of EGFR with HER3 stimulated by amphiregulin (Figs 4b,S8b). However, an inhibitor for HER3 suppressed the heterodimer formation of EGFR with HER3 mediated by neuregulin (Figs 4b,S8b). Taken together, these results indicate that the inhibition of amphiregulin led to the suppression of Akt or ERK signaling, accompanied by disruption of the heterodimer formation of EGFR with HER3.

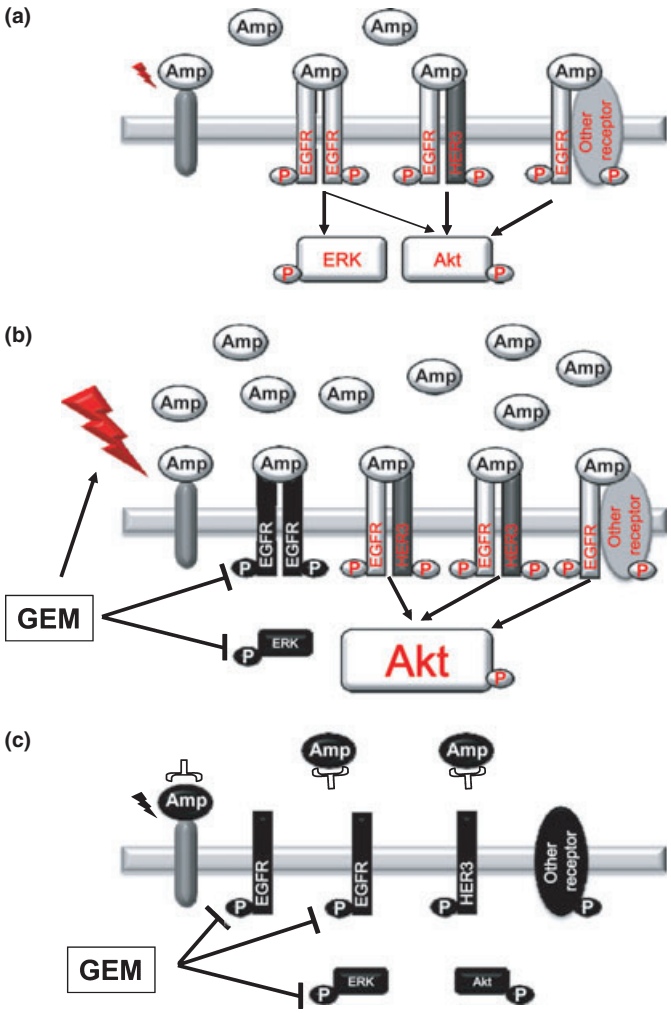

Fig. 6. Associations of therapeutic target molecules including amphiregulin (Amp), epidermal growth factor receptor (EGFR), HER3, and Akt with pancreatic cancer. (a) In pancreatic cancer, the abundant amount of amphiregulin enhances the activation of ERK through phosphorylation of EGFR and the activation of Akt through heterodimer formation of EGFR with HER3. (b) Treatment with gemcitabine (GEM) induces the dephosphorylation cell proliferative signals and stimulates marked secretion of amphiregulin, leading to formation of EGFR/HER3 heterodimer and further activation of Akt as a cell survival signal. (c) The combination of gemcitabine with an inhibitor for amphiregulin completely inhibits ERK and Akt activation. P, phosphorylation.

Synergistic *in vitro* antitumor effects in 3D cell cultures. To reconfirm the antitumor effects of combined treatment with an inhibitor for amphiregulin and gemcitabine, we analyzed the cell behavior in Matrigel 3D cultures following treatment with gemcitabine with or without inhibitors for amphiregulin, EGFR, and HER3. After incubation with control IgG, AsPC-1 cells were tightly aggregated with one another and piled up in the Matrigel 3D cultures (Fig. 5a). In the absence or presence of gemcitabine, the cell number following treatment with an anti-amphiregulin antibody was significantly decreased, compared with those

after treatment with control IgG, cetuximab, or an anti-HER3 antibody (Fig. 5a,b). Furthermore, combined treatment with an anti-amphiregulin antibody + gemcitabine significantly increased the cell apoptotic rate, compared with the rates after any of the other treatments examined (Fig. 5c and Table S2). The amount of amphiregulin was upregulated after combined treatment with cetuximab or anti-HER3 antibody + gemcitabine, compared with the amounts after any of the other treatments examined (Fig. 5d). These results indicate that the synergistic antitumor effects of an inhibitor for amphiregulin + gemcitabine can be verified in 3D cultures, which provides a more physiological and predictive model for tumor development.

Discussion

Amphiregulin is the predominant EGFR ligand expressed in pancreatic cancer. The suppression of amphiregulin blocks EGFR, HER3, and Akt signals, which are involved in the progression of pancreatic cancer (Fig. 6a). Moreover, amphiregulin secretion occurred as a response to gemcitabine treatment promotes cell survival through the activation of PI3 kinase/Akt signaling (Fig. 6b). In principle, a variety of signal transduction pathways arise as a result of EGFR ligands binding to ErbB receptors, which in turn initiates their homodimerization as well as heterodimerization with other ErbB receptors, resulting in the aggressive behavior of cancer cells.[25,26] A recent study showed that a ligand mediating EGFR signaling can simultaneously evoke ERK as well as Akt activation by cross-talk with different kinds of growth factor receptors such as insulin-like growth factor-I receptor or steroid hormone receptor, and increase glucose uptake by complex formation with sodium/glucose cotransporter 1.[27] It is plausible that the existence of these diverse signals mediated by ligand binding to EGFR is one of the reasons why receptor-targeted therapies do not sufficiently inhibit growth or survival signals.

In the presence of a low dose of gemcitabine, a significant percentage of apoptotic cells and a marked increase in amphiregulin expression were observed in AsPC-1 cells (Fig. 2a,c). Enhanced expression of amphiregulin significantly induced the heterodimer formation of EGFR with HER3 as well as EGFR phosphorylation (Fig. 2d,e). In the presence of a high dose of gemcitabine, more than 30% of the cells were apoptotic and a further increase in amphiregulin expression was observed in AsPC-1 cells (Fig. 2a,c). However, the heterodimer formation of EGFR with HER3 was decreased and a loss of EGFR phosphorylation was observed (Fig. 2d,e). The significant apoptosis after treatment with anticancer agents induced damage to various proteins in the cells, possibly resulting in decreased kinase activity. Therefore, although amphiregulin binds to EGFR, the activity of EGFR kinase may be inactivated. Another possibility is that amphiregulin may be unable to bind to EGFR owing to a conformational change of EGFR after the damage caused by anticancer agents.

According to the lines of evidence obtained in the present study, combination chemotherapy involving gemcitabine and an inhibitor for amphiregulin would be clinically valuable for patients with pancreatic cancer (Fig. 6c). To date, the development of novel therapies for pancreatic cancer continues in both the laboratory and subsequent clinical trials.[28–30] In the near future, therefore, combined treatments with an inhibitor of amphiregulin and conventional anticancer agents should be tested in a clinical trial in order to lead to dramatic improvement of the clinical outcomes of patients with pancreatic cancer.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Relative band intensities for the protein expression of epidermal growth factor receptor (EGFR), HER2, HER3, HER4, ERK, and Akt in six pancreatic cancer cell lines. After the highest expression of each molecule was defined as 100% for the densitometry analysis, the expression levels of EGFR, HER2, HER3, HER4, phosphorylated ERK (pERK), and phosphorylated Akt (pAkt) were analyzed. Each experiment was carried out three times. Each value represents the mean and SD ($n = 3$). *$P < 0.05$ *versus* the highest expression level of each molecule.

Fig. S2. Relative band intensities of phosphorylated epidermal growth factor receptor (EGFR), HER3, ERK, and Akt in AsPC-1 pancreatic cancer cells in the presence of various doses of gemcitabine. (a) After the highest expression of each molecule was defined as 100% for the densitometry analysis, the expression levels of phosphorylated EGFR, HER3, ERK, and Akt were analyzed. (b) Expression levels of EGFR bound to HER3, and HER3 bound to EGFR. Each experiment was carried out three times. Each value represents the mean and SD ($n = 3$). *$P < 0.05$ *versus* the lowest expression level of each molecule.

Fig. S3. Alterations in the amount of amphiregulin in the culture medium (a) and the cell apoptotic rate (b) after transfection of siRNAs for a disintegrin and metalloproteinase (ADAM)9, ADAM10, ADAM12, and ADAM17, or in the presence of GM6001 (5 μM) for 48 h in AsPC-1 pancreatic cancer cells. Each value represents the mean and SD ($n = 3$). *$P < 0.05$ *versus* the value for the introduction of a scramble siRNA with gemcitabine treatment; **$P < 0.05$ *versus* the value for the introduction of a scramble siRNA without gemcitabine treatment.

Fig. S4. Relative band intensities for the phosphorylated protein expression levels of Akt (a) and ERK (b) after treatment with gemcitabine plus inhibitors for amphiregulin (10 μg/mL), cetuximab (10 μg/mL), erlotinib (1 μM), or HER3 (10 μg/mL) in AsPC-1 pancreatic cancer cells. After the highest expression of each molecule was defined as 100% for the densitometry analysis, the expression levels of phosphorylated Akt and phosphorylated ERK were analyzed. Each experiment was carried out three times. Each value represents the mean and SD ($n = 3$). *$P < 0.05$ *versus* the expression level of the phosphorylated protein after treatment with gemcitabine (0.1 μM).

Fig. S5. Alterations in the cell apoptotic rate after treatment with an inhibitory anti-amphiregulin antibody and/or gemcitabine in KLM-1 or CAPAN-1 pancreatic cancer cells. Left panels, KLM-1 cells; right panels, CAPAN-1 cells. Differences in the amounts of amphiregulin in the culture medium (a) and the cell apoptotic rate (b) after treatment with various doses of gemcitabine for 48 h in KLM-1 or CAPAN-1 cells. Each value represents the mean and SD ($n = 3$). *$P < 0.05$ *versus* the value of the concentration of amphiregulin or the cell apoptotic rate in the treatment without gemcitabine. (c) Differences in the cell apoptotic rate after treatment with gemcitabine (0.01–1.00 μM) in the absence or presence of an inhibitory anti-amphiregulin antibody (10 μg/mL). Each value represents the mean and SD ($n = 3$). *The combination index value is significantly below 1 ($P < 0.05$).

Fig. S6. Alterations in the cell apoptotic rate and concentration of amphiregulin after transfection of a variety of siRNAs. (a,b) Differences in the cell apoptotic rate (a) and concentration of amphiregulin in the culture medium (b) after treatment with gemcitabine (0.1 μM) plus the introduction of siRNAs for amphiregulin, transforming growth factor (TGF)-α, HB-EGF, epidermal growth factor recptor (EGFR), and HER3 into AsPC-1 pancreatic cancer cells. Each value represents the mean and SD ($n = 4$). *$P < 0.05$ or **$P < 0.05$ *versus* the value for the cell apoptotic rate or concentration of amphiregulin after treatment with an inhibitory anti-amphiregulin antibody with or without gemcitabine treatment (0.1 μM).

Fig. S7. Synergistic antitumor effects for combination treatments with an inhibitory anti-amphiregulin antibody and conventional chemotherapeutic agents in pancreatic cancer. Differences in the amounts of amphiregulin in the culture medium (a) and the cell apoptotic rates (b) after treatment with various doses of paclitaxel, cisplatin, or 5-fluorouracil (5-FU) for 48 h. Each value represents the mean and SD ($n = 3$). *$P < 0.05$ *versus* the value of the concentration of amphiregulin or the cell apoptotic rate without paclitaxel, cisplatin, or 5-FU treatment. Alterations in the expressions (c) and relative band intensities (d) of phosphorylated epidermal growth factor receptor (EGFR), HER3, Akt, and ERK after treatment with various doses of paclitaxel, cisplatin, or 5-FU for 48 h. After the highest expression of each molecule was defined as 100% for densitometric analyses, the expression levels of phosphorylated EGFR, HER3, ERK, and Akt were analyzed. Each experiment was carried out three times. Each value represents the mean and SD ($n = 3$). *$P < 0.05$ *versus* the lowest expression level of each molecule. (e) Differences in the cell apoptotic rates after treatment with paclitaxel, cisplatin, or 5-FU (0.01–1.00 μM) with or without an inhibitory anti-amphiregulin antibody (10 μg/mL). Each value represents the mean and SD ($n = 3$). *The combination index value is significantly below 1 ($P < 0.05$). (f) Analysis of the heterodimer formation of EGFR with HER3 in the presence of various doses of paclitaxel, cisplatin, or 5-FU. (g) Relative band intensities for the expression levels of EGFR bound to HER3, and HER3 bound to EGFR in AsPC-1 cells after treatment with various doses of paclitaxel, cisplatin, or 5-FU. Each experiment was carried out three times. Each band intensity value represents the mean and SD ($n = 3$). *$P < 0.05$ *versus* the lowest expression level of each molecule.

Fig. S8. Relative band intensities for the phosphorylated protein expression levels of Akt and ERK, and the heterodimer formation of epidermal growth factor receptor (EGFR) with HER3 in AsPC-1 pancreatic cancer cells. (a) After the highest expression of each molecule was defined as 100% for the densitometry analysis, the alterations in phosphorylated Akt (upper panels) and phosphorylated ERK (lower panels) after stimulation of amphiregulin (50 ng/mL) or neuregulin (50 ng/mL) in the absence or presence of inhibitory anti-amphiregulin (10 μg/mL), anti-neuregulin (10 μg/mL), or anti-HER3 (10 μg/mL) antibodies were analyzed. (b) After the highest expression of each molecule was defined as 100% for the

densitometry analysis, the relative band intensities of the protein expression of EGFR and HER3 stimulated by amphiregulin (50 ng/mL) or neuregulin (50 ng/mL) in the absence or presence of inhibitory anti-amphiregulin (10 μg/mL), anti-neuregulin (10 μg/mL), or anti-HER3 (10 μg/mL) antibodies were analyzed. Each experiment was carried out three times. Each value represents the mean and SD ($n = 3$). *$P < 0.05$ *versus* the highest expression level of each molecule.

Table S1. Combination index for Fig. 3(c).

Table S2. Combination index for Fig. 5(c).

Table S3. Combination index for Fig. S5(c).

Table S4. Combination index for Fig. S7(e).

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