Table 1. Baseline characteristics and demographics (n = 106)

Characteristic	
Median age (range) (years)	62 (36–78)
Gender, *n* (%)	
Male	56 (52.8)
Female	50 (47.2)
Median bodyweight (range) (kg)	52.3 (33.1–95.0)
Smoking history,† *n* (%)	
Never smoker	39 (36.8)
Past smoker	37 (34.9)
Current smoker	30 (28.3)
ECOG PS, *n* (%)	
0	80 (75.5)
1	26 (24.5)
2	0 (0)
Disease status, *n* (%)	
Metastatic	88 (83.0)
Locally advanced	18 (17.0)
Primary tumor identified, *n* (%)	92 (86.8)
Primary sites, *n* (%)	
Head	46 (43.4)
Body and tail	23 (21.7)
Body	22 (20.8)
Tail	10 (9.4)
Other	5 (4.7)‡
Biliary drainage, *n* (%)	19 (17.9)
Sites of distant metastases, *n* (%)	
Liver	56 (52.8)
Distant lymph nodes	39 (36.8)
Lung	17 (16.0)
Other	26 (24.5)
Prior lines of therapy, *n* (%)	
None	101 (95.3)
One regimen	5 (4.7)§
Median CA19–9 (range) (U/mL)	
Median	776 (0–435 000)
Median CEA (range) (ng/mL)	
Median	4.8 (0.6–1100.1)

†Never smoker, never/hardly smoked; past smoker, passage of at least 1 month since stopping smoking (at the time of registration); current smoker, smoked within 1 month (at the time of registration). ‡Whole of pancreas (n = 1); head and body (n = 3); other (n = 1). §Tegafur, gimeracil, oteracil potassium (S-1) (n = 3); 5-fluorouracil plus leucovorin (n = 2). CA 19–9, carbohydrate antigen 19–9; CEA, carcinoembryonic antigen; ECOG, Eastern Co-Operative Group.

93.4% of the patients; most cases were mild to moderate in severity (87.7%, grade ≤2; 5.7%, grade ≥3). Other common non-hematological AE included anorexia, pruritus, fatigue, nausea and diarrhea. Most patients experienced some degree of hematological toxicity, with grade 3 or 4 neutropenia (neutrophil decreased), leucopenia (white blood cell count decreased) and anemia (hemoglobin decreased) occurring in 34.9%, 29.2% and 14.2% of patients, respectively. Only one treatment-related death occurred (due to gastrointestinal hemorrhage), which was probably due to arterial bleeding caused by the invasion of the primary tumor into the gastrointestinal tract. Although the likelihood of this event being treatment-related was deemed remote, a causal relationship could not be completely excluded because the event occurred during the study treatment administration period.

Treatment-related SAE were reported in 26 (24.5%) patients. These included nine ILD-like events (8.5%), the majority of which (n = 7) were grade 1–2 in severity. Importantly, all of these nine patients recovered or improved, and four of these patients did so without any treatment for ILD-like events. Other

Table 2. Treatment-related adverse events occurring in >30% of patients treated with erlotinib and gemcitabine (n = 106)

	Any grade, *n* (%)	Grade 3, *n* (%)	Grade 4, *n* (%)
Non-hematological			
Rash	78 (73.6)	3 (2.8)	0 (0)
Anorexia	75 (70.8)	15 (14.2)	0 (0)
Pruritus	57 (53.8)	1 (0.9)	0 (0)
Fatigue	56 (52.8)	3 (2.8)	0 (0)
Nausea	56 (52.8)	6 (5.7)	0 (0)
Diarrhea	52 (49.1)	2 (1.9)	0 (0)
Dry skin	49 (46.2)	0 (0)	0 (0)
Stomatitis	38 (35.8)	0 (0)	0 (0)
Pyrexia	32 (30.2)	0 (0)	0 (0)
Hematological			
White blood cell count decreased	85 (80.2)	31 (29.2)	0 (0)
Platelet count decreased	77 (72.6)	9 (8.5)	0 (0)
Hemoglobin decreased	76 (71.7)	13 (12.3)	2 (1.9)
Hematocrit decreased	73 (68.9)	8 (7.5)	0 (0)
Neutrophil decreased	73 (68.9)	32 (30.2)	5 (4.7)
Red blood cell count decreased	72 (67.9)	8 (7.5)	0 (0)
ALT increased	59 (55.7)	10 (9.4)	0 (0)
AST increased	57 (53.8)	4 (3.8)	1 (0.9)
Weight decreased	53 (50.0)	3 (2.8)	0 (0)
Lymphocyte count decreased	46 (43.4)	14 (13.2)	0 (0)
Blood albumin decreased	35 (33.0)	0 (0)	0 (0)
Gamma-glutamyltransferase increased	35 (33.0)	12 (11.3)	1 (0.9)

ALT, alanine amino transferase; AST, aspartate amino transferase.

treatment-related SAE were anorexia (3.8%), vomiting, pyrexia and abnormal hepatic function (1.9% each). The baseline characteristics, treatment and outcomes of patients who developed treatment-related ILD-like events during the study are detailed in Table 3. The onset times of ILD-like events ranged from 7 to 187 days after the start of treatment. In these patients, a relatively long survival was observed (from 119 to 568+ days), and five patients received post-study therapy. All of these nine patients were past or current smokers, and six had emphysema at baseline (not detected prior to treatment, but diagnosed at the extramural review by a radiologist in the DSMC). Multivariate analyses were performed for the occurrence of ILD-like events using the logistic regression model and emphysema at baseline was indicated as a risk factor for onset of ILD-like events (odds ratio [95% CI], 12.13 [1.01–145.7]; P = 0.0491).

Adverse events led to erlotinib discontinuation in 30 patients (28.3%) and gemcitabine discontinuation in 27 patients (25.5%). The main reasons for treatment discontinuation were ILD (n = 6) and anorexia (n = 3); no patient discontinued treatment due to RASH or diarrhea. Due to the onset of AE, a total of 65 patients (61.3%) required one or more interruptions of erlotinib (36 patients [34.0%] for longer than seven consecutive days and 17 patients [16.0%] for longer than 14 consecutive days) and 56 patients (52.8%) had one or more skip of gemcitabine. Modifications in the erlotinib or gemcitabine dosage were required in 17 (16.0%) and 11 (10.4%) patients, respectively, due to AE.

Efficacy. The median OS was 9.23 months (95% CI, 8.31–10.78; Fig. 1A) and the 1-year survival rate was 33% (95% CI, 24–42). Median PFS was 3.48 months (95% CI, 2.63–3.78; Fig. 1B). Among the patients evaluable for tumor response (n = 64), the ORR was 20.3% (13/64; 95% CI, 11.3–32.2) and the DCR was 50.0% (95% CI, 37.2–62.8; CR, n = 0; PR, n = 13; stable disease, n = 19).

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Table 3. Characteristics, treatment and outcomes of patients with treatment-related ILD-like events ($n = 9$)

Event	Gender	Age (years)	Smoking status†	Days on treatment	ILD maximum grade	Suspicious findings of ILD	Steroids	Oxygen	ILD outcome	Presence of emphysema (assessed by radiologist)	Survival outcome (days)	Post-therapy (chemotherapy)
Lymphoid ILD	M	62	Past	82	1	Pyrexia	None	No	Improved	Yes	362	Yes
ILD	M	42	Current	50	3	Pyrexia	Pulse	Yes	Recovered	Yes	517	Yes
Organising pneumonia	M	60	Past	183	2	Respiratory symptoms	None	No	Improved	Yes	568+	Yes
ILD	F	62	Past	113	2	Cough	Oral	No	Recovered	Yes	376	No
ILD	F	74	Past	111	3	Cough, dyspnea	Pulse	Yes	Improved	None	183	No
ILD	M	60	Current	25	1	Pyrexia	Pulse	No	Recovered	None	119	Yes
ILD	M	77	Past	7	1	X-ray	None	No	Recovered	Yes	255	No
ILD	M	55	Past	187	1	CT	None	No	Recovered	Yes	415	No
ILD	F	60	Current	76	2	Cough	Oral	No	Recovered	None	346	Yes

†Past smoker, passage of at least 1 month since stopping smoking (at the time of registration); current smoker, smoked within 1 month (at the time of registration). CT, computed tomography; F, female; ILD, interstitial lung disease; M, male.

The median OS was longer in patients who experienced RASH of grade ≥2 ($n = 67$) than in those with RASH of grade ≤1 ($n = 39$) (10.25 months [95% CI, 8.80–12.12] *vs* 8.31 months [95% CI, 6.18–9.99], respectively; Fig. 1C) and the 1-year survival rate was higher (39% [95% CI, 27–50] *vs* 23% [95% CI, 10–36], respectively). Similarly, the median PFS was longer in patients with RASH of grade ≥2 versus those with RASH grade ≤1 (3.61 months [95% CI, 3.48–5.32] *vs* 1.81 months [95% CI, 1.64–3.48]; Fig. 1D). While there was no notable difference in ORR between patients with RASH grade ≥2 and those with grade ≤1 (21.1% [95% CI, 9.6–37.3] *vs* 19.2% [95% CI, 6.6–39.4]), the DCR was higher in those with more severe RASH (60.5% [95% CI, 43.4–76.0] *vs* 34.6% [95% CI, 17.2–55.7]).

Pharmacokinetics. Plasma sampling for PK analyses was performed in all six patients enrolled in the first step. On day 8, the values of C_{max} were 1760 ± 456.9 ng/mL (mean ± SD) for erlotinib, 169.7 ± 64.5 ng/mL for OSI-420 and 22 700 ± 3272.9 ng/mL for gemcitabine. The AUC_{last} was 29 001 ± 6560 h ng/mL, 2748 ± 788 h ng/mL and 10 717 ± 1458 h ng/mL (mean ± SD), respectively. The mean t_{max} was 8.0 h (range, 2.0–23.9 h), 9.0 h (2.0–23.9 h) and 0.51 h (0.45–0.57 h), respectively. Also on day 8, the mean plasma $t_{1/2}$ was 54.92 h (range, 9.25–144.61 h), 32.79 h (10.36–60.46 h), and 0.63 h (0.31–1.14 h), respectively. The Cl/F of erlotinib and gemcitabine showed interindividual variability; the Cl/F on day 8 was 3972.6 ± 772.1 mL/h (mean ± SD; coefficient of variation 19.4%) and 146 580.4 ± 31 101.3 mL/h (21.2%), respectively.

Biomarker analysis. Of the 106 patients enrolled, *EGFR* mutation status was evaluated in 47 patients (44.3%), all of whom had wild-type *EGFR*. The mutation status of the remaining patients was classified as unknown because samples were not available (30.2%), not examined (9.4%) or the results following sequencing were inconclusive (16.0%).

Discussion

This study was designed to initially assess the safety of erlotinib with gemcitabine for Japanese patients with pancreatic cancer, in whom there had been no prior exposure to either drug. As no significant safety concerns were raised in the first step of the study, enrollment of a further 101 patients was performed. Although the incidence of AE in this study was higher than in the PA.3 study, the incidence of grade 3–4 AE was similar.[28] Despite these results, no new AE specific to Japanese patients were observed. As expected, RASH and gastrointestinal events were among the most common AE in this study, and most of these cases were mild to moderate in severity.

Interstitial lung disease-like events were reported in nine patients (8.5%; grade 1/2/3, 3.8/2.8/1.9%) in the current study, while its incidence was reported to be 2.4% in patients treated in the erlotinib plus gemcitabine arm of the PA.3 study.[28] In addition, in Japanese patients with advanced pancreatic cancer, ILD-like events were reported in two (6.1%) of 33 patients treated with gemcitabine plus S-1, and were reported in three (1.1%) of 264 patients with gemcitabine monotherapy, respectively.[33,34] Likewise, the higher incidence of ILD-like events were documented using S-1 or erlotinib in combination with gemcitabine compared with gemcitabine as monotherapy in patients with pancreatic and biliary tract cancer.[35] On another front, outside of Japan, a high incidence of ILD-like events was reported in gemcitabine and paclitaxel combination therapy in patients with NSCLC.[36] From the above information, considering the higher incidence of ILD when gemcitabine is used in combination, an additive effect from such combinations cannot be ruled out.

In NSCLC, Japanese patients have an increased risk of developing ILD-like events when treated with EGFR TKI.[29,37–39] Fatal cases of ILD-like events have been reported following EGFR TKI administration for the treatment of NSCLC.[37–41] Importantly, however, no patients died due to an ILD-like event in this study. Seven patients experienced ILD-like events of grade 1–2 in severity. This may be due to active management of ILD-like cases during the study period. This management included regular and immediate chest X-rays, in addition to diagnosis with CT scans after any early signs and symptoms were observed (e.g. pyrexia, cough or dyspnea), timely discontinuation of the antitumor drugs (as a precautionary measure in case these drugs were associated with the symptoms) and appropriate treatment for the events (including oral/pulse steroids). By appropriately treating the early symptoms of ILD-like events, patients could restart antitumor therapy (chemotherapy: treatment change). In this study, the onset time for ILD-like events varied markedly between patients (7–187 days). It is therefore necessary to monitor the patients throughout the treatment period.

All of the patients who developed ILD in this study were current or past smokers, and smoking status has been shown to be a risk factor for ILD in the NSCLC population.[38] Results from the multivariate analyses in this study suggest that emphysema is also a risk factor for developing ILD; six of the nine

patients with ILD-like events were diagnosed with emphysema at baseline. Although the number of reports of an ILD-like event may have been artificially elevated due to underlying patient baseline characteristics and the active management of ILD-like events, these results demonstrate the need to consider the risk of ILD-like events in Japanese patients treated with TKI. In particular, it is important that chest CT scans are closely checked for the presence of emphysema or comorbid ILD and that pulmonary status is assessed prior to treatment administration.

This study corroborates the results of the combination of gemcitabine and erlotinib shown in the PA.3 study. The median OS in this study of 9.23 months was longer than those reported in trials with gemcitabine alone. In this study, patients who experienced skin toxicity of grade ≥2 had better outcomes than those with less severe toxicity or the overall study population. Retrospective analyses of data from the PA.3 and AViTA studies have found a significant association between the development of skin toxicity and efficacy in patients with pancreatic cancer treated with erlotinib-based therapy, although the precise mechanisms for the association between skin toxicity and effectiveness are unknown.[28,41,42]

Although the presence of mutations in the tyrosine-kinase region of the *EGFR* gene appears to predict a better response to erlotinib in NSCLC,[43,44] this has not yet been evaluated in pancreatic cancer. *EGFR* mutations are very rare in patients with pancreatic cancer;[45–47] indeed in the present study, no *EGFR* mutations were detected. Further work is required to determine whether *EGFR* mutations can be used as predictive markers for improved survival in Japanese patients receiving erlotinib and gemcitabine as treatment for advanced pancreatic cancer.

In conclusion, the present study shows that erlotinib in combination with gemcitabine is generally well tolerated in Japanese patients with advanced pancreatic cancer. This combination is associated with efficacy and survival outcomes, and the results of this study are consistent with the findings of the global PA.3 study.

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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

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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/

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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

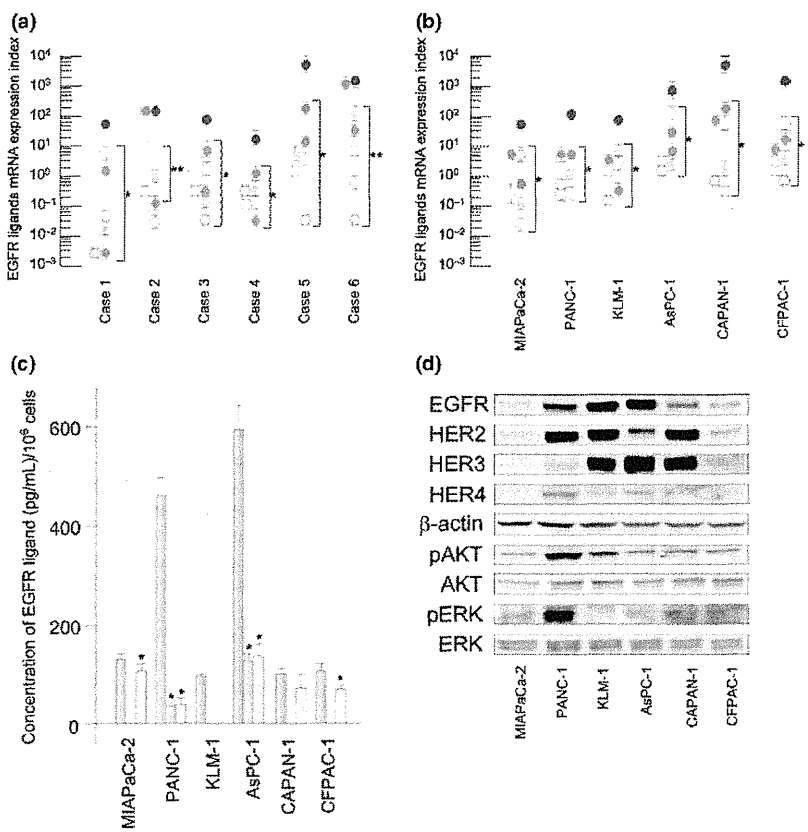

Fig. 1. Cell characteristics in pancreatic cancer. Differences in the expressions of epidermal growth factor receptor (EGFR) ligands in pancreatic cancer patients (a) and pancreatic cancer cell lines (b). Each value represents the mean and SD of the mRNA expression index for an EGFR ligand ($n = 4$). 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 15 000*g* 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 15 000*g* 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,

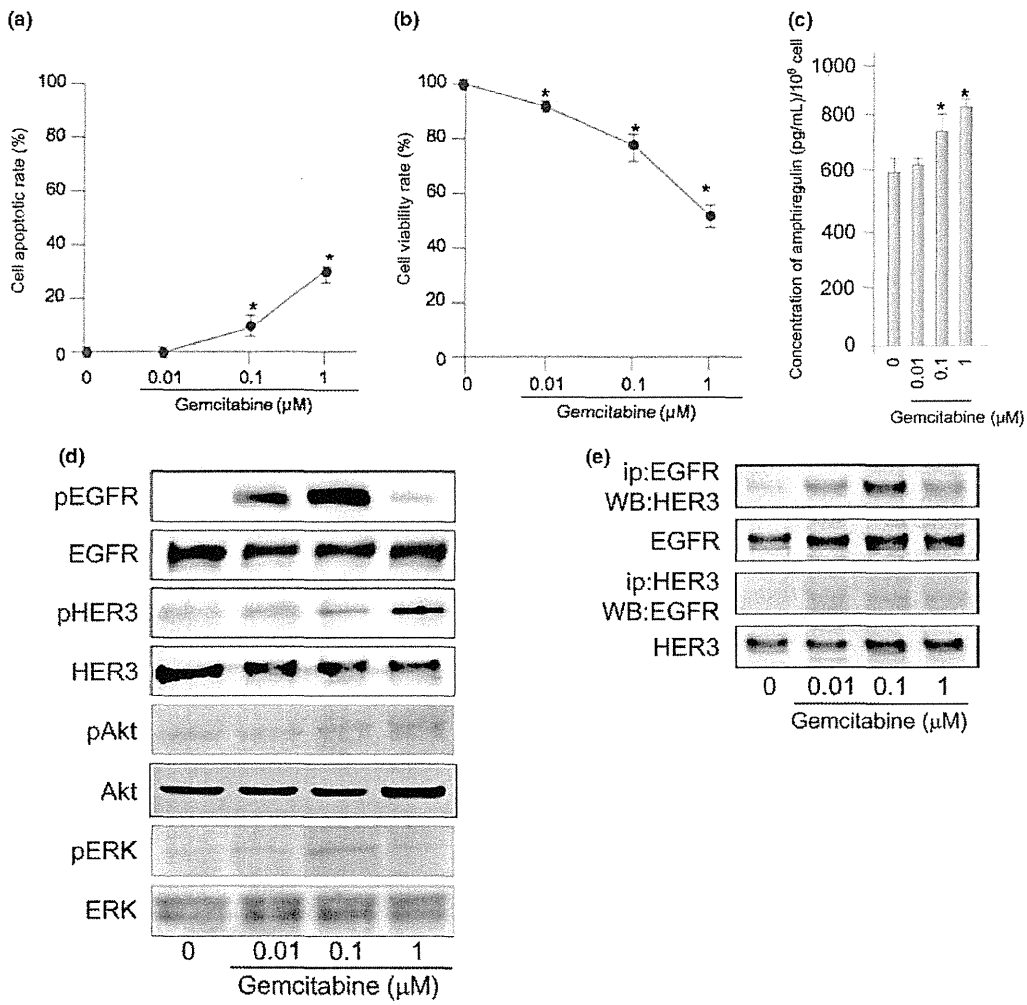

Fig. 2. Cell behavior after treatment with gemcitabine in AsPC-1 pancreatic cancer cells. Differences in the cell apoptotic rate (a), cell viability rate (b), and amount of amphiregulin in the culture medium (c) after treatment with various doses of gemcitabine for 48 h. Each value represents the mean 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

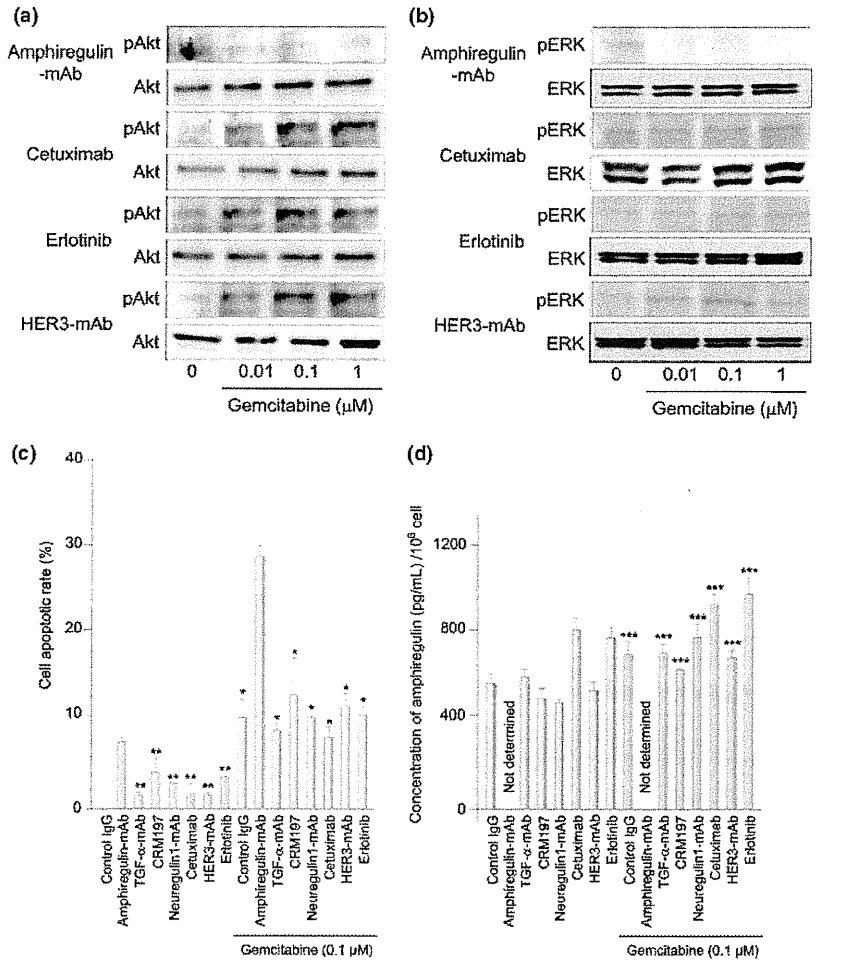

Fig. 3. Antitumor effects of combined treatment with gemcitabine and a variety of inhibitors in AsPC-1 pancreatic cancer cells. Alterations in the expression of phosphorylated Akt (a) and ERK (b) after combined treatment with gemcitabine and an inhibitory anti-amphiregulin antibody (10 μg/mL), cetuximab (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

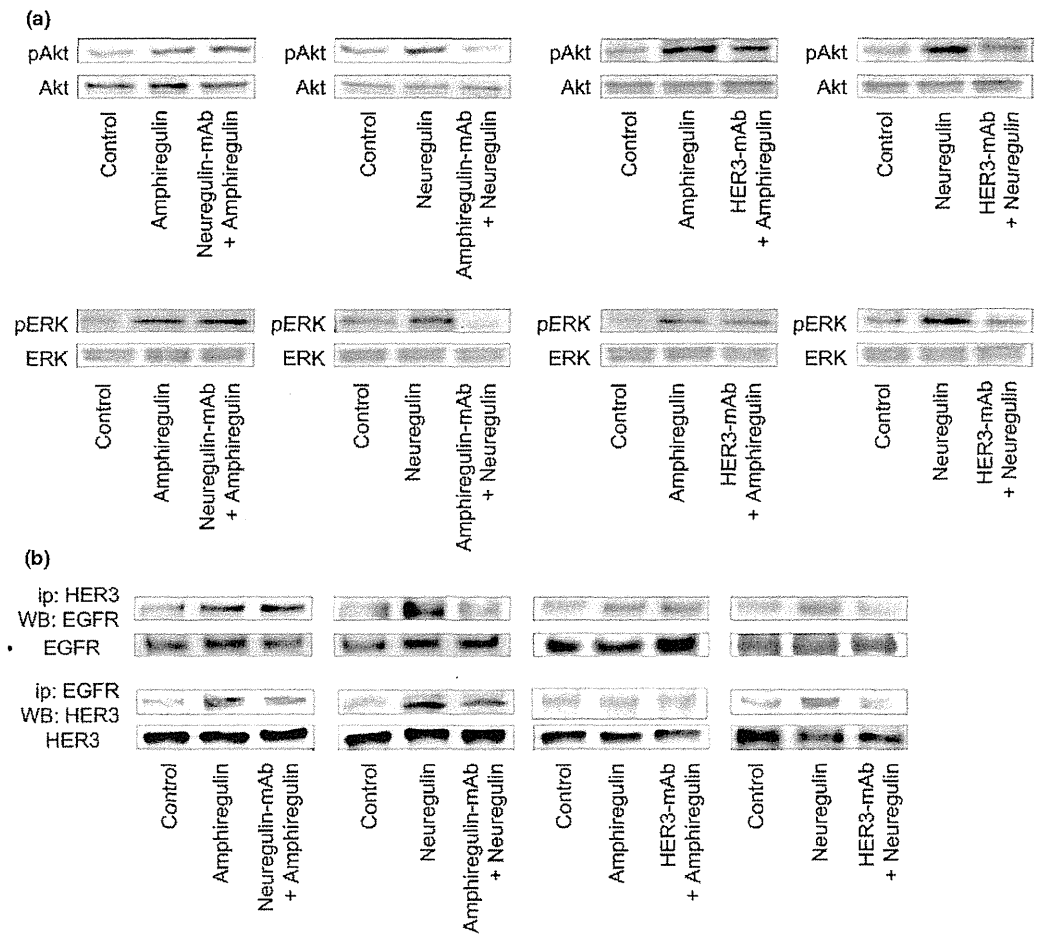

Fig. 4. Activation of Akt and ERK mediated by heterodimer formation of epidermal growth factor receptor (EGFR) with HER3 in AsPC-1 pancreatic cancer cells. (a) Phosphorylation of Akt (upper panels) and ERK (lower panels) stimulated by amphiregulin (50 ng/mL) or neuregulin (50 ng/mL) in the absence or 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

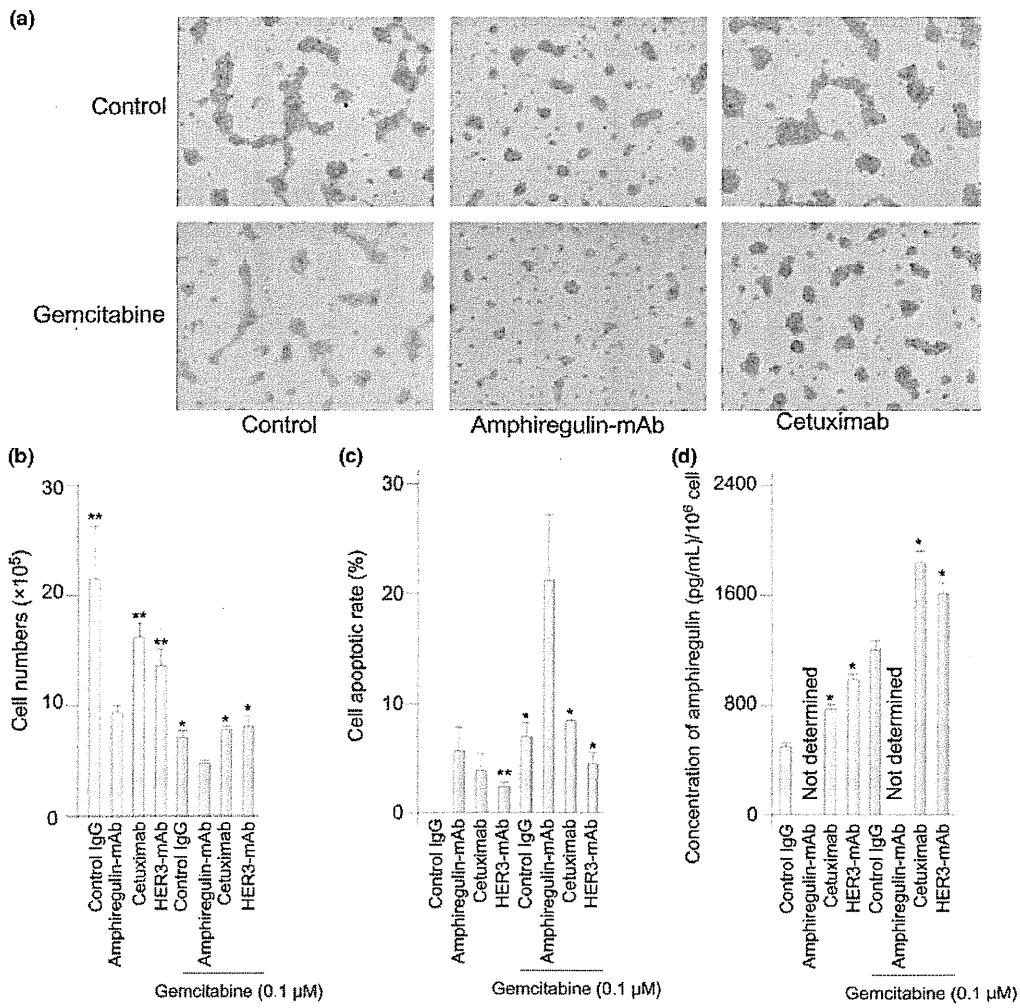

Fig. 5. Antitumor effects of combined treatment with gemcitabine and a variety of inhibitors in AsPC-1 cells using a Matrigel 3D culture system. (a) Appearances of growing cells by phase-contrast microscopy after treatment with control IgG (10 μg/mL), an inhibitory anti-amphiregulin antibody (10 μg/mL), 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

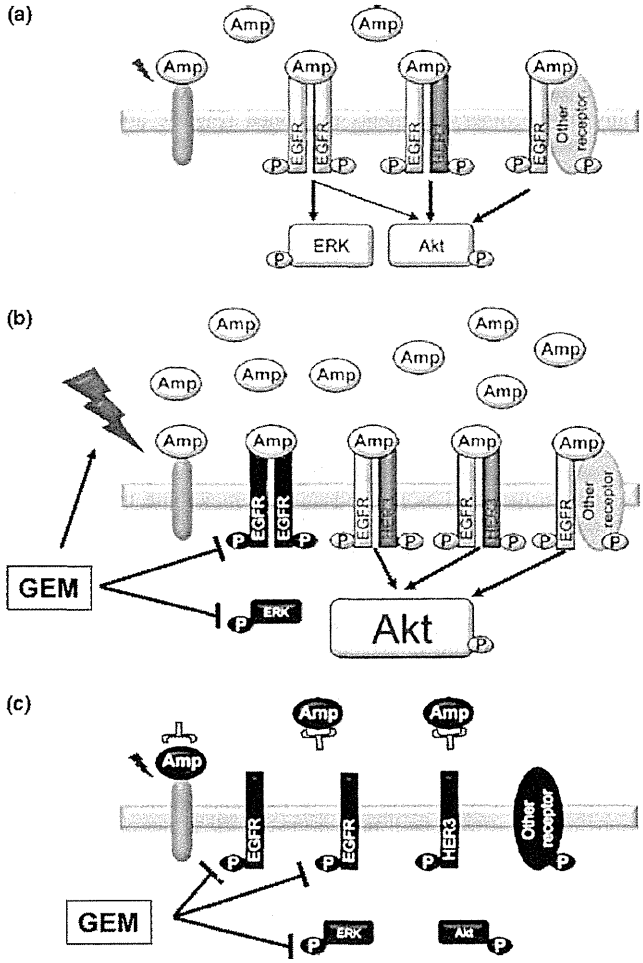

Fig. 6. Associations of therapeutic target molecules including amphiregulin (Amp), epidermal growth factor receptor (EGFR), HER3, and Akt with pancreatic cancer. (a) In pancreatic cancer, the abundant amount of amphiregulin enhances the activation of ERK through phosphorylation of EGFR and the activation 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.

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.

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.

Acknowledgments

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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 receptor (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

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JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

Randomized Phase III Study of Gemcitabine Plus S-1, S-1 Alone, or Gemcitabine Alone in Patients With Locally Advanced and Metastatic Pancreatic Cancer in Japan and Taiwan: GEST Study

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ABSTRACT

Purpose
The present phase III study was designed to investigate the noninferiority of S-1 alone and superiority of gemcitabine plus S-1 compared with gemcitabine alone with respect to overall survival.

Patients and Methods
The participants were chemotherapy-naive patients with locally advanced or metastatic pancreatic cancer. Patients were randomly assigned to receive only gemcitabine (1,000 mg/m^2 on days 1, 8, and 15 of a 28-day cycle), only S-1 (80, 100, or 120 mg/d according to body-surface area on days 1 through 28 of a 42-day cycle), or gemcitabine plus S-1 (gemcitabine 1,000 mg/m^2 on days 1 and 8 plus S-1 60, 80, or 100 mg/d according to body-surface area on days 1 through 14 of a 21-day cycle).

Results
In the total of 834 enrolled patients, median overall survival was 8.8 months in the gemcitabine group, 9.7 months in the S-1 group, and 10.1 months in the gemcitabine plus S-1 group. The noninferiority of S-1 to gemcitabine was demonstrated (hazard ratio, 0.96; 97.5% CI, 0.78 to 1.18; $P < .001$ for noninferiority), whereas the superiority of gemcitabine plus S-1 was not (hazard ratio, 0.88; 97.5% CI, 0.71 to 1.08; $P = .15$). All treatments were generally well tolerated, although hematologic and GI toxicities were more severe in the gemcitabine plus S-1 group than in the gemcitabine group.

Conclusion
Monotherapy with S-1 demonstrated noninferiority to gemcitabine in overall survival with good tolerability and presents a convenient oral alternative for locally advanced and metastatic pancreatic cancer.

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INTRODUCTION

Pancreatic cancer (PC) is currently the eighth leading cause of cancer-related mortality worldwide, with an estimated 266,000 deaths in 2008.[1] Gemcitabine became the standard treatment for advanced PC, improving overall survival (OS) compared with fluorouracil.[2] Although various gemcitabine-based combination regimens have been evaluated, only erlotinib added to gemcitabine showed a survival benefit over gemcitabine, and that was marginal.[3]

Fluorouracil/leucovorin plus irinotecan plus oxaliplatin (FOLFIRINOX), a gemcitabine-free combination regimen, has recently demonstrated a clear survival benefit compared with gemcitabine for patients with metastatic PC who have a performance status of 0 to 1.[4] However, because FOLFIRINOX is associated with significant toxicity, this regimen must be limited to patients with good performance status and requires close monitoring.[5]

In Japan, clinical trials of S-1 (TS-1; Taiho Pharmaceutical, Tokyo, Japan) have been conducted since the early 2000s for patients with PC. S-1

is an oral fluoropyrimidine derivative shown to be effective for gastric and various other types of cancers.[6,7] Phase II studies of S-1 as first-line therapy for metastatic PC resulted in good response rates of 21.1% to 37.5%.[8,9] Consequently, S-1 was approved for the indication of PC in Japan in 2006. Development of gemcitabine plus S-1 (GS) studies have also been initiated, mainly in Japan, and two phase II studies reported high response rates of 44.4% to 48.5% and good median OS of 10.1 to 12.5 months.[10,11]

Because S-1 and GS have shown promising activity in PC, the present randomized phase III study (GEST [Gemcitabine and S-1 Trial] study) was designed to evaluate whether S-1 alone is noninferior to gemcitabine and whether GS is superior to gemcitabine alone for locally advanced and metastatic PC with respect to OS.

PATIENTS AND METHODS

Study Design

This randomized phase III study, sponsored by Taiho Pharmaceutical in Japan and TTY Biopharm in Taiwan, was conducted as a postmarketing study in Japan and as a registration study in Taiwan and was in compliance with the Declaration of Helsinki. Data were collected by a contract research organization contracted by the sponsors and were analyzed by a bio-statistician (Y.O.). An independent data and safety monitoring committee reviewed efficacy and safety data. The study was approved by the ethics committee or institutional review board of each participating center.

Patients

All patients provided written informed consent. Enrollment criteria were locally advanced or metastatic PC, histologically or cytologically proven diagnosis of adenocarcinoma or adenosquamous carcinoma, no prior chemotherapy or radiotherapy for PC, age of more than 20 years (the protocol was amended to restrict the eligible age to < 80 years after four of the first eight patients who were ≥ 80 years experienced serious adverse events), an Eastern Cooperative Oncology Group performance status score of 0 to 1, and adequate organ functions (see Appendix, online only).

Treatment

Random assignment was performed centrally with stratification by extent of disease (locally advanced disease *v* metastatic disease) and institution using the minimization method. Patients allocated to gemcitabine alone received gemcitabine at a dose of 1,000 mg/m^2 intravenously over 30 minutes on days 1, 8, and 15 of a 28-day cycle. Patients allocated to S-1 alone received S-1 orally twice daily at a dose according to the body-surface area (BSA) (< 1.25 m^2, 80 mg/d; ≥ 1.25 to < 1.5 m^2, 100 mg/d; ≥ 1.5 m^2, 120 mg/d) on days 1 through 28 of a 42-day cycle. Patients allocated to GS received gemcitabine at a dose of 1,000 mg/m^2 on days 1 and 8 plus S-1 orally twice daily at a dose according to the BSA (< 1.25 m^2, 60 mg/d; ≥ 1.25 to < 1.5 m^2, 80 mg/d; ≥ 1.5 m^2, 100 mg/d) on days 1 through 14 of a 21-day cycle. The dose levels of S-1 used in the GS group were based on the results of a previous phase II study of GS, in which 1,000 mg/m^2 of gemcitabine was combined with 120 mg/d, 100 mg/d, and 80 mg/d of S-1. In that study, the rate of treatment withdrawal due to adverse events was 41% (22 of 54 patients), the rate of grade 3 or worse neutropenia was 80%, and the dose was reduced in 56% of the patients (30 of 54 patients).[11] Consequently, 20 mg/d lower doses of S-1 than those used in the S-1 monotherapy group were used in the GS group in the present study.

In the event of predefined toxic events, protocol-specified treatment modifications were permitted (see Appendix).

Assessments

Physical examinations, CBCs, and biochemistry tests were usually checked at 2-week intervals in the S-1 group and at each time of administration of gemcitabine both in the gemcitabine group and in the GS group. All adverse events were assessed according to the Common Terminology Criteria for Adverse Events, version 3.0. Computed tomography or magnetic resonance imaging was performed every 6 weeks until disease progression, and response was assessed by the investigators according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.0.[12] Quality of life was assessed using the EuroQol 5 Dimension questionnaire[13] at baseline and 6, 12, 24, 48, and 72 weeks after the study treatment had begun.

Statistical Analysis

The primary end point was OS, defined as time from date of random assignment to date of death from any cause. Secondary end points were progression-free survival (PFS), objective response rate, safety, and quality of life. PFS was counted from the date of random assignment to the date of death without progression or of progression as confirmed by the investigator's assessment. The median OS was assumed to be 7.5 months in the gemcitabine group, 8.0 months in the S-1 group, and 10.5 months in the GS group. To maintain a one-sided significance level of .025 for the entire study while testing two hypotheses (ie, noninferiority and superiority), the one-sided significance

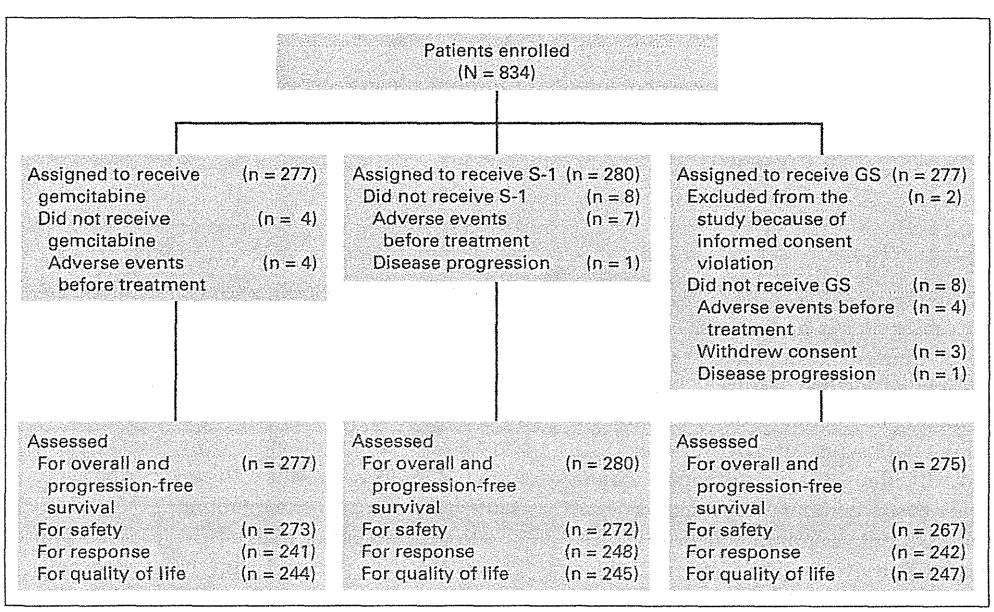

Fig 1. CONSORT diagram. GS, gemcitabine plus S-1.

level for each comparison was set at .0125. The statistical considerations are detailed in the Appendix.

The superiority of GS was evaluated by the stratified log-rank test. To assess the noninferiority of S-1, we used the Cox proportional hazards model to calculate two-sided, 97.5% CIs of the hazard ratio (HR). The noninferiority margin of S-1 was set at 1.33; that is, the null hypothesis was that the median OS with S-1 would be approximately 2 months shorter than with gemcitabine. We decided this setting was justified considering the convenience of S-1 and because there are few effective drugs for the disease. Furthermore, to interpret the obtained data, the Bayesian analysis of the log HR on the basis of the noninformative prior distribution was preplanned. Posterior probability with log HR within a stricter threshold (log 1.15) was also calculated.[14]

In each assigned group, the time-to-event distribution was estimated with the Kaplan-Meier method. The 95% CI of the median survival time was calculated by the method of Brookmeyer and Crowly.[15] In addition, the Greenwood formula[16] was used to calculate the 95% CI for survival rates. In subgroup analyses, interaction tests were performed to assess the homogeneity of the effect of treatment on OS.

The primary end point was analyzed for the full analysis set. All *P* value evaluations were two-tailed. Data analyses were done with SAS, version 9.1.3 (SAS Institute, Cary, NC).

RESULTS

Patients

Between July 2007 and October 2009, a total of 834 patients were enrolled from 75 institutions in Japan and Taiwan (768 in Japan and 66 in Taiwan). Two patients in the GS group were excluded from the study because enrollment was conducted before obtaining written informed consent. The remaining 832 patients were included in the full analysis set and used to calculate OS and PFS (Fig 1). The three treatment groups were well balanced with respect to demographic and baseline characteristics (Table 1).

Study Treatment

The median duration of treatment was 2.6 months in the gemcitabine group, 2.6 months in the S-1 group, and 4.3 months in the GS group. The main reasons for treatment discontinuation were either disease progression (202 patients [72.9%] in the gemcitabine group,

Table 1. Demographics and Baseline Characteristics of Patients (full-analysis set population)

Characteristic	Gemcitabine (n = 277)		S-1 (n = 280)		GS (n = 275)		Total (N = 832)	
	No.	%	No.	%	No.	%	No.	%
Sex								
Male	170	61.4	170	60.7	158	57.5	498	59.9
Female	107	38.6	110	39.3	117	42.5	334	40.1
Age, years								
< 65	134	48.4	145	51.8	137	49.8	416	50.0
≥ 65	143	51.6	135	48.2	138	50.2	416	50.0
ECOG PS								
0	181	65.3	178	63.6	172	62.5	531	63.8
1	96	34.7	102	36.4	103	37.5	301	36.2
Extent of disease								
Locally advanced	66	23.8	68	24.3	68	24.7	202	24.3
Metastatic	211	76.2	212	75.7	207	75.3	630	75.7
Type of tumor								
Adenocarcinoma	272	98.2	276	98.6	272	98.9	820	98.6
Adenosquamous carcinoma	5	1.8	4	1.4	3	1.1	12	1.4
Pancreas excision								
No	254	91.7	264	94.3	248	90.2	766	92.1
Yes	23	8.3	16	5.7	27	9.8	66	7.9
Tumor location*								
Head	122	44.0	110	39.3	116	42.2	348	41.8
Body	88	31.8	124	44.3	102	37.1	314	37.7
Tail	68	24.5	55	19.6	66	24.0	189	22.7
Biliary drainage								
No	202	72.9	217	77.5	209	76.0	628	75.5
Yes	75	27.1	63	22.5	66	24.0	204	24.5
CEA, ng/mL								
Median	5.7		5.6		5.9		5.7	
IQR	3.0-20.1		2.5-18.4		2.5-20.7		2.6-19.5	
CA19-9, U/mL								
Median	1,044		726		441		712	
IQR	52-5,002		64-5,000		45-5,090		55-5,002	
CRP, mg/dL								
Median	0.40		0.50		0.40		0.43	
IQR	0.11-1.38		0.18-1.57		0.15-1.60		0.15-1.57	

Abbreviations: CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; CRP, C-reactive protein; ECOG PS, Eastern Cooperative Oncology Group performance status; GS, gemcitabine plus S-1; IQR, interquartile range.
*Including patients with tumors involving multiple sites.

215 [76.8%] in the S-1 group, and 162 [58.9%] in the GS group) or adverse events (40 patients [14.4%] in the gemcitabine group, 38 [13.6%] in the S-1 group, and 76 [27.6%] in the GS group). The median relative dose-intensity was 83.0% in the gemcitabine group, 96.1% in the S-1 group, and 83.3% for gemcitabine and 87.4% for S-1 in the GS group.

Survival

The median duration of follow-up for surviving patients was 18.4 months (range, 0.3 to 36.9 months) as of July 31, 2010. The analysis of OS was based on 710 deaths (85.3%) among the 832 patients. The median OS was 8.8 months (95% CI, 8.0 to 9.7) in the gemcitabine group, 9.7 months (95% CI, 7.6 to 10.8) in the S-1 group, and 10.1 months (95% CI, 9.0 to 11.2) in the GS group (Fig 2A). OS rates at 12 and 24 months were respectively 35.4% and 9.2% in the gemcitabine group, 38.7% and 12.7% in the S-1 group, and 40.7% and 14.5% in the GS group. The noninferiority of S-1 to gemcitabine with respect to OS was demonstrated (HR, 0.96; 97.5% CI, 0.78 to 1.18; $P < .001$ for noninferiority). The Bayesian posterior probability that the HR of S-1 relative to gemcitabine would be less than 1.15 was calculated to be 98% on the basis of the noninformative prior distribution. However, GS failed to improve OS at a statistically significant level as compared with gemcitabine (HR, 0.88; 97.5% CI, 0.71 to 1.08; $P = .15$).

The analysis of PFS was based on 793 events (95.3%) among the 832 patients. The median PFS was 4.1 months (95% CI, 3.0 to 4.4) in the gemcitabine group, 3.8 months (95% CI, 2.9 to 4.2) in the S-1 group, and 5.7 months (95% CI, 5.4 to 6.7) in the GS group (Fig 2B). PFS rates at 6 and 12 months were respectively 29.8% and 9.1% in the gemcitabine group, 26.9% and 7.2% in the S-1 group, and 47.9% and 20.3% in the GS group. S-1 was shown to be noninferior to gemcitabine with respect to PFS (HR, 1.09; 97.5% CI, 0.90 to 1.33; $P = .02$ for noninferiority), and GS significantly improved PFS compared with gemcitabine (HR, 0.66; 97.5% CI, 0.54 to 0.81; $P < .001$).

Subgroup analyses of survival according to pretreatment characteristics showed no significant interaction between S-1 and gemcitabine in any subgroup (Fig 3A). However, GS showed a favorable HR compared with gemcitabine in the subsets of patients with locally advanced disease or patients with a performance status of 1 (Fig 3B).

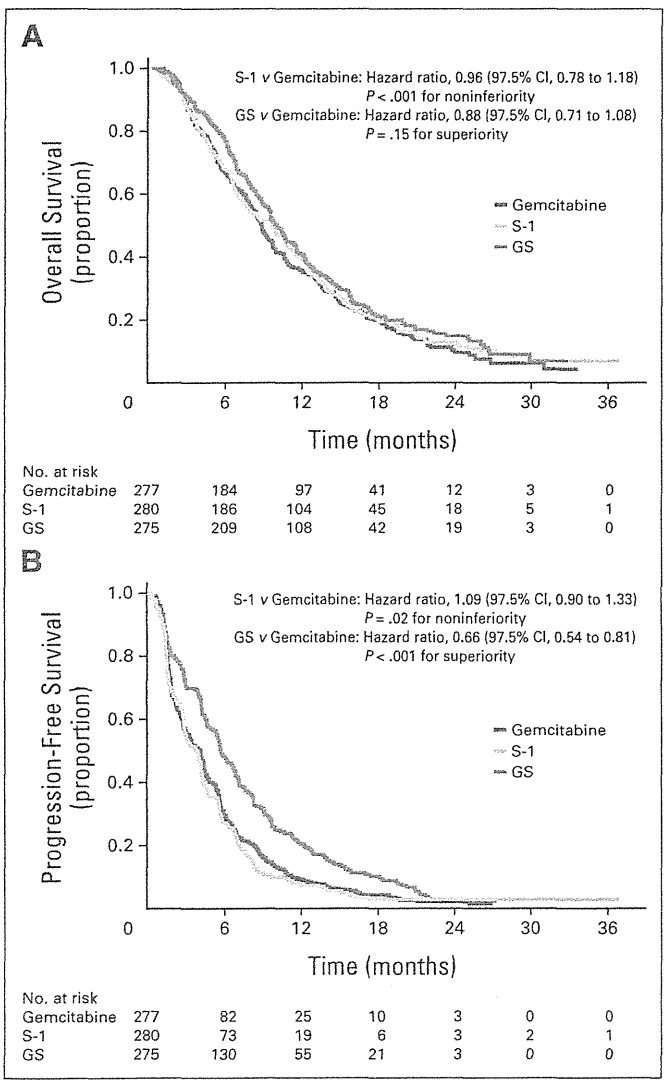

Fig 2. Kaplan-Meier estimates of (A) overall survival and (B) progression-free survival according to treatment group. GS, gemcitabine plus S-1.

Response to Therapy

The objective response rate was 13.3% (95% CI, 9.3 to 18.2) in the gemcitabine group, 21.0% (95% CI, 16.1 to 26.6) in the S-1 group, and 29.3% (95% CI, 23.7 to 35.5) in the GS group (Table 2). The objective response rate was significantly higher in the S-1 group ($P = .02$) and in the GS group ($P < .001$) than in the gemcitabine group.

Second-Line Chemotherapy

Second-line chemotherapy was performed in 184 patients (66.4%) in the gemcitabine group, 185 (66.1%) in the S-1 group, and 172 (62.5%) in the GS group. In the gemcitabine group, 140 patients (50.5%) received S-1 alone or S-1–based regimens, and in the S-1 group 162 (57.9%) received gemcitabine alone or gemcitabine-based regimens as second-line chemotherapy. The most common second-line regimens in the GS group were gemcitabine alone (61 patients), GS (53 patients), S-1 alone (24 patients), irinotecan (six patients), and fluorouracil/leucovorin plus oxaliplatin (four patients). In Japan and Taiwan, the use of treatments such as erlotinib, oxaliplatin, and irinotecan for PC was not approved at the time of this study; hence gemcitabine, S-1, or both were used in most patients as second-line chemotherapy.

Adverse Events and Quality-Adjusted Life-Years

The major grade 3 or worse adverse events are listed in Table 3. Patients in the gemcitabine group had significantly higher incidences of grade 3 or worse leukopenia, neutropenia, thrombocytopenia, elevated AST levels, and elevated ALT levels as compared with patients in the S-1 group. However, the incidence of grade 3 or worse diarrhea was higher in the S-1 group than in the gemcitabine group. Patients in the GS group had significantly higher incidences of grade 3 or worse leukopenia, neutropenia, thrombocytopenia, rash, diarrhea, vomiting, and stomatitis than patients in the gemcitabine group.

There were three deaths considered possibly related to the protocol treatment (interstitial lung disease, sepsis, and acute hepatitis B) in the gemcitabine group, one in the S-1 group (unknown cause), and

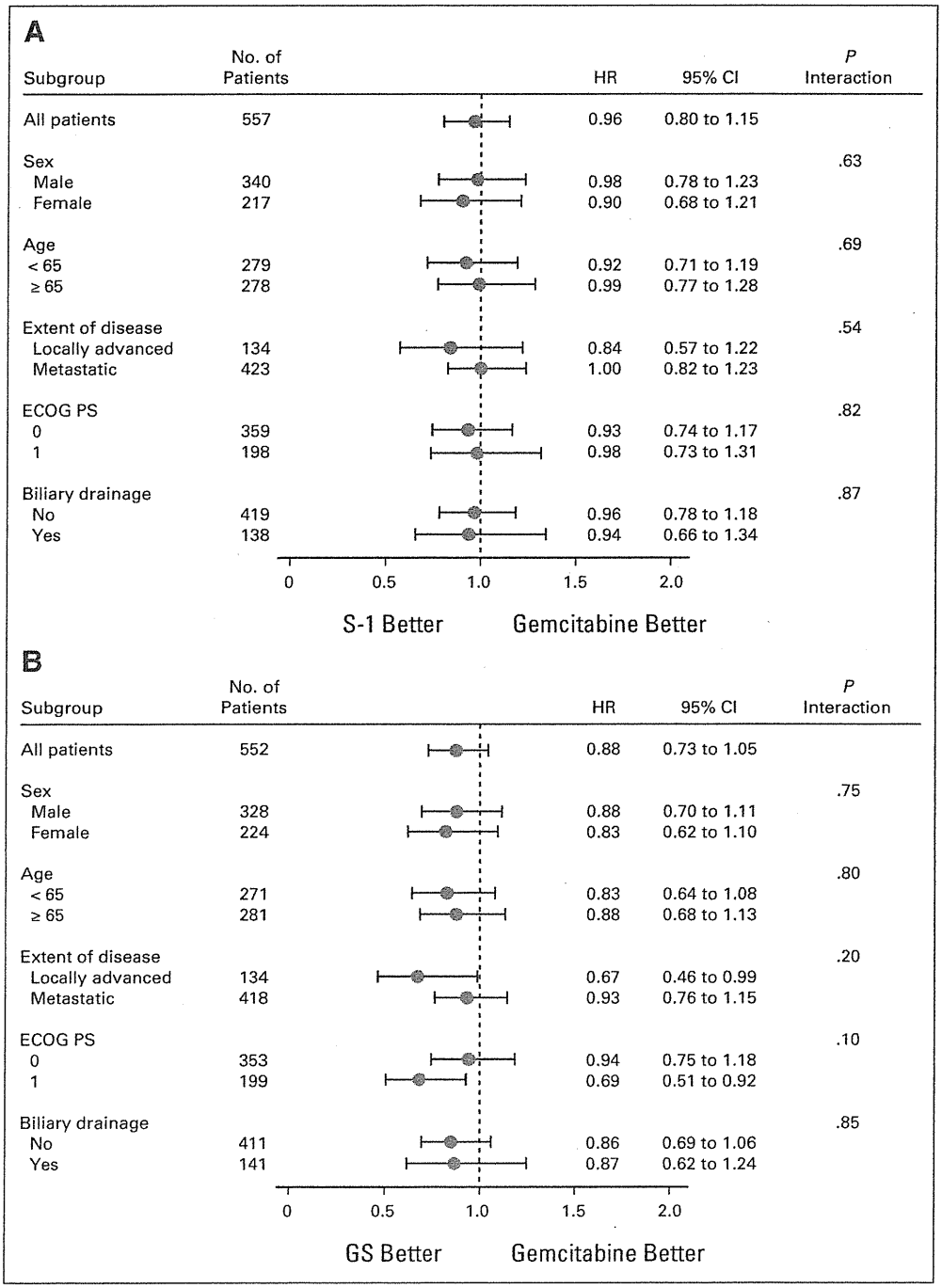

Fig 3. Forest plots of treatment effects on overall survival in subgroup analyses. Forest plots show effects on overall survival of patients in each subgroup. (A) S-1; (B) gemcitabine plus S-1 (GS). Each blue circle shows the treatment response. ECOG PS, Eastern Cooperative Oncology Group performance status; HR, hazard ratio.

four in the GS group (unknown cause associated with myelosuppression, cerebral infarction, cerebrovascular disorder, and interstitial lung disease). The results of quality-adjusted life-years (QALYs) are in the Appendix and the details of quality-of-life assessments will be reported elsewhere.

DISCUSSION

The overall and PFS curves in the S-1 group were nearly identical to those in the gemcitabine group, confirming the noninferiority of S-1 to gemcitabine in terms of OS and PFS (Fig 2A, 2B). Toxicity profiles of these two drugs differed slightly: gemcitabine tended to show hematologic toxicity, whereas S-1 tended to show GI toxicity. However, both S-1 and gemcitabine were generally well tolerated. Furthermore, the results of QALY evaluation demonstrated that S-1 and gemcitabine were equivalent. Hence our results suggest that S-1 can be used as first-line therapy as a convenient oral alternative for locally advanced and metastatic PC. To the best of our knowledge, this is the first phase III study to demonstrate the noninferiority of a single anticancer agent to gemcitabine alone for locally advanced and metastatic PC.

Table 2. Objective Response Rates (patients with measurable lesions)

Variable	Gemcitabine (n = 241)		S-1 (n = 248)		GS (n = 242)		*P* (χ^2 test)	
	No.	%	No.	%	No.	%	Gemcitabine *v* S-1	Gemcitabine *v* GS
Response								
Complete response	1	0.4	0	0	2	0.8		
Partial response	31	12.9	52	21.0	69	28.5		
Stable disease	119	49.4	105	42.3	102	42.1		
Progressive disease	75	31.1	69	27.8	37	15.3		
Objective response rate*	32	13.3	52	21.0	71	29.3	.02	< .001
95% CI	9.3 to 18.2		16.1 to 26.6		23.7 to 35.5			
Disease control rate†	151	62.7	157	63.3	173	71.5	.88	.04
95% CI	56.2 to 68.8		57.0 to 69.3		65.4 to 77.1			

Abbreviation: GS, gemcitabine plus S-1.
*The objective response rate was defined as the proportion of patients who had a complete response or partial response.
†The disease control rate was defined as the proportion of patients who had a complete response, partial response, or stable disease.

At the time of planning this study, the participants of nearly all phase III trials included both patients with locally advanced as well as those with metastatic PC. However, because locally advanced and metastatic diseases are two clinical entities, it is recently recommended that patients with locally advanced disease should be studied separately from those with metastatic disease.[17] Although this study included locally advanced disease, subgroup analysis of extent of disease showed no significant interaction between S-1 and gemcitabine (Fig 3A). Moreover, the OS curve in the S-1 group was still similar to those in the gemcitabine group in both locally advanced and metastatic disease (Fig 4A, 4B). Regarding pathologic diagnosis, our study included adenosquamous carcinoma, although its percentage was very low (1.4% of whole population). When the data were reanalyzed after excluding patients with adenosquamous carcinoma, the results for OS for gemcitabine versus S-1 was unchanged (HR, 0.96; 95% CI, 0.81 to 1.15). The selection of one treatment over the other will depend primarily on patient preference, clinical factors, or drug costs, as biomarkers indicating effective use of S-1 or gemcitabine do not exist at this time.

Regarding GS, the OS did not differ significantly from gemcitabine, although the PFS was significantly longer in the GS group. Second-line chemotherapy mainly with S-1 in the gemcitabine group may be one reason for this discrepancy. The median OS in the gemcitabine group was 8.8 months, which is longer than those previously reported for gemcitabine in other phase III studies for locally advanced and metastatic PC.[2,3,18-24] Although the efficacy of second-line

Table 3. Grade 3 or Worse Adverse Events (safety population)

Event	Gemcitabine (n = 273)		S-1 (n = 272)		GS (n = 267)		*P* (Fisher's exact test)	
	No.	%	No.	%	No.	%	Gemcitabine *v* S-1	Gemcitabine *v* GS
Hematologic								
Leukocytes	51	18.7	10	3.7	101	37.8	< .001	< .001
Neutrophils	112	41.0	24	8.8	166	62.2	< .001	< .001
Platelets	30	11.0	4	1.5	46	17.2	< .001	.05
Hemoglobin	39	14.3	26	9.6	46	17.2	.11	.41
Nonhematologic								
ALT	41	15.0	16	5.9	29	10.9	< .001	.16
AST	41	15.0	21	7.7	32	12.0	.01	.32
Bilirubin	26	9.5	39	14.3	23	8.6	.09	.77
Fatigue	10	3.7	18	6.6	13	4.9	.13	.53
Rash	2	0.7	2	0.7	11	4.1	1.00	.01
Anorexia	20	7.3	31	11.4	25	9.4	.11	.44
Diarrhea	3	1.1	15	5.5	12	4.5	.004	.02
Mucositis/stomatitis	0	0.0	2	0.7	6	2.2	.25	.01
Nausea	5	1.8	5	1.8	12	4.5	1.00	.09
Vomiting	2	0.7	4	1.5	12	4.5	.45	.006
Febrile neutropenia	1	0.4	1	0.4	5	1.9	1.00	.12
Infection with normal ANC	6	2.2	7	2.6	6	2.2	.79	1.00
Pneumonitis	5	1.8	0	0.0	2	0.7	.06	.45

NOTE. Grades of adverse events were defined according to the Common Terminology Criteria for Adverse Events (version 3.0).
Abbreviations: ANC, absolute neutrophil count; GS, gemcitabine plus S-1.

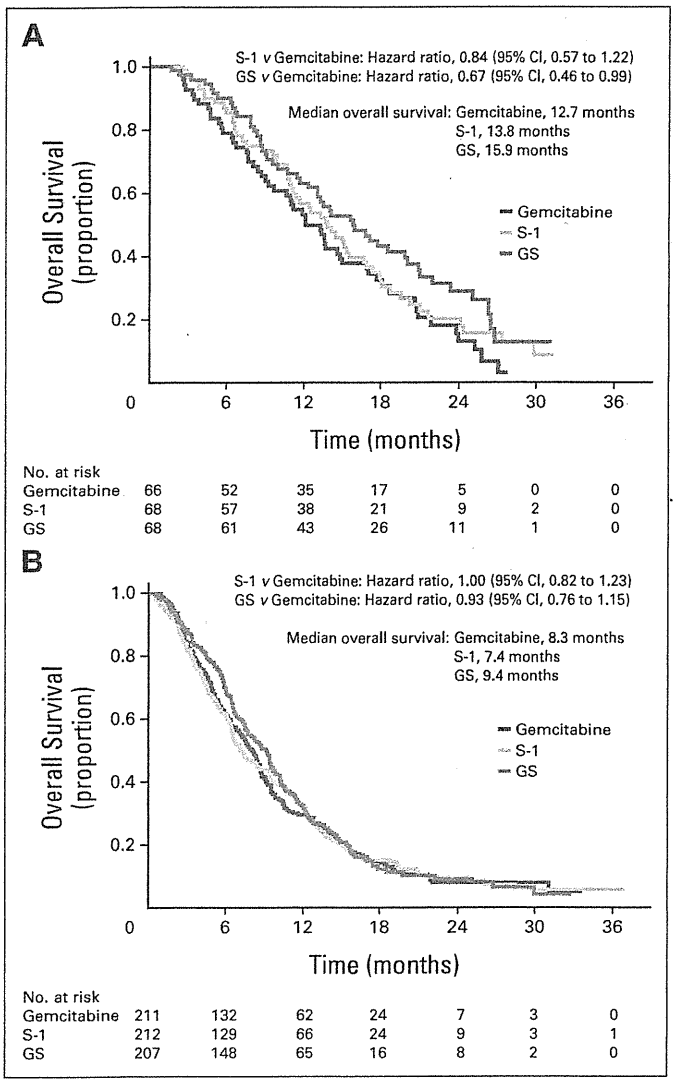

Fig 4. Kaplan-Meier estimates of overall survival in (A) locally advanced disease and (B) metastatic disease. GS, gemcitabine plus S-1.

therapy was not analyzed in this study, a phase II study of second-line S-1 in patients with gemcitabine-refractory PC showed a 15% response rate and 58% disease control rate.[25] Compared with the GS group, which had no promising second-line therapy, the use of S-1 as second-line therapy in the gemcitabine group might have contributed to prolonged survival.

The lack of a significant difference in OS between gemcitabine and GS suggests that gemcitabine and S-1 could be used sequentially rather than concurrently. However, the GS group showed a high response rate and favorable PFS, with a better HR of 0.66 compared with other gemcitabine-based combination regimens in other phase III studies (HR = 0.75 to 1.07).[3,18,20,22,24] Furthermore, the GS group showed a favorable HR for OS in patients with locally advanced disease or patients with a performance status of 1 in the subgroup analyses. Therefore, it is speculated that there may be room to select GS therapy, depending on the profile of the patients and further investigations.

Regarding oral fluoropyrimidines other than S-1, capecitabine has been studied in patients with PC, mainly in the West. In two phase III studies, a combination of gemcitabine plus capecitabine did not significantly prolong survival as compared with gemcitabine alone.[19,20] The results of a meta-analysis of these phase III studies, however, demonstrated that survival was significantly prolonged by combined treatment, with an HR of 0.86,[20] which is similar to the HR for GS in the present study (0.88).

One limitation of our study is that it is uncertain whether our results can be simply extrapolated to Western patients because pharmacokinetics and pharmacodynamics of S-1 between Westerners and East Asians may be different.[26,27] Although S-1 is available for PC only in Japan at the moment, if S-1 is used in Western patients, its effectiveness should be monitored and the dose should be carefully adjusted accordingly. Another potential limitation is that the protocol-specified noninferiority margin of 1.33 may be large. However, the result of point estimate of the HR of S-1 was 0.96 and actual upper limit of the 97.5% CI was 1.18, which was sufficiently lower than the prespecified margin of 1.33. Furthermore, Bayesian posterior probability with log HR within a stricter threshold (log 1.15) was 98%.

Given that most gemcitabine-based combination regimens have not been shown to be significantly superior to gemcitabine alone and that FOLFIRINOX has demonstrated overwhelming superiority to gemcitabine in a phase III study, reporting an HR of 0.57,[4] the development of gemcitabine-free combination regimens for first-line treatment seems to be warranted. However, because FOLFIRINOX requires the placement of a central venous access port for continuous intravenous infusion of fluorouracil, it can be expected that S-1, an oral fluoropyrimidine, will replace the continuous infusion of fluorouracil in the future.

In conclusion, this study has verified the noninferiority of S-1 to gemcitabine, thereby suggesting that S-1 can be used as first-line therapy for locally advanced and metastatic PC. Because S-1 was confirmed to be a key treatment for PC, S-1–based regimens are expected to be developed in the future to improve the management of this formidable disease.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) and/or an author's immediate family member(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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