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.

Acknowledgments

The authors would like to thank all the patients, investigators and site staff involved in the study. We are grateful to Masahiro Fukuoka for acting as a medical advisor for this study. The authors also thank Abdul Al Khateeb of Gardiner–Caldwell Communications for editorial assistance. This study was sponsored by Chugai Pharmaceutical Co., Ltd. Editorial assistance from Abdul Al Khateeb of Gardiner–Caldwell Communications was funded by Chugai Pharmaceutical Co., Ltd.

Disclosure Statement

Junji Furuse received honoraria for lecture fees from Bayer, Eli Lilly Japan, Taiho Pharmaceutical and Eisai; Kazuhiko Nakagawa received honoraria for lecture fees from Eli Lilly Japan, Chugai Pharmaceutical and AstraZeneca; Takuji Okusaka, Akihiro Funakoshi, Tatsuya Ioka, Kenji Yamao, Shinichi Ohkawa, Narikazu Boku, Yoshito Komatsu, Shoji Nakamori, Haruo Iguchi, Tetsuhide Ito and Kohei Nakachi have no conflict of interest.

References

1 Parkin DM, Bray F, Ferlay J *et al.* Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74–108.
2 Japanese Ministry of Health, Labour and Welfare. Statistical investigation result 2005. (In Japanese.) [Cited 16 Feb 2010.] Available from URL: http://www-bm.mhlw.go.jp/toukei/saikin/hw/kanja/05syoubyo/index.html.
3 Japanese Ministry of Health, Labour and Welfare. Table database system. (In Japanese.) [Cited 16 Feb 2010.] Available from URL: http://www.mhlw.go.jp/toukei/youran/indexyk_1_2.html.
4 Burris HA III, Moore MJ, Andersen J *et al.* Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* 1997; **15**: 2403–13.
5 Berlin JD, Catalano P, Thomas JP *et al.* Phase III study of gemcitabine in combination with fluorouracil versus gemcitabine alone in patients with advanced pancreatic carcinoma: Eastern Cooperative Oncology Group Trial E2297. *J Clin Oncol* 2002; **20**: 3270–5.
6 Colucci G, Giuliani F, Gebbia V *et al.* Gemcitabine alone or with cisplatin for the treatment of patients with locally advanced and/or metastatic pancreatic carcinoma: a prospective, randomized phase III study of the Gruppo Oncologia dell'Italia Meridionale. *Cancer* 2002; **94**: 902–10.
7 Rocha Lima CM, Green MR, Rotche R *et al.* Irinotecan plus gemcitabine results in no survival advantage compared with gemcitabine monotherapy in patients with locally advanced or metastatic pancreatic cancer despite increased tumor response rate. *J Clin Oncol* 2004; **22**: 3776–83.
8 Louvet C, Labianca R, Hammel P *et al.* Gemcitabine in combination with oxaliplatin compared with gemcitabine alone in locally advanced or metastatic pancreatic cancer: results of a GERCOR and GISCAD phase III trial. *J Clin Oncol* 2005; **23**: 3509–16.
9 Oettle H, Richards D, Ramanathan RK *et al.* A phase III trial of pemetrexed plus gemcitabine versus gemcitabine in patients with unresectable or metastatic pancreatic cancer. *Ann Oncol* 2005; **16**: 1639–45.
10 Abou-Alfa GK, Letourneau R, Harker G *et al.* Randomized phase III study of exatecan and gemcitabine compared with gemcitabine alone in untreated advanced pancreatic cancer. *J Clin Oncol* 2006; **24**: 4441–7.
11 Heinemann V, Quietzsch D, Gieseler F *et al.* Randomized phase III trial of gemcitabine plus cisplatin compared with gemcitabine alone in advanced pancreatic cancer. *J Clin Oncol* 2006; **24**: 3946–52.
12 Stathopoulos GP, Syrigos K, Aravantinos G *et al.* A multicenter phase III trial comparing irinotecan-gemcitabine (IG) with gemcitabine (G) monotherapy as first-line treatment in patients with locally advanced or metastatic pancreatic cancer. *Br J Cancer* 2006; **95**: 587–92.
13 Herrmann R, Bodoky G, Ruhstaller T *et al.* Gemcitabine plus capecitabine compared with gemcitabine alone in advanced pancreatic cancer: a randomized, multicenter, phase III trial of the Swiss Group for Clinical Cancer Research and the Central European Cooperative Oncology Group. *J Clin Oncol* 2007; **25**: 2212–7.
14 Van Cutsem E, van de Velde H, Karasek P *et al.* Phase III trial of gemcitabine plus tipifarnib compared with gemcitabine plus placebo in advanced pancreatic cancer. *J Clin Oncol* 2004; **22**: 1430–8.
15 Bramhall SR, Rosemurgy A, Brown PD *et al.* Marimastat as first-line therapy for patients with unresectable pancreatic cancer: a randomized trial. *J Clin Oncol* 2001; **19**: 3447–55.
16 Moore M, Hamm J, Dancey J *et al.* Comparison of gemcitabine versus the matrix metalloproteinase inhibitor BAY 12-9566 in patients with advanced or metastatic adenocarcinoma of the pancreas: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* 2003; **21**: 3296–302.
17 Philip PA, Benedetti J, Fenoglio-Preiser C *et al.* Phase III study of gemcitabine [G] plus cetuximab [C] versus gemcitabine in patients [pts] with locally advanced or metastatic pancreatic adenocarcinoma [Pca]: SWOG S0205 study. *J Clin Oncol* 2007; **25** (Suppl 18): 199s (Abstract LBA4509).
18 Kindler HL, Niedzwiecki D, Hollis E *et al.* A double-blind, placebo-controlled, randomizes phase III trial of gemcitabine (G) plus bevacizumab (B) versus gemcitabine plus placebo (P) in patients (pts) with advanced pancreatic cancer (PC): A Preliminary Analysis of Cancer and Leukemia Group B (CALGB). *J Clin Oncol* 2007; **25** (Suppl 18): 199s (Abstract 4508).
19 Van Cutsem E, Vervenne WL, Bennouna J *et al.* Phase III trial of bevacizumab in combination with gemcitabine and erlotinib in patients with metastatic pancreatic cancer. *J Clin Oncol* 2009; **27**: 2231–7.
20 Lynch TJ Jr, Kim ES, Eaby B *et al.* Epidermal growth factor receptor inhibitor-associated cutaneous toxicities: an evolving paradigm in clinical management. *Oncologist* 2007; **12**: 610–21.
21 Perez-Soler R, Saltz L. Cutaneous adverse effects with HER1/EGFR-targeted agents: is there a silver lining? *J Clin Oncol* 2005; **23**: 5235–46.
22 Arteaga C. Targeting HER1/EGFR: a molecular approach to cancer therapy. *Semin Oncol* 2003; **30**: 3–14.
23 Harari D, Yarden Y. Molecular mechanisms underlying ErbB2/HER2 action in breast cancer. *Oncogene* 2000; **19**: 6102–14.
24 Jost M, Gasparro FP, Jensen PJ *et al.* Keratinocyte apoptosis induced by ultraviolet B radiation and CD95 ligation – differential protection through epidermal growth factor receptor activation and Bcl-x(L) expression. *J Invest Dermatol* 2001; **116**: 860–6.
25 Quon H, Liu F, Cummings B. Potential molecular prognostic markers in head and neck squamous cell carcinomas. *Head Neck* 2001; **23**: 147–59.

doi: 10.1111/j.1349-7006.2010.01810.x

26 Ueda S, Ogata S, Tsuda H *et al*. The correlation between cytoplasmic overexpression of epidermal growth factor receptor and tumor aggressiveness: poor prognosis in patients with pancreatic ductal adenocarcinoma. *Pancreas* 2004; **29**: e1–8.

27 Durkin A, Bloomston PM, Rosemurgy AS *et al*. Defining the role of the epidermal growth factor receptor in pancreatic cancer grown *in vitro*. *Am J Surg* 2003; **186**: 431–6.

28 Moore M, Goldstein D, Hamm J *et al*. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* 2007; **25**: 1960–6.

29 Kubota K, Nishiwaki Y, Tamura T *et al*. Efficacy and safety of erlotinib monotherapy for Japanese patients with advanced non-small cell lung cancer: a phase II study. *J Thorac Oncol* 2008; **3**: 1439–45.

30 Dragovich T, Huberman M, Von Hoff DD *et al*. Erlotinib plus gemcitabine in patients with unresectable pancreatic cancer and other solid tumors: phase IB trial. *Cancer Chemother Pharmacol* 2007; **60**: 295–303.

31 Honeywell R, Laan AC, van Groeningen CJ *et al*. The determination of gemcitabine and 2′-deoxycytidine in human plasma and tissue by APCI tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2007; **847**: 142–52.

32 Ling J, Fettner S, Lum BL *et al*. Effect of food on the pharmacokinetics of erlotinib, an orally active epidermal growth factor receptor tyrosine-kinase inhibitor, in healthy individuals. *Anticancer Drugs* 2008; **19**: 209–16.

33 Nakamura K, Yamaguchi T, Ishihara T *et al*. Phase II trial of oral S-1 combined with gemcitabine in metastatic pancreatic cancer. *Br J Cancer* 2006; **94**: 1575–9.

34 Tanaka T, Ikeda M, Okusaka T *et al*. Prognostic factors in Japanese patients with advanced pancreatic cancer treated with single-agent gemcitabine as first-line therapy. *Jpn J Clin Oncol* 2008; **38**: 755–61.

35 Tamiya A, Endo M, Shukuya T *et al*. Features of gemcitabine-related severe pulmonary toxicity patients with pancreatic or biliary tract cancer. *Pancreas* 2009; **38**: 838–40.

36 Bhatia S, Hanna N, Ansari R *et al*. A phase II study of weekly gemcitabine and paclitaxel in patients with previously untreated stage IIIb and IV non-small cell lung cancer. *Lung Cancer* 2002; **38**: 73–7.

37 Ando M, Okamoto I, Yamamoto N *et al*. Predictive factors for interstitial lung disease, antitumor response, and survival in non-small-cell lung cancer patients treated with gefitinib. *J Clin Oncol* 2006; **24**: 2549–56.

38 Kudoh S, Kato H, Nishiwaki N *et al*. Interstitial lung disease in Japanese patients with lung cancer: a cohort and nested case–control study. *Am J Respir Crit Care Med* 2008; **177**: 1348–57.

39 Tsuboi M, Le Chevalier T. Interstitial lung disease in patients with non-small-cell lung cancer treated with epidermal growth factor receptor inhibitors. *Med Oncol* 2006; **23**: 161–70.

40 Yoneda KY, Shelton DK, Beckett LA *et al*. Independent review of interstitial lung disease associated with death in TRIBUTE (paclitaxel and carboplatin with or without concurrent erlotinib) in advanced non-small cell lung cancer. *J Thorac Oncol* 2007; **2**: 537–43.

41 Wacker B, Nagrani T, Weinberg J *et al*. Correlation between development of rash and efficacy in patients treated with the epidermal growth factor receptor tyrosine kinase inhibitor erlotinib in two large phase III studies. *Clin Cancer Res* 2007; **13**: 3913–21.

42 Van Cutsem E, Vervenne WL, Bennouna J *et al*. Rash as a marker for the efficacy of gemcitabine plus erlotinib-based therapy in pancreatic cancer: results from the AViTA study. Proc ASCO Gastrointestinal Cancers Symposium, 2009 (Abstr 117). [Cited 16 Feb 2010.] Available from URL: http://www.asco.org/ASCOv2/Meetings/Abstracts?&vmview=abst_detail_view&confID=63&abstractID=10514.

43 Tsao MS, Sakurada A, Cutz JC *et al*. Erlotinib in lung cancer – molecular and clinical predictors of outcome. *N Engl J Med* 2005; **353**: 133–44.

44 Zhu CQ, da Cunha Santos G, Ding K *et al*. Role of KRAS and EGFR as biomarkers of response to erlotinib in National Cancer Institute of Canada Clinical Trials Group Study BR.21. *J Clin Oncol* 2008; **28**: 4268–75.

45 Immervoll H, Hoem D, Kugarajh K *et al*. Molecular analysis of the EGFR-RAS-RAF pathway in pancreatic ductal adenocarcinomas: lack of mutations in the BRAF and EGFR genes. *Virchows Arch* 2006; **448**: 788–96.

46 Lee J, Jang KT, Ki CS *et al*. Impact of epidermal growth factor receptor (EGFR) kinase mutations, EGFR gene amplifications, and KRAS mutations on survival of pancreatic adenocarcinoma. *Cancer* 2007; **109**: 1561–9.

47 Tzeng CW, Frolov A, Frolova N *et al*. Epidermal growth factor receptor (EGFR) is highly conserved in pancreatic cancer. *Surgery* 2007; **141**: 464–9.

Cancer Science
The official journal of the Japanese Cancer Association JCA

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

(Received February 16, 2010/Revised June 26, 2010/Accepted July 4, 2010/Accepted manuscript online July 7, 2010/Article first published online August 17, 2010)

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

doi: 10.1111/j.1349-7006.2010.01671.x

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

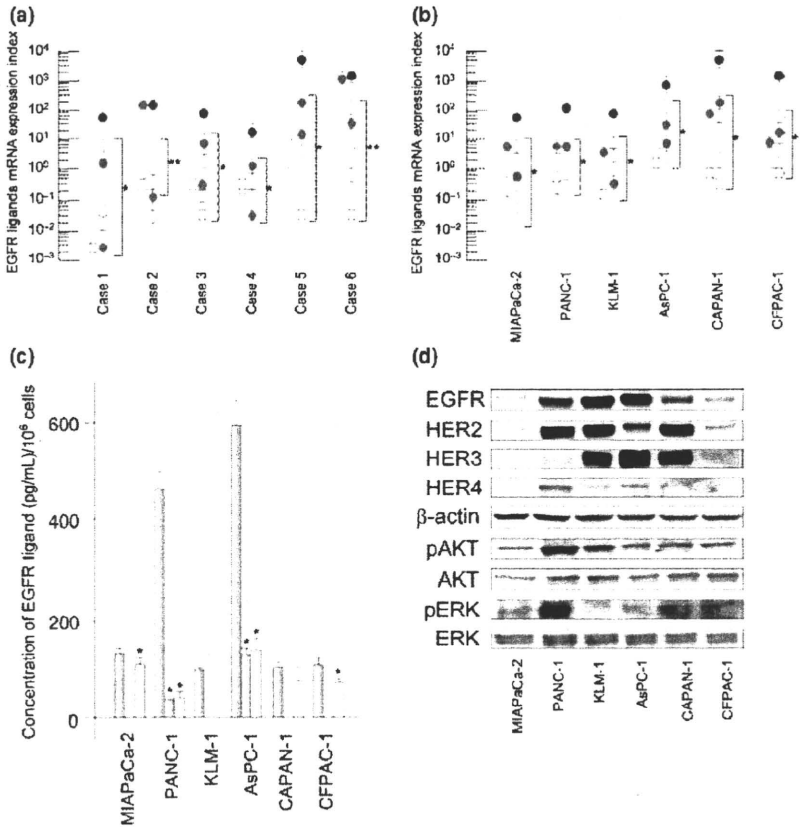


Fig. 1. Cell characteristics in pancreatic cancer. Differences in the expressions of epidermal growth factor receptor (EGFR) ligands in pancreatic cancer patients (a) and pancreatic cancer cell lines (b). Each value represents the mean and SD of the mRNA expression index for an EGFR ligand ($n = 4$).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

doi: 10.1111/j.1349-7006.2010.01671.x

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,

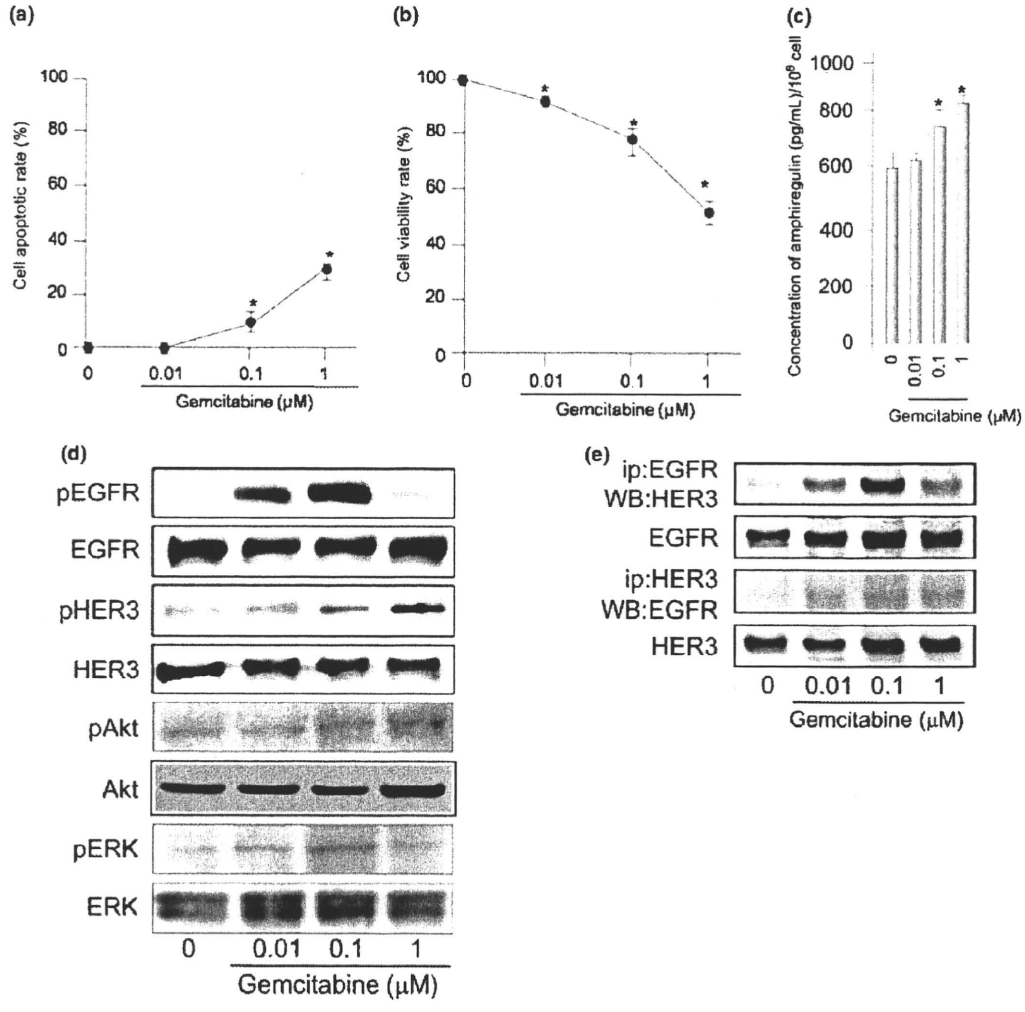


Fig. 2. Cell behavior after treatment with gemcitabine in AsPC-1 pancreatic cancer cells. Differences in the cell apoptotic rate (a), cell viability rate (b), and amount of amphiregulin in the culture medium (c) after treatment with various doses of gemcitabine for 48 h. Each value represents the mean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

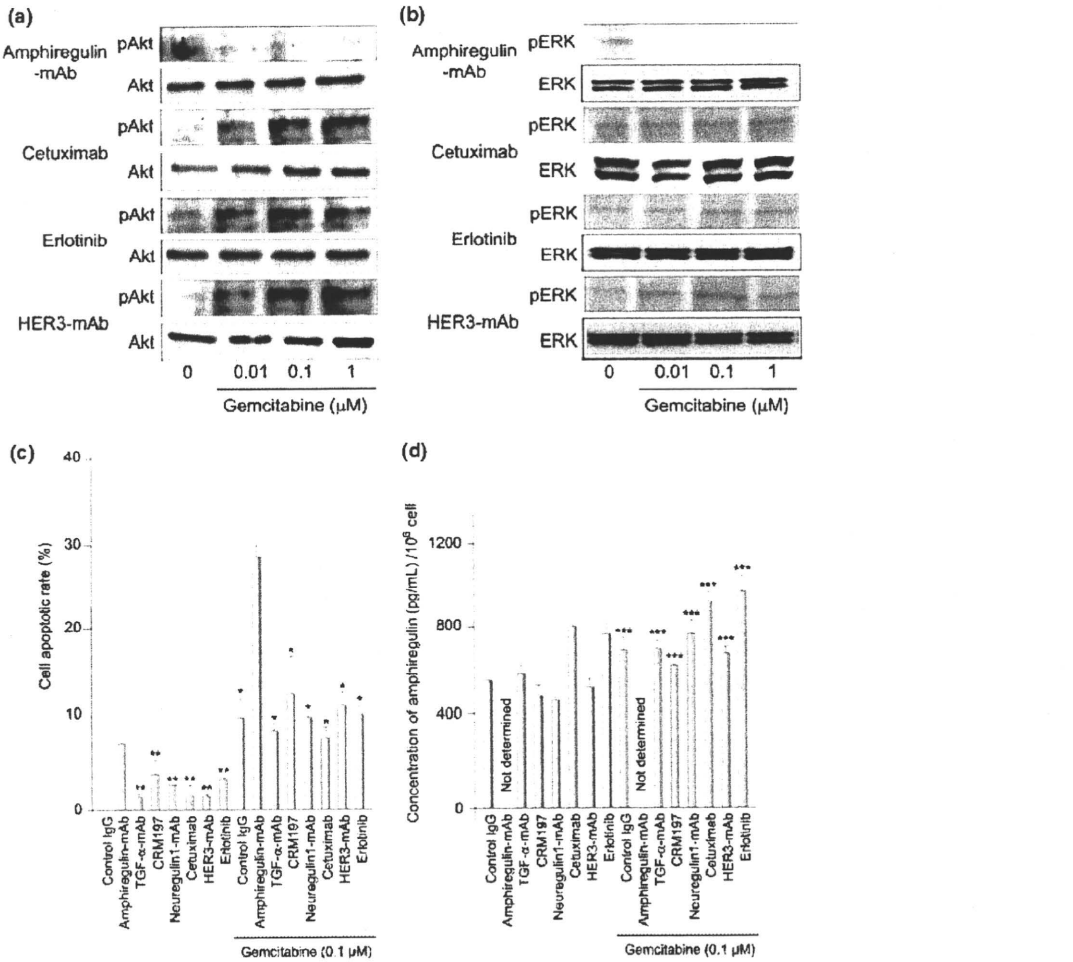


Fig. 3. Antitumor effects of combined treatment with gemcitabine and a variety of inhibitors in AsPC-1 pancreatic cancer cells. Alterations in the expression of phosphorylated Akt (a) and ERK (b) after combined treatment with gemcitabine and an inhibitory anti-amphiregulin antibody (10 μg/mL), cetuximab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

doi: 10.1111/j.1349-7006.2010.01671.x

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

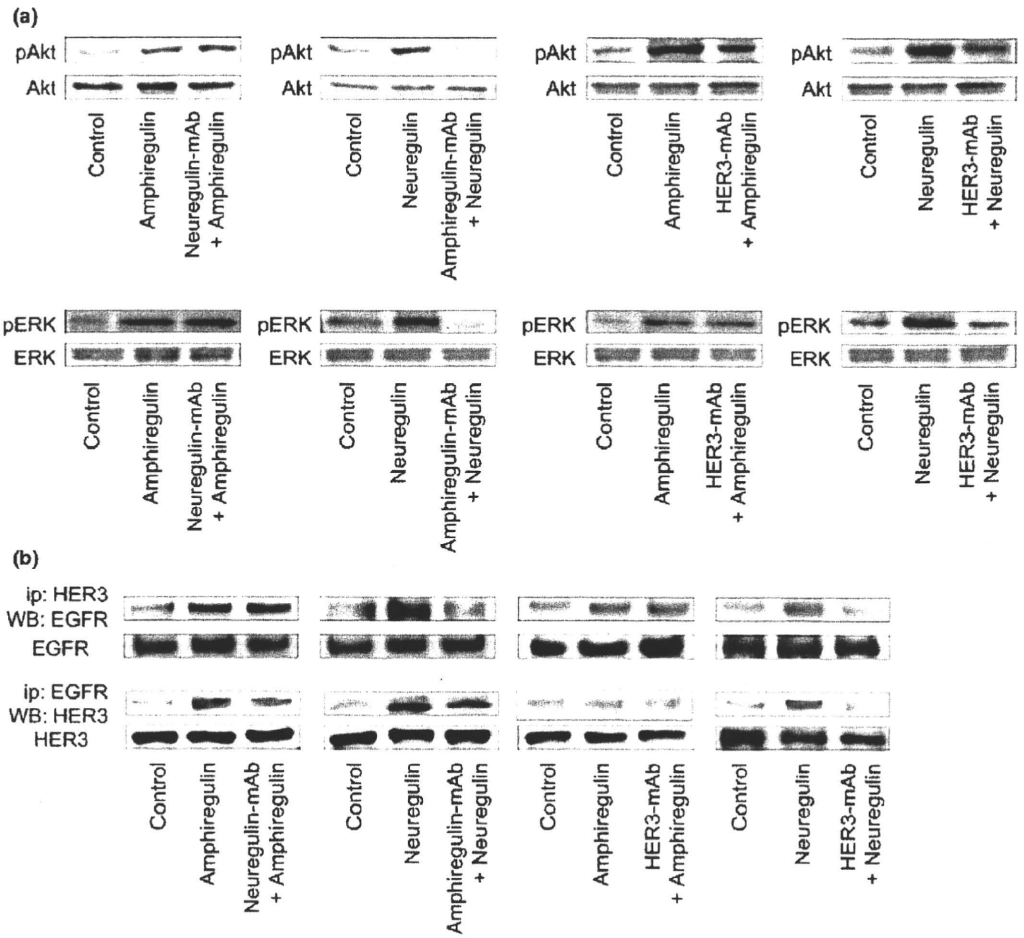


Fig. 4. Activation of Akt and ERK mediated by heterodimer formation of epidermal growth factor receptor (EGFR) with HER3 in AsPC-1 pancreatic cancer cells. (a) Phosphorylation of Akt (upper panels) and ERK (lower panels) stimulated by amphiregulin (50 ng/mL) or neuregulin (50 ng/mL) in the absence or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

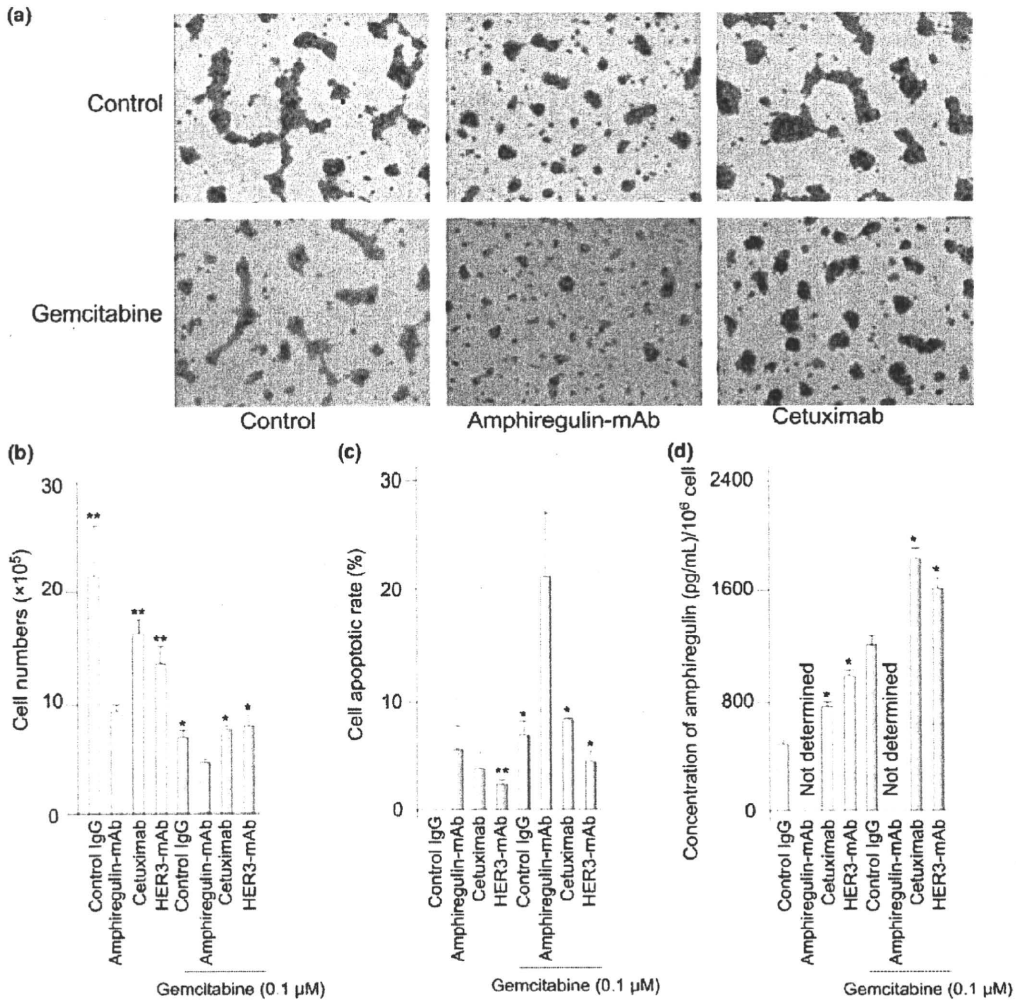


Fig. 5. Antitumor effects of combined treatment with gemcitabine and a variety of inhibitors in AsPC-1 cells using a Matrigel 3D culture system. (a) Appearances of growing cells by phase-contrast microscopy after treatment with control IgG (10 μg/mL), an inhibitory anti-amphiregulin antibody (10 μg/mL),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

doi: 10.1111/j.1349-7006.2010.01671.x

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

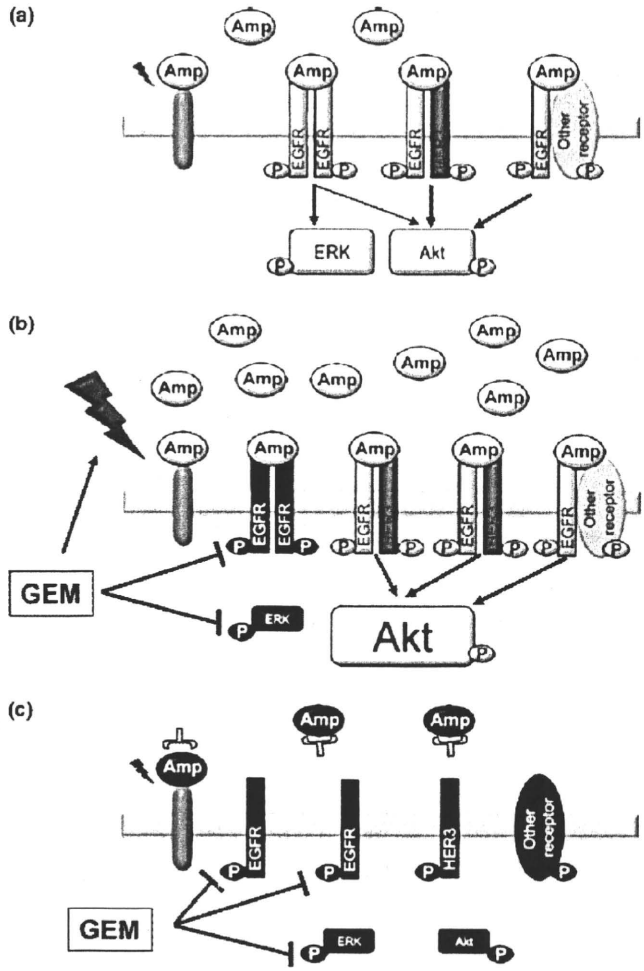


Fig. 6. Associations of therapeutic target molecules including amphiregulin (Amp), epidermal growth factor receptor (EGFR), HER3, and Akt with pancreatic cancer. (a) In pancreatic cancer, the abundant amount of amphiregulin enhances the activation of ERK through phosphorylation of EGFR and the activation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

This work was supported in part by funds from the Central Research Institute of Fukuoka University (Fukuoka, Japan), a grant-in-aid from the Kakihara Science and Technology Foundation (Fukuoka, Japan), Kyowa Hakko Kirin (Tokyo, Japan), a Young Investigator Research Award from the Fukuoka University School of Medicine Eboshi Association (Fukuoka, Japan) and the International Research Fund for Subsidy of Kyushu University School of Medicine Alumni (Fukuoka, Japan).

References

1 Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. *CA Cancer J Clin* 2009; **59**: 225–49.
2 Li D, Xie K, Wolff R, Abbruzzese JL. Pancreatic cancer. *Lancet* 2004; **363**: 1049–57.
3 Burris HA III, Moore MJ, Andersen MJ *et al.* Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreatic cancer: a randomized trial. *J Clin Oncol* 1997; **15**: 2403–13.
4 Moore MJ, Goldstein D, Hamm J *et al.* Erlotinib+gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada clinical trials group. *J Clin Oncol* 2007; **25**: 1960–6.
5 Welch SA, Moore MJ. Combination chemotherapy in advanced pancreatic cancer: time to raise the white flag? *J Clin Oncol* 2007; **25**: 2159–61.
6 Bondar VM, Sweeney-Gotsch B, Andreeff M, Mills GB, McConkey DJ. Inhibition of the phosphatidylinositol 3′-kinase-AKT pathway induces apoptosis in pancreatic carcinoma cells *in vitro* and *in vivo*. *Mol Cancer Ther* 2002; **1**: 989–97.
7 Simon PO Jr, McDunn JE, Kashiwagi H *et al.* Targeting AKT with the proapoptotic peptide, TAT-CTMP: a novel strategy for the treatment of human pancreatic adenocarcinoma. *Int J Cancer* 2009; **125**: 942–51.
8 Frolov A, Schuller K, Tzeng CW *et al.* ErbB3 expression and dimerization with EGFR influence pancreatic cancer cell sensitivity to erlotinib. *Cancer Biol Ther* 2007; **6**: 548–54.
9 Sergina NV, Rausch M, Wang D *et al.* Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. *Nature* 2007; **445**: 437–41.
10 Yotsumoto F, Yagi H, Suzuki SO *et al.* Validation of HB-EGF and amphiregulin as targets for human cancer therapy. *Biochem Biophys Res Commun* 2008; **365**: 555–61.
11 Shoyab M, McDonald VL, Bradley JG, Todaro GJ. Amphiregulin: a bifunctional growth-modulating glycoprotein produced by the phorbol 12-myristate 13-acetate-treated human breast adenocarcinoma cell line MCF-7. *Proc Natl Acad Sci USA* 1988; **85**: 6528–32.
12 Sunnarborg SW, Hinkle CL, Stevenson M *et al.* Tumor necrosis factor-alpha converting enzyme (TACE) regulates epidermal growth factor receptor ligand availability. *J Biol Chem* 2002; **277**: 12838–45.
13 Berasain C, Garcia-Trevijano ER, Castillo J *et al.* Novel role for amphiregulin in protection from liver injury. *J Biol Chem* 2005; **280**: 19012–20.
14 Berasain C, Garcia-Trevijano ER, Castillo J *et al.* Amphiregulin: an early trigger of liver regeneration in mice. *Gastroenterology* 2005; **128**: 424–32.
15 Luetteke NC, Qiu TH, Fenton SE *et al.* Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. *Development* 1999; **126**: 2739–50.
16 Troyer KL, Luetteke NC, Saxon ML, Qiu TH, Xian CJ, Lee DC. Growth retardation, duodenal lesions, and aberrant ileum architecture in triple null mice lacking EGF, amphiregulin, and TGF-alpha. *Gastroenterology* 2001; **121**: 68–78.
17 Wagner M, Greten FR, Weber CK *et al.* A murine tumor progression model for pancreatic cancer recapitulating the genetic alterations of human disease. *Genes Dev* 2001; **15**: 286–93.
18 Wagner M, Weber CK, Bressau F *et al.* Transgenic overexpression of amphiregulin induces a mitogenic response selectively in pancreatic duct cells. *Gastroenterology* 2002; **122**: 1898–912.
19 Ebert M, Yokoyama M, Kobrin MS *et al.* Induction and expression of amphiregulin in human pancreatic cancer. *Cancer Res* 1994; **54**: 3959–62.
20 Yagi H, Yotsumoto F, Sonoda K *et al.* Synergistic anti-tumor effect of paclitaxel with CRM197, an inhibitor of HB-EGF, in ovarian cancer. *Int J Cancer* 2009; **124**: 1429–39.
21 Miyamoto S, Hirata M, Yamazaki A *et al.* Heparin-binding EGF-like growth factor is a promising target for ovarian cancer therapy. *Cancer Res* 2004; **64**: 5720–7.

doi: 10.1111/j.1349-7006.2010.01671.x

22 Shafer A, Zhou C, Paola A, Boggess JF, Bae-Jump VL. Rapamycin potentiates the effects of paclitaxel in endometrial cancer cells through inhibition of cell proliferation and induction of apoptosis. *Int J Cancer* 2010; **126**: 1144–54.
23 Stathis A, Moore MJ. Advanced pancreatic carcinoma: current treatment and future challenges. *Nat Rev Clin Oncol* 2010; **7**: 163–72.
24 Constantinou M, Tsai JY, Safran H. Paclitaxel and concurrent radiation in upper gastrointestinal cancers. *Cancer Invest* 2003; **21**: 887–96.
25 Burgess AW, Cho HS, Eigenbrot C *et al.* An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors *Mol Cell* 2003; **12**: 541–52.
26 Lemmon MA. Ligand-induced ErbB receptor dimerization. *Exp Cell Res* 2009; **315**: 638–48.
27 Weihua Z, Tsan R, Huang WC *et al.* Survival of Cancer cells in maintained by EGFR independent of its kinase activity. *Cancer Cell* 2008; **13**: 385–93.
28 Chau I, Cunningham D, Russell C *et al.* Gastrazole (JB95008), a novel CCK2/gastrin receptor antagonist, in the treatment of advanced pancreatic cancer: results from two randomized controlled trials. *Br J Cancer* 2006; **94**: 1107–15.
29 Van Cutsem E, Vervenne WL, Bennouna J *et al.* Phase III trial of bevacizumab in combination with gemcitabine and erlotinib in patients with metastatic pancreatic cancer. *J Clin Oncol* 2009; **27**: 2231–7.
30 Bramhall SR, Rosemurgy A, Brown PD *et al.* Marimastat as first-line therapy for patients with unresectable pancreatic cancer: a randomized trial. *J Clin Oncol* 2001; **19**: 3447–55.

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

臨牀消化器内科 2011 Vol.26 No.1

特集 膵癌 up-to-date

12 化学療法(術後補助療法)

阪本 良弘* 小菅 智男* 上野 秀樹**
奈良 聡* 江崎 稔* 島田 和明*

Key words: 膵癌, 術後補助化学療法, ランダム化比較試験, ゲムシタビン, TS-1

要 旨

膵癌の術後補助療法に関するエビデンスが近年確立されつつある. 米国では化学放射線療法が術後補助療法の中心だが, 欧州や本邦では化学療法が中心である. 欧州の CONKO-001 や本邦の JSAP-02(小菅班)ではゲムシタビン(GEM)による化学療法施行群において手術単独群よりも有意に良好な無再発生存率が得られ, さらに前者では全生存率の延長が確認された. ESPAC-3 では GEM と 5-FU による術後補助化学療法の比較が行われ, 両群の生存曲線はほぼ一致したものの, 有害事象は GEM 群で低率なことから, GEM による化学療法が標準治療として位置づけられた. 現在, 本邦でも新たな補助化学療法の試験が進行中である.

はじめに

膵癌の術後補助療法に関するエビデンスは, 米国, 欧州, 本邦において行われてきたランダム化比較試験(randomized clinical trial ; RCT)の結果から, 最近になってようやく確立されつつある. 現在, ゲムシタビン(GEM)による術後全身化学療法が欧州と日本では術後補助療法の中心である. 本稿では膵癌術後補助療法の歴史的変遷と 2010 年現在のエビデンスについて概説する.

Ⅰ. 化学放射線療法 vs. 化学療法

この項のポイント
- 米国と欧州では術後補助療法に対する歴史的アプローチが異なる. 米国では化学放射線療法が主体となり, 欧州と本邦では化学療法が主体となった.

膵癌の術後補助療法に関する最初の RCT を行ったのは米国の Gastrointestinal Tumor Study Group(GITSG)である(表). GITSG は 5-FU を用いた化学放射線療法の有用性を多施設共同の RCT によって検討した. 1 群わずか 21 例と 22 例による小規模な試験の結果, 化学放射線療法群が手術単独群に比較して 2 倍の生存期間を得たと報告した[1].

欧州では European Organisation for Research and Treatment of Cancer(EORTC)が中心となって, 膵頭部領域癌に対する 5-FU を用いた化学放射線療法群(n = 104)と手術単独群(n = 103)を比較した多施設共同 RCT が行われた. 浸潤性膵管癌症例に限って生存曲線をみる

*国立がん研究センター中央病院肝胆膵外科
(〒 104-0045 東京都中央区築地 5-1-1)
**同 肝胆膵内科

0911-601X/11/JCOPY

表　膵管癌の術後補助療法に関するランダム化比較試験の結果

報告者	国　名	発表年	対象期間	治療方法	n	登録数/年	全5年生存率(%)	MST(月)	p値
Kalser (GITSG)[1]	米国	1985	1974～1982	手術単独	22	5.4	NA	11	0.03
				手術＋5-FU＋RT	21			20	
Klinkenbijl (EORTC)[2]	オランダ	1999	1987～1995	手術＋5-FU＋RT	60	14.3	20	17.1	0.099
				手術単独	54		10	12.6	
Neoptolemos (ESPAC-1)[3]	欧州	2004	1994～2000	(手術＋5-FU＋LV)＋/－CRT	147	41.3	21	20.1	0.009
				手術＋/－CRT	142		8	15.5	
Kosuge (JSAP-01)[4]	日本	2006	1992～2000	手術＋5-FU＋Cis	45	9.9	26.4	12.5	0.94
				手術単独	44		14.9	15.8	
Regine (RTOG)[7]	米国	2008	1998～2002	GEM→5-FU＋RT→GEM	221	110	NA	18.8	0.15
				5-FU→5-FU＋RT→5-FU	221			16.9	
Oettle (CONKO-001)[8]	ドイツ	2007	1998～2004	手術＋GEM	179	50.6	22.5	22.1	0.06
				手術単独	175		11.5	20.2	
Ueno (JSAP-02)[9]	日本	2009	2002～2005	手術＋GEM	58	40.0	NA	22.3	0.19
				手術単独	60			18.4	
Neoptolemos (ESPAC-3)[10]	欧州	2009	2000～2007	手術＋5-FU	551	155	NA	23.0	0.56
				手術＋GEM	537			23.6	

RT：放射線療法，CRT：化学放射線療法，NA：not available，LV：leucovorin，GEM：gemcitabine，Cis：cisplatin，MST：全生存期間中央値

と化学放射線療法群(n＝60)が手術単独群(n＝54)を上回ったが，統計学的な有意差は認めなかった[2]．

その後，英国を中心に European Study Group for Pancreatic Cancer (ESPAC) による大規模な国際多施設共同 RCT (ESPAC-1) が行われた[3]．541 例の浸潤性膵管癌症例を 73 例の化学放射線治療群(20 Gy＋5-FU 500 mg/m^2×3 を 2 コース)，75 例の化学療法群(ロイコボリン＋5-FU 425 mg/m^{2}を 6 コース)，72 例の化学放射線治療＋化学療法群，69 例の手術単独群の 4 群に割り付けた．5-FU による化学療法群が非化学療法群に比較して有意に予後良好(5 年生存率 21% vs. 8%，p＝0.009)である一方，化学放射線療法群は非化学放射線療法群よりも予後不良(5 年生存率 10% vs. 20%，p＝0.05)であった．そこで，ESPAC-1 では膵癌術後の補助療法として，化学療法は有効だが，化学放射線療法はむしろ弊害があると結論づけた．EORTC による試験と ESPAC-1 の結果から，欧州は，米国の支持する化学放射線療法の有用性を否定し，化学療法を補助療法の主軸に位置づけた．

日本では 5-FU とシスプラチンによる化学療法群と手術単独群を比較した RCT が行われたが，生存率や再発率に有意な差は認めなかった[4]．

さらに，ESPAC-1 までに報告された RCT

の結果に基づいてメタアナリシスが行われ，5-FUを中心とする化学療法は死亡のリスクを25%低下させるが，化学放射線療法には同様の効果はないこと，切除断端陽性例には化学放射線療法が有用かもしれないことなどが報告された[5]．

Ⅱ．ゲムシタビン(GEM)の登場

この項のポイント
- 21世紀には，従来の5-FUに代わってGEMが膵癌の化学療法および補助化学療法の標準的治療薬となった．

1997年に北米のグループが行った切除不能進行膵癌に対する第Ⅲ相試験においてGEM投与群は，5-FU投与群よりも症状緩和効果，生存期間とも有意に優れていたことが示された(5.7カ月 vs. 4.4カ月，p＝0.0025)[6]．以降，GEMが5-FUに代わって進行膵癌に対する標準的な治療薬となり，わが国でも2001年4月に保険適応が認められた．

米国ではGITSGの結果から，5-FUと放射線治療を中心に補助療法を組み立てることにこだわった．最近，Radiation Therapy Oncology Group(RTOG)が442症例の切除可能な膵癌症例を解析対象とした大規模なRCTを行った．この試験は5-FUを含んだ化学放射線療法の前後に5-FUを投与した群とGEMを投与した群を比較したものである．全症例の解析では5-FU群とGEM群の生存期間に有意差を認めなかったが，膵頭部癌のみを対象としたサブセット解析ではGEM群が有意に予後良好であるとした(生存期間20.6カ月 vs. 16.9カ月，p＝0.033)[7]．ただし，これは当初の評価項目を変更して導かれたものであり，解釈が難しい．

一方，欧州はGEMの6カ月投与による補助化学療法群と手術単独群とを比較した．大規模な多施設RCT(CONKO-001)が行われ，GEM群では無再発生存期間が有意に改善したことが報告された(13.4カ月 vs. 6.9カ月，p<0.001)．論文発表時点では，全生存期間については有意差を認めなかったが(22.1カ月 vs. 20.2カ月，p＝0.06)[8]，その後2008年の米国臨床腫瘍学会(ASCO)においては，観察期間の延長により，生存期間にも有意差が確認されたと追加報告された．

同様の臨床試験が，本邦でもJSAP-02(小菅班)として行われた[9]．GEMの3カ月投与群と手術単独群が比較され，無再発生存期間は11.4カ月 vs. 5.0カ月でGEM群が有意に優れていた(p＝0.01)が，全生存期間には有意差を認めなかった(22.3カ月 vs. 18.4カ月，p＝0.19)．有害事象としてはGrade 3以上の好中球減少が70%に認められた．本治療成績はCONKO-001の成績と非常に類似しており，試験結果の再現性が認められたことにより，GEMは日欧における事実上の標準治療と位置づけられることになった．

さらに欧州では5-FUとGEMの有用性を比較するESPAC-3試験が行われた[10]．2000年から2007年において16カ国が参加し，1,088例を5-FU群(n＝551)とGEM群(n＝537)にランダム化した大規模な多施設共同の試験であったが，結果は生存期間の中央値が5-FU群23.0カ月に対してGEM群23.6カ月であり，生存期間には差を認めなかった．しかし，治療との因果関係が否定できない重篤な有害事象がGEM群で有意に低率であったことから(7.5% vs. 14%，p<0.001)，GEMのほうが術後補助化学療法として推奨できるとされた．

Ⅲ．多剤併用による膵癌治療を応用した補助療法

この項のポイント
- GEMに経口フッ化ピリミジン系代謝拮抗薬を併用した補助化学療法に関する臨床試験が本邦および欧州で現在予定中である．

1．切除不能膵癌に対するGEMとの併用療法の進歩

現時点で切除不能膵癌の治療においてGEMよりも優れていることが第Ⅲ相試験で証明された単剤化学療法は存在しない．しかし，2006年に膵癌に対して保険適応となったTS-1が注目されている．TS-1は胃癌の補助療法において有意な効果を認めており標準的補助治療薬となっている[11]．現在，GEM単独療法をコントロールアームとしてTS-1単独療法の非劣性，およびGEM＋TS-1併用療法の優越性を検証するための第Ⅲ相試験(GEST)が日本台湾共同で行われている．すでに800を超える症例の登録が完了し，2年の経過観察期間に移った．この試験の結果によっては膵癌の標準治療法が変わる可能性があり，結果が待たれるところである．

切除不能の膵癌に対してGEMとの併用により延命に上乗せ効果を示した薬剤は，フッ化ピリミジン系代謝拮抗薬であるカペシタビン(ゼローダ®)[12]と分子標的薬のエルロチニブ(タルセバ®)[13]のみである．しかし，カペシタビンについては上乗せ効果を認めなかったという報告がある．また，ほかの分子標的薬のベバシツマブ(アバスチン®)やセツキシマブ(アービタックス®)には上乗せ効果は認められなかった．その他，イリノテカンなどのトポイソメラーゼⅠ阻害薬やシスプラチンやオキサリプラチンなどの白金製剤においても上乗せ効果は証明されていない．

また，GEMとの併用療法ではないが，本年のASCOにおいて，フランスから遠隔転移を伴った膵癌症例に対するFOLFIRINOX(5-FU/ロイコボリン，イリノテカン，オキサリプラチンの3者併用療法)とGEMの第Ⅲ相試験の結果が発表され，FOLFIRINOXの毒性は非常に強いものの，高い奏効率(27.6% vs. 10.9%，$p<0.001$)と全生存期間の有意な延長が認められたと報告された(10.5カ月 vs. 6.9カ月，$p<0.001$)．毒性の問題があるため，今後どのように使われるようになるかが注目される．

2．GEMとの併用療法による補助療法の臨床試験

現在，膵癌の術後補助療法として，GEM vs. TS-1のランダム化比較試験が本邦において進行中であり(JASPAC-01)，症例の登録を終了している[14]．また，JSAP-04では補助療法としてのGEM vs. GEM＋TS-1のランダム化比較試験を予定している．また，ESPAC-4はGEMの単剤群とGEM＋カペシタビンの併用療法を比較する試験であり，一群で540例の登録を予定している．今後はこれらの試験の結果が待たれるところである．

おわりに

膵癌の根治切除後の予後は未だ不良であり，標準的な補助療法を確立し，普及させることは急務である．欧州と本邦ではGEMによる化学療法が事実上の標準的な補助療法である．本邦ではTS-1を併用あるいは単剤として使用した補助化学療法試験が進行中であり，近い将来に膵癌の治療には新しいエビデンスが得られる可能性がある．

文　献

1) Kalser, M. H. and Ellenberg, S. S. : Pancreatic cancer. Adjuvant combined radiation and chemotherapy following curative resection. Arch. Surg. 120 ; 899-903, 1985
2) Klinkenbijl, J. H., Jeelel, J., Sahmoud, T., et al. : Adjuvant radiotherapy and 5-fluorouracil after curative resection of cancer of the pancreas and periampullary region. Phase Ⅲ trial of the EORTC Gastrointestinal Tract Cancer Cooperative Group. Ann. Surg. 230 ; 776-784, 1999
3) Neoptolemos, J. P., Stocken, D. D., Friess, H. H., et al. : A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. N. Engl. J. Med. 350 ; 1200-1210, 2004
4) Kosuge, T., Kikuchi, T., Mukai, K., et al. : A multicenter randomized controlled trial to evaluate the effect of adjuvant cisplatin and 5-fluorouracil therapy after curative resection in cases of pancreatic cancer. Jpn. J. Clin. Oncol. 36 ; 159-165, 2006
5) Stochen, D. D., Büchler, M., Dervenis, C., et al. : Meta-analysis of randomised adjuvant therapy trials of pancreatic cancer. Br. J. Cancer. 25 ; 1372-1381, 2005
6) Burris, H. A. 3rd, Moore, M. J., Andersen, J., et al. : Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer : a randomized trial. J. Clin. Oncol. 15 ; 2403-2413, 1997
7) Regine, W. F., Winter, K. A., Abrams, R. A., et al. : Fluorouracil vs gemcitabine chemotherapy before and after fluorouracil-based chemoradiation following resection of pancreatic adenocarcinoma : a randomized controlled trial. JAMA 5 ; 1019-1026, 2008
8) Oettle, H., Post, S., Neuhaus, P., et al. : Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer. A randomized controlled trial. JAMA 297 ; 267-277, 2007
9) Ueno, H., Kosuge, T., Matsuyama, Y., et al. : A randomised phase Ⅲ trial comparing gemcitabine with surgery-only in patients with resected pancreatic cancer : Japanese Study Group of Adjuvant Therapy for Pancreatic Cancer. Br. J. Cancer 101 : 908-915, 2009
10) Neoptolemos, J., Büchler, M., Stocken, D. D., et al. : ESPAC-3(vs) : A multicenter, international, open-label, randomized, controlled phase Ⅲ trial of adjuvant 5-fluorouracil/folinic acid (5-FU/FA) versus gemcitabine (GEM) in patients with resected pancreatic ductal adenocarcinoma. J. Clin. Oncol. 27 ; 18s, 2009 (supple ; abstr LBA4505).
11) Sakuramoto, S., Sasako, M., Yamaguchi, T., et al. : Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. N. Engl. J. Med. 357 ; 1810-1820, 2007
12) Cunningham, D., Chau, I., Stocken, D. D., et al. : Phase Ⅲ randomized comparison of gemcitabine versus gemcitabine plus capecitabine in patients with advanced pancreatic cancer. J. Clin. Oncol. 27 ; 5513-5518, 2009
13) Moore, M. J., Goldstein, D., Hamm, J., et al. : Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer : a phase Ⅲ trial of the National Cancer Institute of Canada Clinical Trials Group. J. Clin. Oncol. 20 ; 1960-1966, 2007
14) Maeda, A., Boku, N., Fukutomi, A., et al. : Randomized phase Ⅲ trial of adjuvant chemotherapy with gemcitabine versus S-1 in patients with resected pancreatic cancer : Japan adjuvant study group of pancreatic cancer (JASPAC-01). Jpn. J. Clin. Oncol. 38 : 227-229, 2008

Summary

Clinical Evidence from Postoperative Adjuvant Therapy for Pancreatic Cancer

Yoshihiro Sakamoto*, Tomoo Kosuge*, Hideki Ueno**, Satoshi Nara*, Minoru Esaki* and Kazuaki Shimada*

Clinical evidence to support postoperative adjuvant therapy for pancreatic cancer has recently been established. In the USA, adjuvant chemoradiation therapy is the dominant form of treatment. In Europe and Japan, adjuvant chemotherapy is the primary treatment. In the CONKO-001 and JSAP-02 trials, disease-free survival rates were significantly better in the adjuvant gemcitabine (GEM)

group than in the surgery alone group and in the former trial, overall survival rates improved in the GEM group. In the ESPAC-3 trial (a randomized trial comparing adjuvant GEM to 5-FU groups) the survival rates of both groups were similar. However, adverse events were significantly lower in the GEM group than in the 5-FU group. Thus, adjuvant chemotherapy using GEM has been recognized as the standard therapy for pancreatic cancer. Clinical trials of adjuvant therapy are ongoing in Japan.

Key words: pancreatic cancer, postoperative adjuvant chemotherapy, randomized clinical trial, gemcitabine, TS-1

**Hepatobiliary and Pancreatic Surgery Division, **Hepatobiliary and Pancreatic Oncology Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*

特集

胆と膵 Vol. 31 (4) p. 387～393, 2010

膵・胆道癌における分子標的治療の動向

胆道癌に対するEGFR/ VEGFRを標的とした分子標的治療の可能性*

吉川大太郎[1,2]・尾島　英知[3]・小菅　智男[4]・河野　透[1]
古川　博之[1]・柴田　龍弘[2]

要約：胆道癌に対するEGFR/ VEGFRを標的とした分子標的治療の可能性について検討した。臨床病理学的検討により，胆道癌においてEGFRおよびVEGFの発現が比較的高頻度に認められ，両分子が胆道癌の進展に関わっていることが示唆された。胆道癌マウスモデルと新規EGFR/ VEGFR阻害剤であるvandetanibを用いた検討により，vandetanibが胆道癌に対して抗腫瘍効果を発揮することが示唆された。またKRAS遺伝子およびEGFR遺伝子の異常が，胆道癌に対するEGFR阻害剤の感受性予測マーカーとなる可能性が示唆された。胆道癌治療における化学療法の役割は大きいが，有効な化学療法は依然として少ないのが現状である。EGFRおよびVEGFR経路を標的とした分子標的治療は，一つの有望な治療手段となる可能性があると考えられた。胆道癌に対する分子標的治療の開発はいままさに黎明期であり，わが国でもその臨床的有効性が検討されていくことが期待される。

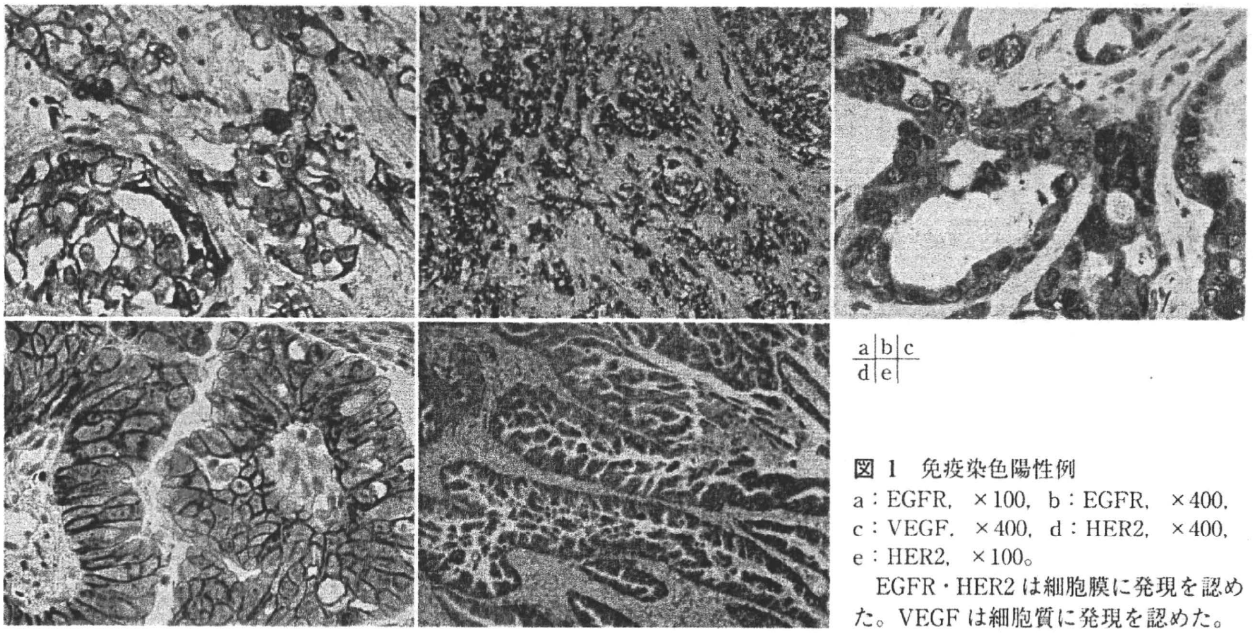
Key words：EGFR, VEGF (R), molecular-targeted therapy, cholangiocarcinoma

はじめに

胆道癌を治癒できる可能性のある唯一の治療法は外科的完全切除であるが，診断時に切除不能である症例も多く，切除されたとしても再発率が高い。したがって化学療法が重要になるが，有効な化学療法も十分確立されていない。今のところ日本で認可されている薬剤のうち，gemcitabine（以下GEM）・S-1がkey drugであると考えられるが，2009年には英国と日本から進行胆道癌に対するgemcitabine-cisplatin療法の有効性が報告され[1,2]，今後の標準化学療法となっていくと考えられる。日本は，世界的にみても胆道癌の罹患率が高い国の一つであり[3]，胆嚢・胆管癌だけでも年間約1万5千人が死亡している。まさに有効な「次の一手」を開発していくことが求められている。

近年，乳癌に対する抗HER2ヒト化モノクロナール抗体trastuzumab（Herceptin™）や，非小細胞肺癌（NSCLC）に対するEGFRチロシンキナーゼ阻害剤gefitinib（Iressa™），結腸癌に対する抗ヒトEGFRモノクロナール抗体cetuximab（Erbitax™）・抗VEGFヒト化モノクロナール抗体bevacizumab（Avastin™）など，分子標的治療薬が各種癌治療の臨床に適応され効果をあげている。分子標的治療薬は，文字通り標的となる分子の異常（発現や遺伝子増幅/変異など）が存在して初めて効果を示す薬剤であり，分子標的治療薬の導入に当たっては，標的分子の異常について十分検討する必要がある。特に胆道癌のような症例集積が困難な癌腫であればなおのこと，基礎的検討を十分行い標的を絞り込むことが重要である。われわれは胆道癌に対してEGFR（epidermal growth factor receptor）経路およびVEGFR（vascular endothelial growth fac-

* Potentiality of the Molecular-targeted Therapy for EGFR/ VEGFR against Biliary Tract Cancer
1）旭川医科大学外科学講座消化器病態外科学分野（〒078-8510 旭川市緑が丘東2条1-1-1）
2）国立がんセンター研究所ゲノム構造解析プロジェクト
3）同　病理部
4）国立がんセンター中央病院肝胆膵外科



a b c
d e

図 1 免疫染色陽性例
a：EGFR，×100，b：EGFR，×400，
c：VEGF，×400，d：HER2，×400，
e：HER2，×100。
EGFR・HER2 は細胞膜に発現を認めた。VEGF は細胞質に発現を認めた。

tor receptor）経路を標的とした治療を導入することを目標とし，臨床検体を用いた臨床病理学的検討と胆道癌マウスモデルを用いた VEGFR/ EGFR 阻害剤の効果について検討を行ってきた[4,5]。本稿ではその結果を紹介し，今後の展望について概説する。

Ⅰ．胆道癌における EGFR/ VEGF 発現の臨床病理学的意義

EGFR・HER2 等の ErbB family や VEGFR family といった receptor tyrosine kinase の活性化は，下流の細胞内シグナル伝達経路を活性化し癌の進展に関わっている。2000 年頃より，胆道癌において EGFR 発現[6~8]や EGFR 遺伝子変異[9,10]，VEGF（vascular endothelial growth factor）発現[11,12]が比較的高頻度に認められるという報告がなされてきたが，その臨床病理学的意義については不明であった。そこで国立がんセンター中央病院で切除された胆道癌 236 例（肝内胆管癌（IHCC）106 例，肝外胆管癌（EHCC）130 例）のホルマリン固定標本を用いて EGFR・VEGF・HER2 の免疫染色を施行し，臨床病理学的意義について統計学的解析を行った。

図 1 に陽性例の写真を示す。IHCC において，EGFR・VEGF・HER2 は各々 29 例（27.4%）・57 例（53.8%）・1 例（0.9%）に陽性となり，EHCC においてはおのおの 25 例（19.2%）・77 例（59.2%）・11 例（8.5%）に陽性となった。

注目すべきは，EGFR 陽性例のほとんどが腫瘍浸潤部の中～低分化の成分に陽性となる（52/ 54）のに対して，HER2 は高分化の成分に優先的に陽性となる点であった（11/ 12）。また臨床病理学的因子との相関をみても，HER2 発現は高分化型癌（P＝0.0078），非浸潤癌（P＝0.0242）に有意に発現していた。これらの結果は，HER2 発現が胆道癌の危険因子である肝内結石症や原発性硬化性胆管炎（PSC）の胆道上皮と高分化癌に認められ，胆道癌の carcinogenesis における early event であるという報告[13]と相違しない結果であった。また，HER2 は IHCC や EHCC に比べて胆嚢癌に有意に多く発現するという報告があるが[8]，胆嚢癌の切除例には早期癌が多く含まれる可能性もあると思われ，このことが症例の偏りによるものなのか，胆嚢癌が他と異なる性質をもつのか，今のところ不明であり検討の余地がある。

IHCC において VEGF 発現は肝内転移と相関を認めた（P＝0.0224）。EHCC において EGFR 発現は，肉眼型（乳頭型 0%，非乳頭型 24.0%，P＝0.0120）・リンパ節転移（P＝0.0006）・リンパ管浸潤（P＝0.0371）・神経周囲浸潤（P＝0.0459）といった腫瘍進展を表す因子と相関した。

予後因子解析では，IHCC において肉眼型（腫瘤形成型，HR 2.96，95% CI 1.52～4.69，P＝0.0006）・肝内転移（HR 2.91，95% CI 1.60～5.29，P＝0.0005）・リンパ節転移（HR 1.96，95% CI 1.04～3.69，P＝0.0133）といった因子とともに，EGFR 発現が独立した予後因子となった（HR 2.67，95% CI 1.52～4.69，P＝0.0006）。

以上の結果から，胆道癌において HER2 が carcinogenesis の early event として発現するのに対して，EGFR および VEGF は late event として浸潤・進展に