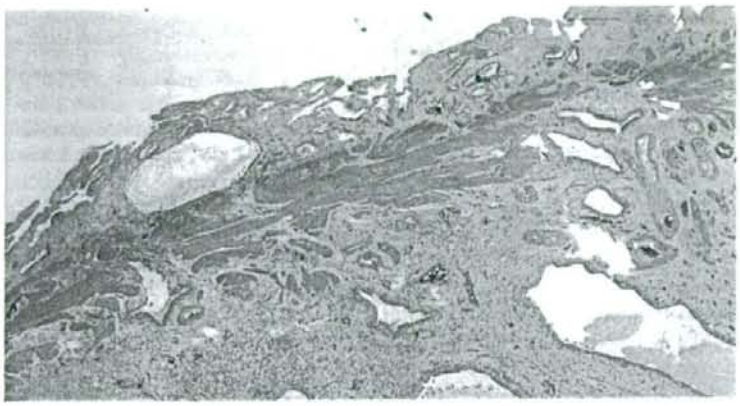

Fig. 4. This Loupe image shows growing and dilating Rokitansky-Aschoff sinuses from the muscularis propria to the subserosa, and the growing smooth muscle fiber and collagen fiber surrounding them (H&E)

resected specimens from the patients with carcinoma of the papilla of Vater and found tha the common channel was the most frequent site for the possible origin of carcinoma (level IV). These results suggest that the common channel is the most important site in the pathogenesis of carcinoma of the papilla of Vater. With regard to the incidence of "adenoma" surrounding carcinoma of the papilla of Vater, the values have been reported to range from 82% to 91%[27] (level IV). Therefore, the adenoma-carcinoma sequence is very important in the pathogenesis of carcinoma of the papilla of Vater. In addition, familial adenomatous polyposis (FAP) is notable for the risk of adenoma in the ampulla of Vater.

Side memo 1
Primary sclerosing cholangitis (PSC)

Definition: recurrent or persistent chronic inflammatory disease of extra- and intrahepatic bile ducts resulting in obliterative fibrosis. No effective treatment has been discovered to date, and liver transplant is required in the terminal stage. Although autoimmune abnormality is suspected, the underlying pathogenesis is still unknown.

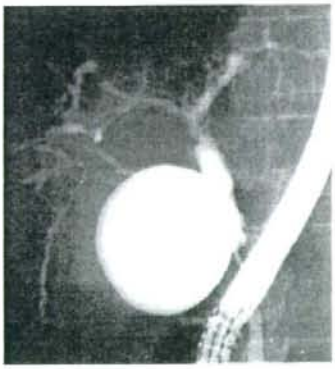

Fig. 5. Endoscopic retrograde cholangiopancreatography image of PSC. Multifocal stricturing of intrahepatic and extrahepatic bile ducts, and shaggy appearance of extrahepatic bile ducts

Diagnostic criteria

1. Typical radiological findings
   Typical radiological findings of bile duct are;
   Multifocal stricturing
   — Beaded appearance; short or/and annular strictures interspersed among normal or slightly inflamed ducts. This appearance is thought to reflect the fibrous stricture portions and the normal segments of the bile duct. One-fifth of the cases present band-like strictures that are extremely short, and one-fourth present diverticulum-like outpouchings
   — Pruned-tree appearance; diminished arborization of the intrahepatic ducts
   — Shaggy appearance; diffuse mural irregularities of extrahepatic bile ducts
2. Typical clinical presentation
   Important clinical history: inflammatory bowel disease, bile obstruction
   Blood examination: high alkaline phosphatase (ALP; two or three times upper limit of normal) for 6 months or more
3. Deductive diagnosis
   Exclude secondary sclerotic cholangitis as follows:
   — Infectious cholangitis due to AIDS
   — Malignant neoplasms or similar disease in the bile duct (excluding PSC accompanied by early-stage cholangiocarcinoma)
   — Previous surgery of the biliary tract (excluding cholecystectomy)
   — Bacterial cholangitis accompanying biliary tract stricturing or biliary calculus
   — Ischemic cholangitis due to floxuridine

Side memo 2
Diagnostic criteria of pancreaticobiliary maljunction (PBM; see Fig 6)

Definition: PBM is a congenital anomaly consisting of a union of the pancreatic and bile ducts located outside the duodenal wall.

Diagnostic criteria: PBM is diagnosed by either radiological or anatomical findings

1) Radiological findings
It is necessary to confirm the lack of sphincter action at the union of the pancreatic and bile ducts. However, because clarification of the lack of sphincter action is often difficult, the following radiological findings of endoscopic retrograde cholangiopancreatography (ERCP), percutaneous transhepatic cholangiography (PTHC), intraoperative cholangiography, or similar methods are used to verify whether there is a long common channel or a complicated confluence of the pancreatic and bile ducts.
2) Anatomical findings
Confirm the abnormal anatomical confluence of the pancreatic and bile ducts outside the duodenal wall or confirm the complicated confluence by surgery, autopsy, or other procedures.

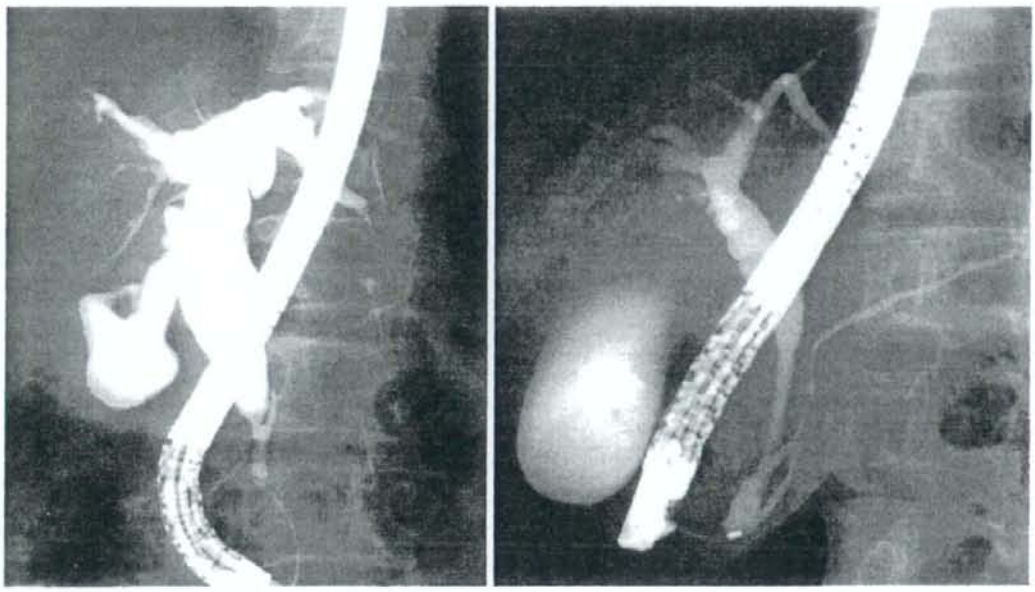

Fig. 6a,b. Endoscopic retrograde cholangiopancreatography (ERCP) image of pancreaticobiliary maljunction (PBM): a patient with congenital bile duct dilatation (Kotani IV a type). **a** The pancreatic duct joins the biliary duct. **b** Pancreaticobiliary maljunction without bile duct dilatation (Fig 6a, with permission from Koyanagi K, Aoki T, editors. *Pancreaticobiliary maljunction*. Tokyo: Igaku Tosho Shuppan: 2002. p 25, Fig. 4[29])

CQ 2 Is prophylactic treatment necessary for pancreaticobiliary maljunction (PBM)?

PBM with bile duct dilatation is a risk factor for bile duct and gallbladder cancer. Prophylactic excision of the gallbladder and common bile duct should be recommended for PBM with bile duct dilatation (recommendation grade C1)

PBM without bile duct dilatation is a risk factor for gallbladder cancer. A prophylactic cholecystectomy is the appropriate surgical procedure for PBM without bile duct dilatation. (recommendation grade B)

As mentioned above, a retrospective nationwide survey (1990 to 1999) of PBM revealed that the incidence of biliary tract cancer was 10.6% in the group with bile duct dilatation; of these biliary tract cancers, 64.9% were gallbladder carcinoma and 33.6% were bile duct cancer.[3] Therefore, PBM with bile duct dilatation should be considered as a risk factor for bile duct cancer and gallbladder cancer, and in these patients, prophylac-

tic excision of the gallbladder and extrahepatic bile duct should be considered. Currently, standard prophylactic treatment of PBM with bile duct dilatation consists of cholecystectomy, extrahepatic biliary duct resection, and hepatocholangiojejunostomy.[28]

In the PBM patients without bile duct dilatation, the incidence of biliary tract cancer was 37.9%, and 93.2% of these patients had gallbladder cancer[3] (level IV). These reports suggest that PBM without bile duct dilatation should be considered as a risk factor for gallbladder cancer. Therefore, in these patients, excision of the gallbladder should be considered as a minimal prophylactic treatment. Controversy remains as to whether prophylactic bile duct resection is necessary in PBM patients without bile duct dilatation. It has been reported that the long-term results of a prophylactic cholecystectomy without bile duct resection for PBM patients without bile duct dilatation were acceptable[29,30] (level IV). Also, it has been reported that *K-ras* gene mutation is frequent in the mucosa of the bile duct in PBM patients with bile duct dilatation, while no mutation is observed in PBM patients without bile duct dilatation[31] (level IV). These reports support a prophylactic cholecystectomy without bile duct resection as being the best treatment option for PBM patients without bile duct dilatation. In contrast, it has been reported that the incidence of bile duct cancer in PBM patients without bile duct dilatation is lower than that in PBM patients with bile duct dilatation, but the incidence seems higher than that in healthy controls[3,32] (level IV). Moreover, it has been reported that *K-ras* and *p53* gene mutations are found frequently even in the nondilated bile duct in patients with PBM[33] (level IV). These reports support the possibility that complete excision of the common bile duct may be needed to decrease the risk of bile duct cancer.

CQ 3 Is a cholecystectomy required for asymptomatic cholecystolithiasis?

A cholecystectomy is not advised for asymptomatic cholecystolithiasis (recommendation grade C2)

It has been well established that gallstones are associated with gallbladder cancer[1,8] (level V). Not only is there an increased prevalence of gallbladder cancer in patients with gallstones but up to 75% of patients with gallbladder cancer have been noted to have had gallstones.[1] It has also been reported that a stone size of more than 3 cm, a family history of gallbladder cancer, and the duration of cholelithiasis are potential risk factors for the development of gallbladder cancer.[1,14-16] However, there is no evidence of a direct causal relationship between gallstones and gallbladder cancer. Maringhini et al.[34] followed-up, for more than 31 000 person-years a total of 2583 residents of Rochester, Minnesota, who had gallstones. Gallbladder cancer developed in 5 patients after a median follow-up of 13.3 years. The incidence of gallbladder cancer was significantly higher than expected for men but not for women (153 vs 18 per 100 000 person-years). However, these authors do not recommend cholecystectomy, because the absolute incidence and the total numbers of men and women who developed gallbladder cancer were low. Moreover, Gracie and Ransohoff[17] followed up the subsequent history of 123 patients with asymptomatic gallstones for 10 years or longer, and revealed that there was no case of gallbladder cancer reported among that group. This low rate of cancer development in asymptomatic patients with gallstones is similar to that noted in other reports (0.01%/year-0.02%/year)[1,35] and, in general, this low rate has lent support to the practice of not performing prophylactic cholecystectomy in patients with asymptomatic cholelithiasis.

Controversy remains as to whether patients with calcification of the wall of the gallbladder, referred to as "porcelain gallbladder" carry a risk of gallbladder cancer. It has been reported that porcelain gallbladder is often complicated by gallbladder carcinoma[18,19] (level IV). However, Towfigh et al.[20] have reported that porcelain gallbladder is not associated with gallbladder carcinoma (level IV). The pathogenic mechanism underlying the relationship of calcification to gallbladder cancer also remains unknown.

CQ 4 Is a cholecystectomy necessary for polypoid lesions of the gallbladder (see side memo 3)

When a polypoid lesion of the gallbladder is sessile, has a diameter greater than 10 mm, and/or grows rapidly, it is highly likely to be cancerous and should be resected (recommendation grade B)

Currently, adenoma and dysplasia have been regarded as a precancerous lesions of the gallbladder.[21] Intestinal and gastric metaplasia seems to be the pathway through which the epithelial dysplasia is produced.[21,22] Yamagiwa[22] examined 110 cases of resected gallbladder carcinoma and revealed that dysplasia adjacent to carcinoma was found in 46 of the 110 cases, and this change was frequently found in lesions at the early stage and in well-differentiated carcinoma (level V).

Kubota et al.[23] evaluated 72 patients who underwent cholecystectomy for polypoid lesions of the gallbladder. In that study, histological examination showed cholesterol polyps in 47 patients, adenoma in 8, cancers in 16,

and an inflammatory polyp in 1. The respective diameters of the adenomas and cancers were 6.9 mm (range, 4 to 13 mm) and 25.7 mm (range, 5 to 50 mm); 75% of the adenomas and 13% of the cancers had a diameter of less than 10 mm (level IV). Chijiiwa and Tanaka[36] also examined 44 patients who underwent cholecystectomy for polypoid lesions of the gallbladder, and reported that the sex ratio, symptoms, and the presence of gallstones were not significantly different between patients with carcinoma and those with benign polypoid lesions, and that the size (>10 mm), number of polypoid lesions (single), and age (> or =60 years) were significant indicators of carcinoma.

It has been reported that when a polypoid lesion of the gallbladder is sessile, has a diameter greater than 10 mm, and/or grows rapidly, it is highly likely to be cancerous[23–25,36–39] (level IV). In such cases, surgical resection should be considered.

Side memo 3
Polypoid lesions of the gallbladder (see Figs. 7–10)

Definition: "Polypoid" is the general term for torous lesions protruding into the lumen of the gallbladder, no matter whether they are neoplastic or nonneoplastic. The majority of polypoid lesions are nonneoplastic lesions such as cholesterol polyp, adenomyomatosis, or inflammatory polyp. Benign neoplastic lesions include adenoma and metaplastic polyp, while malignant neoplastic lesions include gallbladder cancer

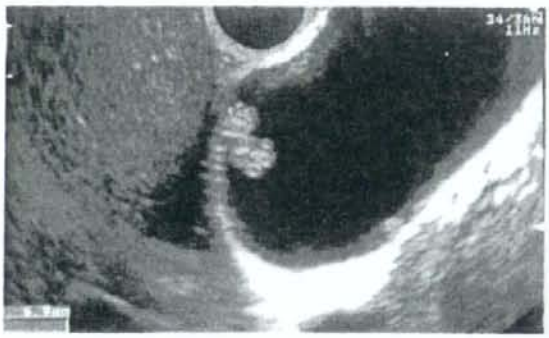

Fig. 7. Ultrasound image of a cholesterol polyp: sessile polyp that has a higher echoic signal than the liver

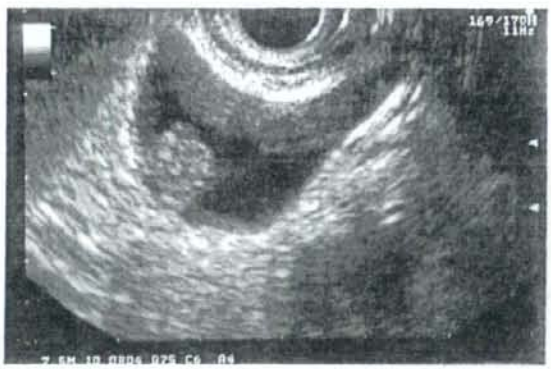

Fig. 8. Ultrasound image of adenoma of the gallbladder: this image indicates a semipedunculate polyp with isoechoic signal

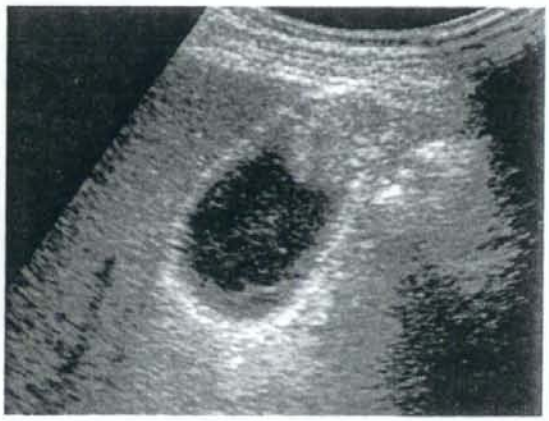

Fig. 9. Ultrasound image of early-stage gallbladder carcinoma: a pedunculated protrusion with a relatively high echoic signal. Depth of tumor invasion is limited to the mucosal layer

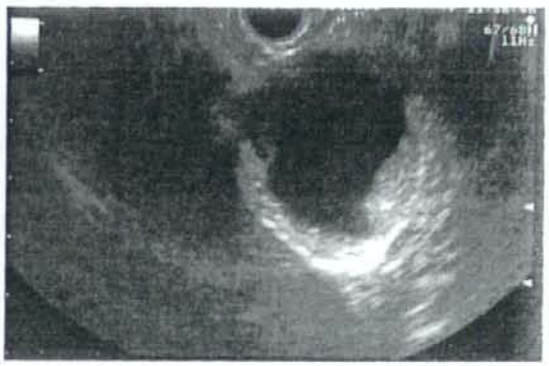

Fig. 10. Ultrasound image of gallbladder carcinoma: sessile protruding lesion. Depth of tumor invasion is to the subserosa

Acknowledgment. We would like to express our deep gratitude to the members of the the Japanese Association of Biliary Surgery, the Japanese Society of Hepato-Biliary-Pancreatic Surgery, and the Japan Society of Clinical Oncology, who provided us with great support and guidance in the preparation of the Guidelines. This process was conducted as part of the Integrated Research Project for Assessing Medical Technology 2005 and 2006 sponsored by the Japanese Ministry of Health, Labour, and Welfare.

We truly appreciate the following active working members who developed the draft of the evidence-based clinical practice Guidelines for the treatment of biliary tract cancer (Japanese version, 2007): Masahiro Kai (Miyazaki), Yasutoshi Kimura (Sapporo), Shigeaki Sawada (Toyama), Hiroaki Shimizu (Chiba), Hisatoshi Nakagawara (Kanazawa), Kohei Nakachi (Kashiwa), and Hiroyuki Yoshitome (Chiba). We also appreciate very much the following members who reviewed and approved the final Japanese version of the guidelines: Hiromitsu Saisyo (Ichikawa), Munemasa Ryu (Chiba), Satoru Shikata (Kyoto), and Yuji Nimura (Nagoya).

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British Journal of Cancer (2008) 99, 305–313

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FGF10/FGFR2 signal induces cell migration and invasion in pancreatic cancer

S Nomura[1], H Yoshitomi*[,1], S Takano[1], T Shida[1], S Kobayashi[1], M Ohtsuka[1], F Kimura[1], H Shimizu[1], H Yoshidome[1], A Kato[1] and M Miyazaki[1]

[1]*The Department of General Surgery, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba city, Chiba 260-8670, Japan*

Pancreatic cancer has one of the highest mortalities among all malignancies and there is an urgent need for new therapy. This might be achieved by resolving the detailed biological mechanism, and in this study we examined how pancreatic cancer cells develop aggressive properties by focusing on signalling through the fibroblast growth factor (FGF)10 and FGF receptor (FGFR)2, which play important roles in pancreatic organogenesis. Immunostaining of pancreatic cancer tissues showed that FGFR2 was expressed in cancer cells, whereas FGF10 was expressed in stromal cells surrounding the cancer cells. Patients with high FGFR2 expression in cancer cells had a shorter survival time compared to those with low FGFR2 expression. Fibroblast growth factor 10 induced cell migration and invasion of CFPAC-1 and AsPC-1 pancreatic cancer cells through interaction with FGFR2-IIIb, a specific isoform of FGFR2. Fibroblast growth factor 10 also induced expression of mRNA for membrane type 1-matrix metalloproteinase (MT1-MMP) and transforming growth factor (TGF)-β1, and increased secretion of TGF-β1 protein from these cell lines. These data indicate that stromal FGF10 induces migration and invasion in pancreatic cancer cells through interaction with FGFR2, resulting in a poor prognosis. This suggests that FGF10/FGFR2 signalling is a promising target for new molecular therapy against pancreatic cancer.
British Journal of Cancer (2008) **99,** 305–313. doi:10.1038/sj.bjc.6604473 www.bjcancer.com
Published online 1 July 2008

Keywords: pancreatic cancer; fibroblast growth factor 10; fibroblast growth factor receptor 2; cancer stromal cell

Pancreatic cancer has one of the highest mortalities among all malignancies, and is the fourth most common cause of cancer death in the United States and the fifth in Japan (Li *et al*, 2004; Willett *et al*, 2005). Although significant advances are now being made into the management of the disease, the 5-year survival rate still remains poor (Willett *et al*, 2005; Ghaneh *et al*, 2007). Therefore, there is an urgent need for new therapies for pancreatic cancer, based on an improved understanding of the molecular biology of the disease. The high mortality rate of pancreatic cancer is, in part, owing to difficulties of early diagnosis, the high incidence of metastatic disease at the time of diagnosis, and rapid progression of the disease. In addition, although newer adjuvant modalities are greatly increasing the prognosis (Ghaneh *et al*, 2008), most patients who undergo the surgery eventually relapse and die of the disease, even with curative resection (Li *et al*, 2004; Willett *et al*, 2005).

An understanding of the mechanisms underlying the biological aggressiveness of pancreatic cancer may be key for development of new therapies. Therefore, in this study we examined the molecular mechanisms underlying cellular invasion and metastasis of pancreatic cancer cells. Cellular and genetic studies have shown that tumour growth is not determined by malignant cancer cells alone, but also by cells in the tumour stroma. Supply of oxygen and nutrients by endothelial cells of blood vessels are critical for maintenance of the tumour microenvironment, and stromal fibroblasts are the principal source of extracellular matrix, which serves as a scaffold for cancer cells (Kalluri and Zeisberg, 2006). In addition, recent studies have revealed more active roles of stromal cells in tumour initiation and progression through direct interaction with tumour cells. For example, stromal cell-derived factor-1 (SDF-1/CXCL12) released from fibroblasts promotes cancer cell proliferation through a specific receptor, CXCR4, in several types of malignancies, including breast cancer (Orimo *et al*, 2005) and pancreatic cancer (Koshiba *et al*, 2000; Marchesi *et al*, 2004). Immune cells also play important roles in cancer progression (Pollard, 2004); for example, tumour-associated macrophages induced by colony-stimulating factor 1 promote invasiveness of cancer cells (Lin *et al*, 2001). Given this background, we hypothesized that stromal cell–cancer cell interactions have an important role in acquisition of the aggressive character by pancreatic cancer, and we examined signalling molecules that may be associated with this mechanism.

The molecular mechanisms underlying carcinogenesis are often similar to those in organogenesis. Interactions between stromal and parenchymal cells are important during organ development, and signals from stromal cells regulate epithelial cell growth and differentiation in pancreatic development. The classical tissue recombinant study by Golosow and Grostein (1962) showed that growth and morphogenesis of the developing pancreas depend on mesenchymal interactions, and more recently advances in molecular biology have allowed the molecular basis of this interaction to be established. We have shown that signals from endothelial cells and mesenchymal cells surrounding the

*Correspondence: Dr H Yoshitomi; E-mail: yoshitomi@faculty.chiba-u.jp
Revised 7 May 2008; accepted 20 May 2008; published online 1 July 2008

pancreatic bud are crucial for initiation of pancreatic development from endoderm (Yoshitomi and Zaret, 2003; Jacquemin *et al*, 2006). Especially, we found that fibroblast growth factor-10 (FGF10) from mesenchymal cells maintained expression of Ptf1a, a critical transcription factor for initiation of pancreatic development, in pancreatic progenitor cells (Jacquemin *et al*, 2006). Mice deficient in FGF10 or the FGF receptor-2 (FGFR2)/IIIb isoform, the specific receptor for FGF10 (Igarashi *et al*, 1998), show impaired pancreatic development (Bhushan *et al*, 2001; Pulkkinen *et al*, 2003). However, it is unknown if FGF10/FGFR2-IIIb-signalling is associated with carcinogenesis in pancreatic cancer. In this study, we show that FGF10/FGFR2-signalling has an important role in pancreatic cancer progression, and we suggest that these results may lead to a new therapy and a better prognosis for patients with pancreatic cancer.

MATERIALS AND METHODS

Patients and tissue samples

Pancreatic cancer tissues were obtained from 76 pancreatic cancer patients who underwent curative surgical resection in the Department of General Surgery, Chiba University Hospital, Chiba, Japan, from June 2001 to April 2006. All patients were diagnosed histologically as primary invasive pancreatic ductal carcinoma. The patient characteristics are summarised in Table 1. The study protocol was approved by the Ethics Committee of our institute and written informed consent was obtained from all patients.

Immunohistochemistry

Paraffin-embedded tissues were cut into 4 μm serial sections and deparaffinised. The sections were placed in citrate buffer (10 mmoll^{-1} pH 6.0) with 0.2% Tween 20 and boiled in a microwave oven (two times × 6 min) to retrieve the antigen. They were then rinsed and blocked in 10% H_2O_2 solution with methanol for 10 min. Next, the sections were incubated with goat anti-human FGF10 polyclonal antibody (R&D Systems, Minneapolis, MN, USA) at 1 : 20 dilution, mouse anti-human FGFR2 monoclonal antibody (R&D Systems) at 1 : 10 dilution, or rabbit anti-human CD3 monoclonal antibody (ready-to-use without dilution) (Thermo Fisher Scientific Anatomical Pathology, Fremont, CA, USA) overnight at 4°C. They were then rinsed in PBS and incubated for 60 min with a secondary antibody labelled with streptoavidin–biotin–peroxidase for goat polyclonal antibody (DAKO LSAB + ™ System, DAKO, Glostrup, Denmark), or dextran polymer-peroxidase for mouse monoclonal antibody (DAKO EnVision™ System, DAKO). For detection of anti-CD3 antibody, CSA II biotin-free catalysed amplification system with rabbit link (DAKO) was used. The bound complex was visualised using diaminobenzidine liquid chromogen (Dako) and counterstained with haematoxylin. Goat polyclonal IgG (Santa Cruz Biotechnology. Santa Cruz, CA, USA) at an optimal dilution was used as a negative control.

Cell lines and culture conditions

AsPC-1 cells were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) and MIA PaCa-2, PANC-1 and CFPAC-1 cells

Table 1 Characteristics of pancreatic cancer patients in IHC analysis

		FGFR2			FGF10		
	Total	Low	High	*P*-value	Low	High	*P*-value
	76	37	39		34	42	
Sex				NS			NS
M	44	18	26		19	25	
F	32	19	13		15	17	
Age (years)				NS			NS
Mean	65.0	65.0	64.5		65.0	64.5	
± s.d.	± 9.2	± 9.3	± 9.4		± 9.3	± 8.8	
Stage				NS			NS
IA	2	2	0		2	0	
IB	2	2	0		2	0	
IIA	15	9	6		4	11	
IIB	53	22	31		24	29	
III	4	2	2		2	2	
Histology				NS			NS
Tubular adenocarcinoma							
Well.	7	3	4		4	3	
Mod.	47	26	21		19	28	
Poor.	13	5	8		9	4	
Invasive carcinoma derived from intraductal tumour	4	2	2		0	4	
Anaplastic carcinoma	3	1	2		2	1	
Adenosquamous carcinoma	2	0	2		0	2	
Resection status							
Negative	51	26	25	NS	23	29	NS
Positive	25	11	14		12	13	
Adjuvant chemotherapy							
–	21	9	12	NS	9	12	NS
+	55	28	27		25	30	

Mod = moderately differentiated; NS = no significant; Poor = poorly differentiated; s.d. = standard deviation; Well = well differentiated. Patient stage was determined according to UICC TNM classification.

were cultured with DMEM medium (Sigma-Aldrich, St Louis, MO, USA). All cell lines were incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Each medium contained 10% fetal bovine serum (Invitrogen), 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin sulphate (Sigma-Aldrich).

For some experiments, cells were seeded (1×10^6 cells in 2 ml medium per well) in 6-well plates, and cultured for 24 h. Then cells were cultured with serum-free medium for another 24 h and changed to new serum-free medium with recombinant human FGF10 (R&D systems) or/and FGFR2-IIIb/IgG Fc Chimera (R&D systems) for indicated time in each experiments. Cells and medium were harvested for further experiments.

Transforming growth factor β 1 (TGF-β1) concentration was measured using Quantikine Human TGF-β1 immunoassay kit (R&D systems).

Reverse transcription–PCR

Total RNA was extracted from cultured cells, pancreatic cancer tissues and adjacent normal tissues with an RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions. cDNA was synthesised from 1 μg of total RNA with SuperScript™ III First-Strand Synthesis SuperMix for reverse transcription (RT)–PCR (Invitrogen). Polymerase chain reaction was performed with the following primer sets: FGF10, forward 5-ACATTGTGCCTCAGCCTTTC-3, reverse 5-CCCCTTCTTGTT CATGGCTA-3; FGFR2-IIIb, forward 5-TATATAGGGCAGGCAAC CA-3, reverse 5-GCTGAAGTCTGGCTTCTTGG-3; FGFR2-IIIc, forward 5-GTGCTTGGCGGGTAATTCTA-3, reverse 5-GCTGAAGTCT GGCTTCTTGG-3; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5-GTCAGCCGCATCTTCTTTTG-3, reverse 5-TTCACACCCATGACGAACAT-3. The RT–PCR conditions for FGF10, FGFR2-IIIb, FGFR2-IIIc and GAPDH were as follows: 94°C for 2 min, 40 cycles at 94°C for 15 s, 58°C for 30 s, and 72°C for 30 s, with an extension step of 7 min at 72°C at the end of the last cycle.

Quantitative RT–PCR

Quantitative RT–PCR was performed as described previously (Mitsuhashi *et al*, 2003). Primers for 18 genes related to cancer invasion and motility have been described by Ide *et al* (2006). The mRNA levels of these genes (E-Cadherin, N-Cadherin, Snail, MMP-1, MMP-2, MMP-7, MMP-9, MT1-MMP, TIMP-2, uPA, TGF-β1, HGF, c-Met, RhoA, CD44, Integrin-α4, Integrin-β4, and VEGF-A) were determined as the absolute number of copies normalised against the GAPDH mRNA copy number (Mitsuhashi *et al*, 2003). These experiments were performed three times independently.

Cell migration assay

A migration assay was performed in 12-well plates using a Quantitative Cell Migration™ Assay Kit (Chemicon International, Temecula, CA, USA) with an 8.0 μm pore size collagen-coated chamber membrane. The cells were seeded (1×10^5 cells in 0.3 ml of serum-free medium) in the upper chamber and cultured for 24 h for attachment. The medium was then replaced by fresh serum-free medium for another 24 h, before addition of recombinant human FGF10 (100 ng ml^{-1}) (R&D Systems) to the lower chamber. In some experiments, recombinant human FGFR2-IIIb/IgG Fc Chimera (500 ng ml^{-1}) (R&D Systems) was also added to the lower chamber. The cells were incubated for 12 h and the number of cells that passed through the membrane was counted according to the manufacturer's instructions. All experiments were performed in triplicate and independently at least three times.

Cell invasion assay

An invasion assay was performed in 24-well plates using a BD Biocoat™ Matrigel™ Invasion Chamber (BD Biosciences, Bedford, MA, USA) with an 8.0 μm. pore size polyethylene terephthalate (PET) membrane coated with Matrigel. The inserts were rehydrated by adding 0.5 ml of warm culture medium at 37°C for 2 h. The cells were seeded (5×10^5 cells in 0.5 ml of serum-free medium) in the upper chamber and cultured as described in the method for the migration assay. The number of seeded cells, culture conditions and other items were also similar to those for the migration assay. The cells were incubated for 24 h and the number of cells that passed through the membrane was counted according to the manufacturer's instructions. All experiments were performed in triplicate and independently at least three times.

Statistical analysis

Values are expressed as means ± s.d. The distribution of categorical data for FGFR2 immunostaining in tissue samples and for clinicopathological characteristics were assessed by a χ^2 test and a Fisher's exact test. Survival time was calculated using the Kaplan–Meier method and compared by log-rank test. Cell migration and cell invasion data were analysed using a Student's *t*-test and a Mann–Whitney *U*-test. Statistical significance was assumed for $P < 0.05$.

RESULTS

Expression of FGFR2 in pancreatic cancer cells and FGF10 in cancer stromal cells

To examine the expression pattern of FGFR2 and FGF10 in pancreatic cancer tissues, we performed immunohistochemical staining of 76 tissue samples of invasive pancreatic ductal carcinoma and of normal pancreatic tissues. FGF receptor-2 immunoreactivity was weak to moderate in pancreatic ductal cells in normal tissues (Figure 1A; arrow) and acinar cells (Figure 1A; arrowhead), as well as in islet cells (data not shown), as described previously (Ishiwata *et al*, 2002). On the other hand, immunostaining of FGF10 did not occur in normal pancreatic tissue, as also described previously (Ishiwata *et al*, 2002) (Figure 1B).

In pancreatic cancer tissues, cancer cells expressed FGFR2 at various levels (Figure 1C), but did not express FGF10 (Figure 1D). Pancreatic cancer tissue often contains a few islet cells (Figure 1E and 1F; arrowheads) and we compared the expression levels of FGFR2 in cancer cells and islets. In 39 cases (51.3%), FGFR2 immunoreactivity in cancer cells was stronger than in islets (high expression group, Figure 1E). In the other 37 cases (48.7%), FGFR2 immunoreactivity was not found or was faint in cancer cells, and was weaker than in normal islet cells (low expression group, Figure 1F). There was no correlation between FGFR2 immunoreactivity levels and tumour histological findings (Table 1).

Pancreatic cancer cells did not express FGF10 in any samples (Figure 1D), but scattered cells in stroma surrounding the cancer cells showed strong expression of FGF10 (Figure 1D; arrows). Fibroblast growth factor 10-positive stromal cells in cancer tissues were found in 42 cases (55.3%), and interestingly, were mainly localised close to cancer cells (Figure 1D and 1E). Moreover, of the 42 cancer tissue samples with FGF10-positive stromal cells, 29 (69.0%) showed high FGFR2 expression in cancer cells, and there was a significant correlation between the presence of FGF10-positive stromal cells and high FGFR2 expression in cancer cells ($P = 0.013$).

Next we examined which kind of cancer stromal cells expressed FGF10. We stained sequential sections with antibody against FGF10 and stromal cell markers (CD68; macrophage marker, α-smooth muscle actin; activated fibroblast marker, CD3; T-cell

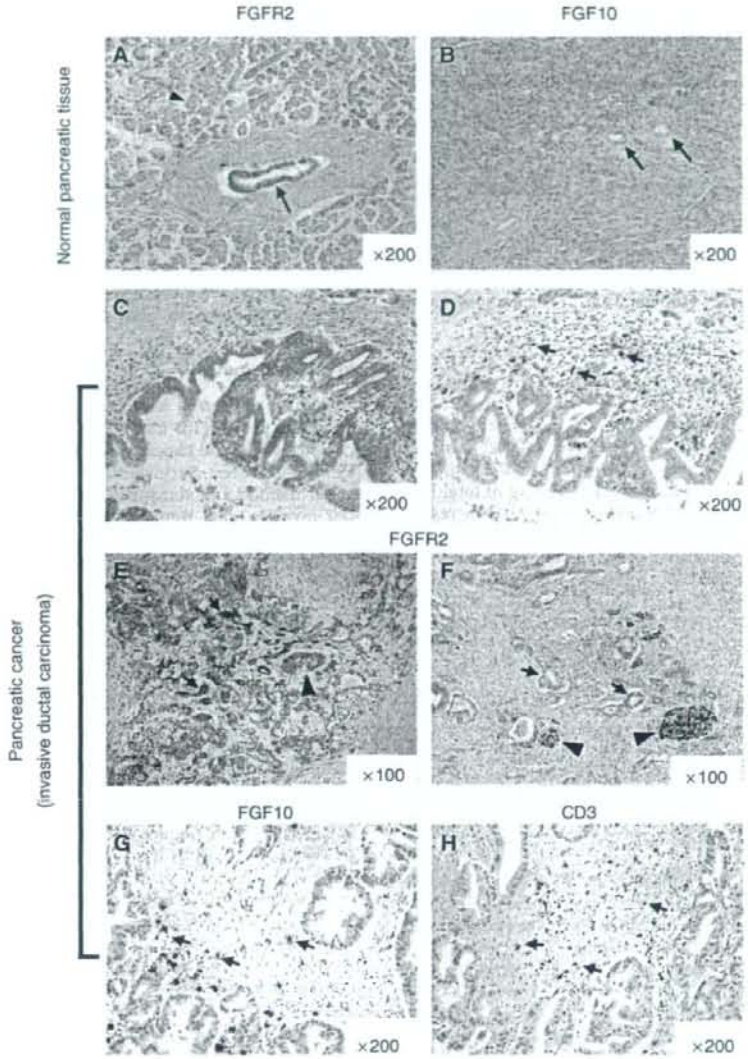

Figure 1 Expression patterns of FGFR2 and FGF10 in normal pancreas and pancreatic cancer. The magnification is shown in the right bottom corner of each figure. (**A** and **B**) Immunostaining of FGFR2 (**A**) and FGF10 (**B**) in normal pancreas, showing that FGFR2 is expressed weakly in ductal cells (**A**, arrow) and acinar cells (**A**, arrow head), and that no obvious FGF10 staining is found in normal pancreatic tissue, including ductal cells (**B**, arrows). (**C** and **D**) Immunostaining of FGFR2 (**C**) and FGF10 (**D**) in pancreatic cancer tissues, showing that FGFR2 is expressed in cancer cells (**C**), whereas FGF10 is expressed in scattered cells in the stroma surrounding cancer cells (**D**, arrows). (**E** and **F**) Immunostaining of FGFR2 in pancreatic cancer cells. (**E**) Representative result from the FGFR2 high expression group, indicating higher FGFR2 expression in cancer cells (arrows) compared with islets (arrow head). (**F**) Representative result from the FGFR2 low expression group, showing lower FGFR2 expression in cancer cells (arrows) compared with islet (arrow heads). (**G** and **H**) Immunostaining of FGF10 (**G**) and CD3 (**H**), marker for T cell. Fibroblast growth factor 10 and CD3 are both expressed in scattered cells with similar cell shape in the stroma surrounding cancer cells (arrows).

marker). Within them, CD3-positive stromal cells, T-cells, were located similar to FGF10-positive stromal cells and also has similar cell shapes (Figure 1G and 1H; arrows). However, due to the technical difficulties, we could not demonstrate that FGF10-expressing cells were identical with T-cells.

Overall, the results show that FGFR2 is expressed in pancreatic cancer tissue, and that its ligand, FGF10, is expressed in stromal cells.

FGFR2 expression levels correlates with prognosis of pancreatic cancer patients

Next, we examined whether strong expression of FGFR2 in cancer cells correlated with patient prognosis. The 76 patients were divided into two groups according to the expression level of FGFR2 in cancer cells (the high and low expression groups in Table 1). The expression level of FGFR2 was not correlated with

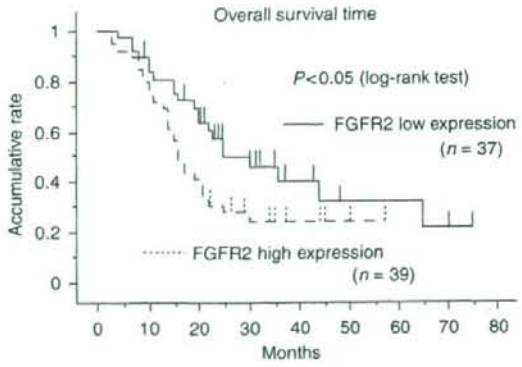

Figure 2 Kaplan–Meier survival curves for patients with high and low FGFR2 expression. Patients with low FGFR2 expression had a significantly longer overall survival time compared to those with high FGFR2 expression ($P<0.05$ by log-rank test).

Table 2 Clinico-pathological features of pancreatic cancer patients in FGFR2-IHC analysis

		FGFR2 expression of IHC		
	Total	Low expression	High expression	
	76	37	39	*P*-value
T (1,2,3/ 4)	42/34	23/14	19/20	NS
N (0/ 1,2,3)	20/56	14/23	6/33	0.0263
M (0/ 1)	67/9	35/2	32/7	NS
ly (0/ 1,2,3)	14/62	9/28	5/34	NS
v (0/ 1,2,3)	31/45	19/18	12/27	NS
ne (0/ 1,2,3)	11/65	7/30	4/35	NS
Size (</≥; 30 mm)	37/39	23/14	14/25	0.022
IA, IB, IIA/IIB, III	19/57	13/24	6/33	0.0469
Poor/others	13/63	8/29	5/34	NS

ly = lymphatic invasion; M = distant metastasis; N = nodal metastasis; ne = neural invasion; NS = no significant; Poor = poorly differentiated adenocarcinama; T = tumour depth; v = venous invasion. Patient stage was determined according to UICC TNM classification.

clinicopathological factors such as sex, age and pathological stage (Table 1). There were no statistical differences in the resection status of both groups. Also, the ratios of patients who received adjuvant chemotherapy (using gemcitabine) were similar in both groups, indicating that patients in both groups were treated with similar therapeutic approaches after surgery (Table 1). Interestingly, Kaplan–Meier analysis showed that patients with high FGFR2 expression had a significantly shorter overall survival time compared to those with low expression levels (Figure 2) ($P=0.047$ by log-rank test). Moreover, patients with high FGFR2 expression had significantly more nodal invasion, a larger tumour size, and a worse UICC Stage (Table 2; $P=0.0263$, $P=0.0469$, and $P=0.022$, respectively, by χ^2-test). There was no significant correlation between survival time and the presence of FGF10 in stromal cells in cancer tissue (data not shown).

FGF10/FGFR2-IIIb signalling induces cell migration and invasion

The expression pattern of FGF10 and FGFR2 in cancerous tissue and the poor prognosis of patients with high FGFR2 expression in cancer cells indicate that a stromal cell–epithelial cell interaction through FGF10/FGFR2 signalling might induce the malignant properties of pancreatic cancer. To examine this hypothesis, we analysed the effects of FGF10 on the proliferation, invasion and migration of pancreatic cancer cells. For this purpose, four pancreatic cancer cell lines were used: MIA PaCa-2, PANC-1, CFPAC-1 and AsPC-1 cells.

First, we examined whether these cell lines expressed FGFR2 and FGF10. Reverse transcriptase–PCR analysis showed that all four cell lines did not express FGF10 mRNA, consistent with the results of immunostaining showing FGF10 expression in stromal cells, but not in cancer cells, in pancreatic cancer tissue (Figure 3A). Fibroblast growth factor 10 activity is dependent on its binding to the FGFR2-specific isoform, FGFR2-IIIb (Igarashi *et al*, 1998). Therefore, a primer set for FGFR2-IIIb was designed with a 5 primer for its specific exon in the FGFR2 gene. Reverse transcriptase–PCR analysis with this primer showed that CFPAC-1 and AsPC-1 cells expressed the FGFR2-IIIb isoform, whereas the other two cell lines did not do so (Figure 3A).

To examine cancer cell proliferation, the cells were stimulated with various concentrations of FGF10 ($10-200\,ng\,ml^{-1}$), but no effect of FGF10 was observed in any of the four cell lines (data not shown). Next, we examined whether FGF10 affects migration or invasion of pancreatic cancer cells. Representative results from the cell migration and invasion assays are shown in Figure 3B. Interestingly, FGF10 stimulated cell migration and invasion of cells that expressed FGFR2-IIIb (CFPAC-1 and AsPC-1), but not of cells without expression of the specific receptor (MIA PaCa-2 and PANC-1) (Figure 3C and 3D). For CFPAC-1 cells, migration was almost doubled (Figure 3C) and invasion was increased by 1.5 times (Figure 3D) following stimulation with FGF10 ($100\,ng\,ml^{-1}$). Similar results were obtained for AsPC-1 cells (Figure 3C and 3D).

To confirm that the effects of FGF10 on pancreatic cells were mediated through FGFR2-IIIb, we used a molecular hybrid including the FGFR2-IIIb extracellular domain and the carboxy-terminal Fc region of human IgG (recombinant human FGFR2-IIIb/IgG Fc Chimera). Such hybrids inhibit signalling through the FGFR2-IIIb receptor by antagonising ligand binding (Jung *et al*, 1999). Stimulated migration of CFPAC-1 cells by $100\,ng\,ml^{-1}$ FGF10 was completely inhibited by addition of FGFR2-IIIb/IgG Fc Chimera ($500\,ng\,ml^{-1}$) (Figure 3E; compare the second and third columns). The effects of the chimera were due to inhibition of signalling through FGFR2-IIIb, rather than to a direct effect of the chimera on the cells, as addition of chimera alone did not affect cell migration (Figure 3E; compare the first and fourth columns). Invasion of CFPAC-1 cells stimulated by FGF10 was also inhibited by FGFR2-IIIb/IgG Fc Chimera (Figure 3F). Overall, these results indicate that FGF10 stimulates migration and invasion of cancer cells through its specific receptor, FGFR2-IIIb.

Upregulation of MT1-MMP and TGF-β1 mRNA by FGF10

To examine the molecular mechanisms through which FGF10 signalling induces migration and invasion of pancreatic cancer cells with FGFR2-IIIb expression, we analysed whether FGF10 stimulation induced mRNA expression of 18 genes related to cell mobility in CFPAC-1 cells (see Materials and methods for the 18 genes). CFPAC-1 cells were cultured with recombinant human FGF10 ($100\,ng\,ml^{-1}$) for 12, 24 and 48 h, and then total RNA were extracted and the mRNA levels of the 18 genes were analysed by quantitative RT–PCR. Among these genes, FGF10 upregulated the expression levels of MT1-MMP (Figure 4A) and TGF-β1 (Figure 4B) mRNAs in a time-dependent manner. In CFPAC-1 cells, the mRNA expression levels for both genes were almost 10 times higher after FGF10 stimulation for 48 h compared with the level before stimulation. The concentration of TGF-β1 in culture medium was also upregulated in time-dependent manner in CFPAC-1 cells (Figure 4C) and also in AsPC-1 cells (data not shown). The secretion of TGF-β1 by FGF10 stimulation in CFPAC-1 cells was inhibited by FGFR2-IIIb/IgG chimera (Figure 4D), indicating FGF10-induced TGF-β1 secretion through FGFR2.

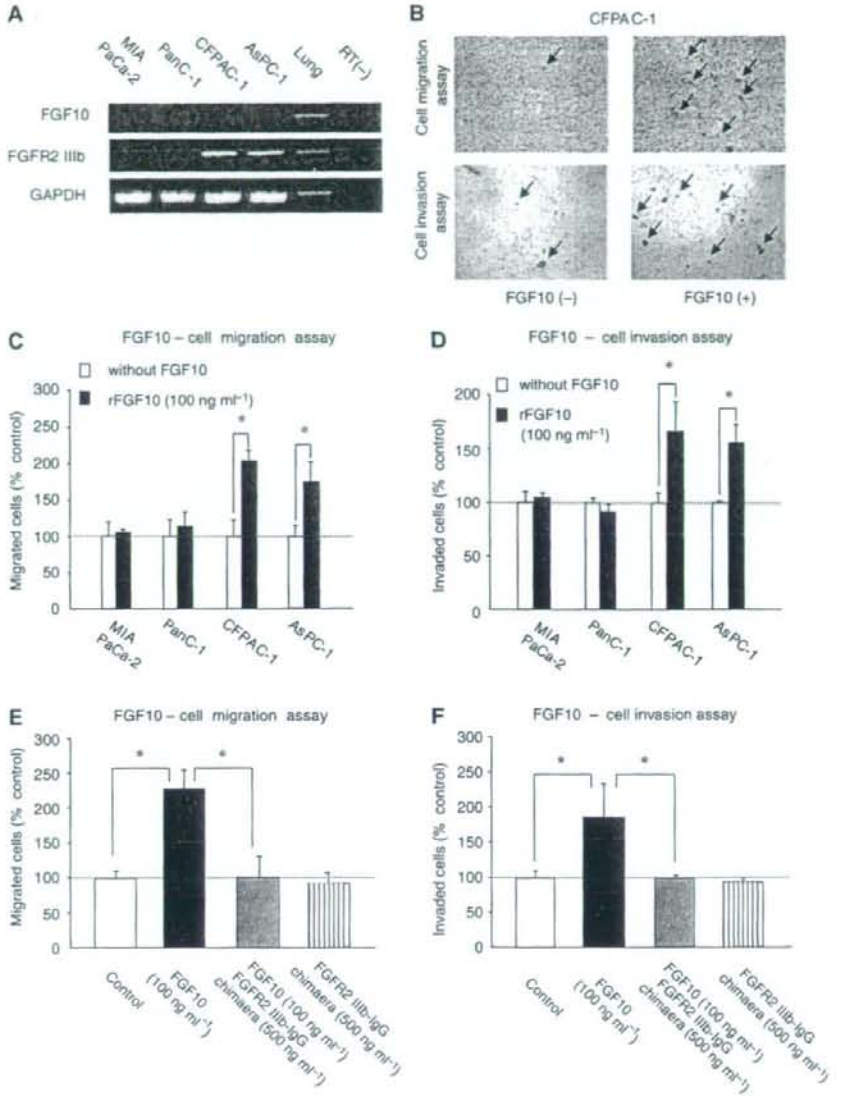

Figure 3 Fibroblast growth factor 10 induces cell migration and invasion in pancreatic cell lines with FGFR2-IIIb expression. (**A**) RT–PCR analysis of FGF10 and FGFR2-IIIb in four pancreatic cell lines and cDNA obtained from normal lung tissue as a positive control. None of the cell lines express FGF10. MIA PaCa-2 and PanC-1 cells do not express FGFR2-IIIb, but CFPAC-1 and AsPC-1 do express this gene. (**B**) Representative results of cell migration (upper panels) and invasion (lower panels) for CFPAC-1 cells. Representative migrated and invaded cells are indicated with arrows. (**C** and **D**) Cell migration (**C**) and invasion (**D**) assay of all four cell lines cultured without (white column) or with (black column) FGF10 (100 ng ml^{-1}). FGF10-induced cell migration and invasion in CFPAC-1 and AsPC-1 cells, but not in MIAPaCa-2 and PanC-1 cells. The numbers of migrated or invaded cells cultured with FGF10 are shown relative to a value of 100% for cell migration without ligand. (**E** and **F**) Inhibition of FGFR2-IIIb signalling by an FGFR2-IIIb/IgG chimera in CFPAC-1 cells. Migration (**E**) and invasion (**F**) assay. The numbers of migrated or invaded cells are shown relative to a value of 100% for cells cultured without FGF10 or chimera (control; white column). FGF10-induced migration and invasion in CFPAC-1 cells (black column). Addition of the FGFR2-IIIb/IgG chimera completely eliminated the effects of FGF10 (grey column), whereas the chimera itself did not affect cell migration and invasion of CFPAC-1 cells (striped column). *$P<0.05$.

DISCUSSION

In this study, we investigated the molecular mechanisms underlying the aggressiveness of pancreatic cancer, and found that FGF10/FGFR2-IIIb-signalling plays an important role in inducing migration and invasion of pancreatic cancer cells. FGF receptor-2-IIIb is a splice variant of FGFR2 that is predominantly expressed by cells of epithelial origin and is involved in proliferation of these cells, whereas other FGFR2 variant transcripts are detected in mesenchymal cells (Miki *et al*, 1992). Ishiwata *et al* (1998) first showed that the FGFR2-IIIb isoform of the FGF receptor is expressed in pancreatic cancer cells. We confirmed this result by

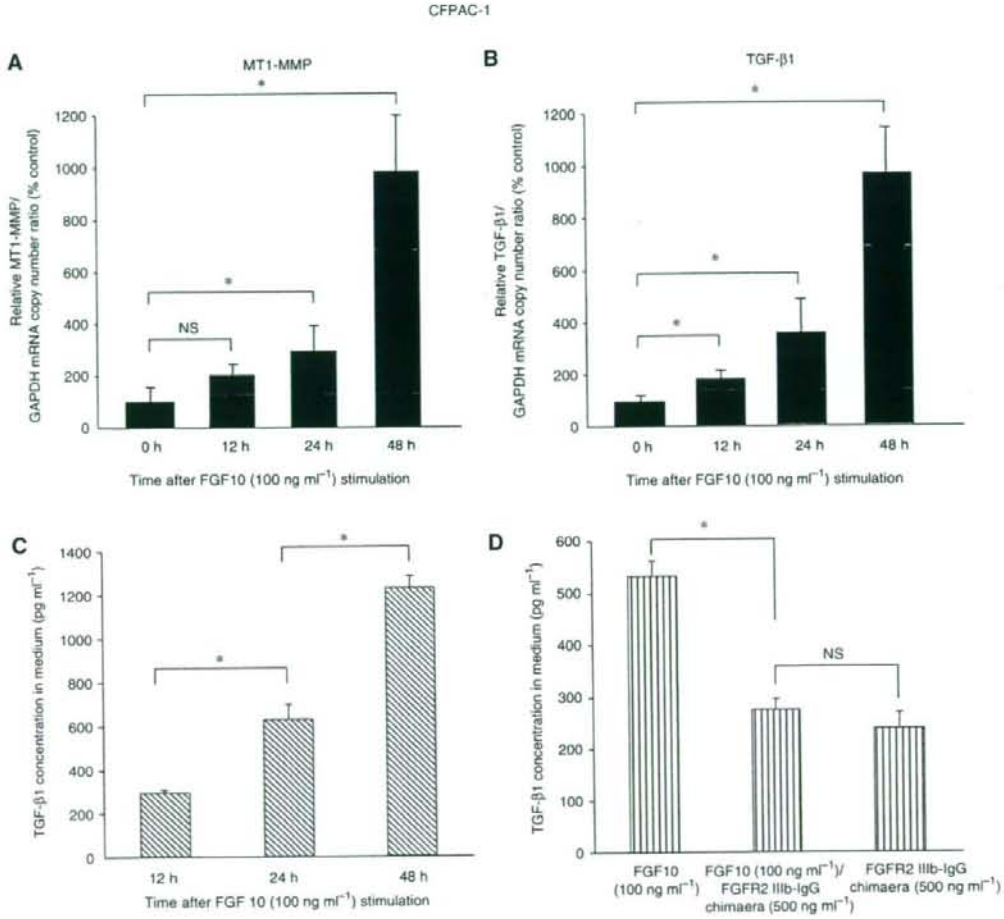

Figure 4 Fibroblast growth factor 10 induces expression of mRNA for MT1-MMP and TGF-β1 in CFPAC-1 cells. The figure shows the relative copy numbers of MT1-MMP (**A**) and TGF-β1 (**B**) mRNA in CFPAC-1 cells cultured with 100 ng ml^{-1} of FGF10 for the indicated times. The MT1-MMP and TGFβ1 mRNA/GAPDH mRNA copy number ratios are shown relative to those of cells without FGF10 stimulation (0 h). The concentration of TGF-β1 protein in medium also increased in time-dependent manner in CFPAC-1 cells 48 h after addition of FGF10 (100 ng ml^{-1}) (**C**). FGF receptor-2-IIIb/IgG chimera (500 ng ml^{-1}) inhibited this TGF-β1 secretion by FGF10, whereas chimera alone did not affect TGF-β1 secretion by itself (**D**). *$P<0.05$; NS=not significant.

immunostaining of 76 resected pancreatic cancer tissues, and moreover, showed that the expression level of FGFR2-IIIb is correlated with prognosis and the incidence of nodal involvement. These findings indicate that signalling through FGFR2-IIIb may induce malignant potential, and especially, may increase the metastatic ability of pancreatic cancer cells.

FGFR2-IIIb was first identified as a high-affinity receptor of FGF7 (keratinocyte growth factor) (Miki *et al*, 1992), and it can also be activated by FGF10, which has strong sequence homology with FGF7 (Igarashi *et al*, 1998). Our results show that FGF10 is expressed in stromal cells scattered around pancreatic cancer cells, suggesting a possible interaction with cancer cells expressing FGFR2-IIIb. To our knowledge, this is the first report of the expression pattern of FGF10 in pancreatic cancer. We also show that FGF10-positive cells and CD3-positive T cells exist in a similar location and have a similar shape. According to these data, we hypothesize that FGF10 is expressed in T cells surrounding pancreatic cancer cells. Supporting this, during wound healing, T cells bearing $\gamma\delta$ T cell receptors are an important source of FGF7 and FGF10, which activate epithelial cell proliferation (Jameson *et al*, 2002). Further studies are required for confirming this hypothesis.

Despite the high homology, the function of FGF10 differs slightly from that of FGF7. We found that FGF10 induced cell migration and invasion in pancreatic cancer cells, but had no effect on cell proliferation. Alderson *et al* (2002) also found that FGF10 does not affect cell proliferation in several types of cancer cells, whereas Niu *et al* (2007) recently showed that FGF7 stimulates cell proliferation, in addition to cell migration and invasion, in pancreatic ductal epithelial cells. These two genes also have different expression patterns in pancreatic cancer. Cho *et al* (2007) showed that FGF7 is expressed in pancreatic cancer cells themselves and acts in an autocrine manner, whereas our results showed FGF10 expression in stromal cells of pancreatic cancer, indicating a paracrine FGF10/FGFR2-IIIb interaction. Further studies are required to understand how these ligands, which share the same receptor on cancer cells, are orchestrated to induce malignant properties in pancreatic cancer.

To understand how FGF10/FGFR2-IIIb signalling induces cell migration and invasion of pancreatic cancer cells, we examined whether FGF10 influences the expression of genes related to cell mobility. Interestingly, FGF10 induced expression of membrane type 1-matrix metalloproteinase (MT1-MMP) and transforming growth factor (TGF)-β1 mRNA in CFPAC-1 cells, and these genes may lead, at least in part, to cell migration and invasion of cancer cells. The metalloproteinases are known to involve cell invasion ability. Within several types of metalloproteinases and their inhibitor that we examined (MMP-1, 2, 7, 9, MT1-MMP, TIMP-2), only the mRNA expression of MT1-MMP was upregulated by FGF10 in CFPAC-1 cells. Membrane type 1-matrix metalloproteinase was originally found as a metalloproteinase with a transmembrane domain in homology screening for the MMP conserved domain (Sato *et al*, 1994). The MT1-MMP protein induces invasive activity by degrading extracellular matrix surrounding epithelial cells through its proteinase activity, or by activating other proteinases (Seiki, 2003). In pancreatic cancer, enhanced MT1-MMP expression is particularly observed in metastatic lesions (Maatta *et al*, 2000). These facts may indicate that induction of MT1-MMP is one of the mechanisms through which FGF10 induces cell invasion activity. We are trying to examine if the proteinase activity of MT1-MMP is increased by FGF10 stimulation in pancreatic cancer cells.

TGF-β1 is also an important regulator of cell invasion and migration activity, and is frequently overexpressed in pancreatic cancer, with the expression level associated with an advanced tumour stage and a poor prognosis (Friess *et al*, 1993a, b). Moreover, TGF-β1 is an important regulator of the epithelial mesenchymal transition, in which epithelial cells disassemble their junctional structures, start expressing mesenchymal cell proteins, remodel the extracellular matrix, and become migratory (Moustakas and Heldin, 2007). As a result, cancer cells acquire metastatic properties (Oft *et al*, 1998; McEarchern *et al*, 2001), suggesting that FGF10/FGFR2-IIIb-signalling may promote migration of pancreatic cancer cells through induction of TGF-β1 expression. We also found that FGF10 induced not only mRNA expression of TGF-b1 but also the secretion of this protein through signalling through FGFR2-IIIb in CFPAC-1 cells. This strongly supports the hypothesis and it should be confirmed in future.

In conclusion, our results indicate an important role for the interaction between stromal cells and parenchymal cells mediated by FGF10/FGFR2-IIIb signalling in pancreatic cancer. This suggests that FGF10 and FGFR2-IIIb are promising candidates as target molecules for new therapy against pancreatic cancer, and that therapeutic agents directed against these molecules may improve the prognosis of patients with this disease.

ACKNOWLEDGEMENTS

This work is partly supported by grants from Ministry of Education, Culture, Science, Sports, and Technology of Japan. We thank Noboru Mitsuhashi MD PhD for comments on this paper.

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British Journal of Cancer (2008) 99, 314–320

www.bjcancer.com

Heterogeneity and clinical significance of *ETV1* translocations in human prostate cancer

G Attard[1,2,10], J Clark*[,1,10], L Ambroisine[3], IG Mills[4], G Fisher[3], P Flohr[1], A Reid[1,2], S Edwards[1], G Kovacs[5], D Berney[6], C Foster[7], CE Massie[4], A Fletcher[1], JS De Bono[1,2], P Scardino[8,9], J Cuzick[3] and CS Cooper[1] on behalf of the Transatlantic Prostate Group

[1]Institute of Cancer Research, Male Urological Cancer Research Centre, 15 Cotswold Road, Sutton, Surrey SM2 5NG, UK; [2]The Royal Marsden NHS Trust Foundation Hospital, Downs, Road, Sutton, Surrey SM2 5PT, UK; [3]Department of Mathematics and Statistics, Cancer Research UK Centre for Epidemiology, Wolfson Institute of Preventive Medicine, St Bartholomew's Medical School, Queen Mary, University of London, Charterhouse Square, London EC1M 6BQ, UK; [4]Department of Oncology, Uro-Oncology Research Group, Cancer Research UK Cambridge Research Institute, Li Ka Shing Centre, University of Cambridge, Robinson Way, Cambridge CB2 0RE, UK; [5]Molekulare Onkologie, Heidelberg Klinikum, Ruprecht-Karls-Universitat, Im Neuenheimer Feld 365, Heidelberg 69120, Germany; [6]The Orchid Tissue Laboratory, St Bartholomew's Hospital, London EC1A 7BE, UK; [7]Department of Pathology, The University of Liverpool, Duncan Building, Royal Liverpool University Hospital, Daulby Street, Liverpool L69 3GA, UK; [8]Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, NY 10021, USA; [9]Department of Urology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA

A fluorescence *in situ* hybridisation (FISH) assay has been used to screen for *ETV1* gene rearrangements in a cohort of 429 prostate cancers from patients who had been diagnosed by trans-urethral resection of the prostate. The presence of *ETV1* gene alterations (found in 23 cases, 5.4%) was correlated with higher Gleason Score ($P=0.001$), PSA level at diagnosis ($P=<0.0001$) and clinical stage ($P=0.017$) but was not linked to poorer survival. We found that the six previously characterised translocation partners of *ETV1* only accounted for 34% of *ETV1* re-arrangements (eight out of 23) in this series, with fusion to the androgen-repressed gene *C15orf21* representing the commonest event (four out of 23). In 5′-RACE experiments on RNA extracted from formalin-fixed tissue we identified the androgen-upregulated gene *ACSL3* as a new 5′-translocation partner of *ETV1*. These studies report a novel fusion partner for *ETV1* and highlight the considerable heterogeneity of *ETV1* gene rearrangements in human prostate cancer.
British Journal of Cancer (2008) **99,** 314–320. doi:10.1038/sj.bjc.6604472 www.bjcancer.com
Published online 1 July 2008

Keywords: prostate cancer; *ETV1*; *ACSL3*; *ACSL3:ETV1* fusion

Recently, fusion of the prostate-specific androgen-regulated *TMPRSS2* gene to the ETS family transcription factor gene *ERG* was reported as a common event in prostate cancer (Tomlins *et al*, 2005, 2006; Clark *et al*, 2006; Iljin *et al*, 2006; Perner *et al*, 2006; Soller *et al*, 2006; Wang *et al*, 2006a; Yoshimoto *et al*, 2006; Hermans *et al*, 2006). Less frequently *TMPRSS2* becomes fused to *ETV1* and *ETV4*. In all these cases a *TMPRSS2-ETS* chimaeric gene is generated resulting in high-level expression of the fused 3′-*ETS* gene sequences. The reported incidence of *TMPRSS2:ETV1* fusion in these studies (1–2%) was, however, considerably lower than the observed incidence of *ETV1* gene overexpression (~10% in prostate cancer). This prompted Tomlins *et al* (2007) to search for alternative mechanisms of *ETV1* overexpression. They identified five new 5′-fusion *ETV1* partners including the prostate-specific androgen-induced gene *SLC45A3/Prostein*, an endogenous retroviral element *HERV-K*, a prostate-specific androgen-repressed gene *C15orf21*, and a strongly expressed housekeeping gene *HNRPA2B1*. Additionally they found that in the two prostate cancer cell lines LNCaP and MDA-PCa2B, outlier expression of *ETV1* was caused through the entire *ETV1* gene becoming juxtaposed to sequences at 14q13.3–14q21.1. By characterising the expression of four contiguous genes within this region (*SLC25A21*, *MIPOL1*, *FOXA1* and *TTC6*), as well as that of *ETV1*, in LNCaP cells they demonstrated that this region exhibited prostate-specific expression that was coordinately regulated by androgens in a castration-resistant cell line model without formation of a fusion gene. In that study only single cases of each fusion were reported, with the exception of the juxtaposition of *ETV1* sequences to 14q13.3–14q21.1 where two cases were observed. It was therefore not possible to assess the relative importance of the different fusion partners in their small tumour set.

For *ERG* gene re-arrangements several studies have demonstrated links to clinicopathological indicators (Perner *et al*, 2006; Wang *et al*, 2006a; Demichelis *et al*, 2007; Nam *et al*, 2007). In a watchful waiting cohort of 111 patients, Demichelis *et al* (2007) reported a significant link between the presence of *ERG* alterations and prostate cancer-specific death. In a series of 165 patients who underwent prostatectomy, Nam *et al* (2007) found that the presence of a *TMPRSS2:ERG* fusion was associated with a greater probability of biochemical relapse. Additionally, we have recently demonstrated that loss of 5′-*ERG* sequences coupled with duplication of *TMPRSS2:ERG* fusion sequences predicts extremely poor cancer-specific survival independently of Gleason score and PSA

*Correspondence: Dr J Clark; E-mail: jeremy.clark@icr.ac.uk
[10]Clark and Attard are joint first authors
Revised 12 May 2008; accepted 20 May 2008; published online 1 July 2008

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Annals of Surgical Oncology **15**(11):3157–3168
DOI: 10.1245/s10434-008-0061-5

Annexin II Overexpression Predicts Rapid Recurrence after Surgery in Pancreatic Cancer Patients Undergoing Gemcitabine-Adjuvant Chemotherapy

Shigetsugu Takano, MD, PhD,[1,2] Akira Togawa, MD, PhD,[1] Hideyuki Yoshitomi, MD, PhD,[1] Takashi Shida, MD, PhD,[1] Fumio Kimura, MD, PhD,[1] Hiroaki Shimizu, MD, PhD,[1] Hiroyuki Yoshidome, MD, PhD,[1] Masayuki Ohtsuka, MD, PhD,[1] Atsushi Kato, MD, PhD,[1] Takeshi Tomonaga, MD, PhD,[2] Fumio Nomura, MD, PhD,[2] and Masaru Miyazaki, MD, PhD[1]

[1]The Department of General Surgery, Graduate School of Medicine, Chiba University, Chiba, Japan
[2]Molecular Diagnosis, Graduate School of Medicine, Chiba University, Chiba, Japan

Background: Gemcitabine has been shown to exhibit significant clinical activity against pancreatic cancer and has become a first-line chemotherapeutic for this disease in recent years. However, there are still many patients who do not respond to this treatment and it is expected to improve the clinical outcome if we can develop a method to predict the efficacy of gemcitabine before treatment. The purpose of this study was to determine novel factors that make pancreatic cancer resistant to gemcitabine.

Materials and methods: Using the high-resolution proteomic approach, agarose two-dimensional gel electrophoresis, we compared protein profiling of a gemcitabine-resistant pancreatic cancer cell line with its wild-type.

Results: We identified Annexin II as an up-regulated protein in the gemcitabine-resistant pancreatic cancer cell line. Immunohistochemistry demonstrated that Annexin II was mainly expressed at the cell surface of pancreatic cancer cells. Interestingly, Annexin II overexpression in cancer cells was significantly associated with rapid recurrence after gemcitabine adjuvant chemotherapy in postoperative patients ($P = .0078$), and its staining was also an independent prognostic indicator of recurrence in pancreatic cancer patients who underwent adjuvant gemcitabine treatment after curative surgery on multivariate analysis ($P = .0047$). In addition, inhibition of Annexin II expression by siRNA in pancreatic cancer cell lines increased the cytotoxic efficacy of gemcitabine. These results indicate that Annexin II overexpression may induce gemcitabine resistance in pancreatic cancer resulting in rapid recurrence.

Conclusions: Analysis of Annexin II expression in cancer tissues may predict the clinical outcome of gemcitabine treatment, leading to the development of a new method for tailor-made treatment for this disease.

Key Words: Annexin II—Pancreatic cancer—Gemcitabine—Resistance—Prognosis—Tailor-made therapy.

Pancreatic cancer remains one of the most lethal cancers among all malignancies. This is because of the difficulties of early diagnosis, low resectability rate, rapid recurrence after surgery, and resistance against chemotherapy or radiation therapy.[1,2] In particular, classical chemotherapeutic agents that are efficient toward other cancers have no effect on this disease.

Recent advances in chemotherapy have improved the prognosis of pancreatic cancer. Gemcitabine is a

Published online August 20, 2008.
Address correspondence and reprint requests to: Hideyuki Yoshitomi, MD, PhD; E-mail: yoshitomi@faculty.chiba-u.jp

novel pyrimidine analog, which exhibits anticancer activity toward several tumors,[3–5] and has become a standard chemotherapy in patients with advanced pancreatic cancer since Burris et al. reported its clinical benefit for this disease.[6] However, the clinical benefits with gemcitabine are limited with only few months' improvement of median survival time in patients with unresectable pancreatic cancer. Therefore, the combination of gemcitabine with other reagents has been attempted to increase its efficacy.[7] To achieve this, erlotinib, an epidermal growth factor receptor (EGFR) inhibitor, which has been approved as a new targeted agent for advanced pancreatic cancer in combination with gemcitabine, is already shown to improve the survival of patients with unresectable pancreatic cancer.[8]

Thus, the detection of specific drug-resistant factors against gemcitabine is very important for the development of new therapeutic methods. This may lead to "tailor-made chemotherapeutics" contributing to a patient's chance of longer survival and may also lead to "cost-efficient therapy." Although in vitro studies have revealed several gemcitabine-resistant factors in pancreatic cancer,[9,10] there are few factors that have demonstrated clear correlation with the clinical outcomes of gemcitabine treatment.

Proteomic technologies have been used to identify proteins that are useful as cancer biomarkers and therapeutic targets. Within these proteomics technologies, two-dimensional gel electrophoresis (2-DE) has been widely used in analysis of protein profiling; in particular, the agarose 2-DE method has previously been shown to have a higher loading capacity than 2-DE with immobilized pH gradient (IPG) gel for isoelectric focusing.[11] These advantages of the agarose 2-DE method have enabled wide-span protein profiling and have led to the identification of cancer-specific factors by detecting differentially expressed proteins in cancer and adjacent normal tissues.[12,13]

In this study, we successfully identified a novel potential gemcitabine-resistant factor through the comparison of protein profiling between a gemcitabine-resistant human pancreatic cancer cell line, which we previously established,[14] and its wild-type using the agarose 2-DE method. Immunohistochemistry (IHC) of pancreatic cancer tissues demonstrated that the level of expression of this factor correlated with rapid recurrence after surgery in patients undergoing postoperative gemcitabine adjuvant therapy. Moreover, inhibition of this molecule increased the cytotoxicity of gemcitabine to its resistant pancreatic cancer cells in vitro. Our study may lead to new concepts in selecting suitable chemotherapeutics for each patient and contribute to realizing cost-efficient therapy for pancreatic cancer patients.

MATERIALS AND METHODS

MIA PaCa-2 Cultures and Establishment of Gemcitabine-Resistant Cells

The human pancreatic cancer cell lines MIA PaCa-2 (WT-MIA PaCa-2) and the established gemcitabine-resistant MIA PaCa-2 (GEM-MIA PaCa-2) were cultured and maintained as previously described.[14] Gemcitabine was obtained from Eli Lilly Japan (Kobe, Japan).

Patients and Samples

Pancreatic cancer and adjacent normal pancreas tissues were obtained from 62 pancreatic cancer patients who had undergone pancreatectomy in the Department of General Surgery, Chiba University, Japan, between June 2001 and April 2006. All patients were diagnosed histologically with primary invasive pancreatic ductal carcinoma for which surgery was curative. The Ethics Committee of our institute approved this protocol and written informed consent was obtained from each patient before surgery. Inclusion in this study required that each of the following criteria were met: (1) All patients were treated with gemcitabine as first-line adjuvant chemotherapy after curative surgery. Each chemotherapy cycle consisted of three weekly infusions of gemcitabine 1000 mg/m^2, followed by a one-week pause. The patients received at least adjuvant chemotherapy with four cycles of gemcitabine. (2) Neither chemotherapy nor radiotherapy was performed prior to surgery. Each patient's disease-free survival time was determined from the time of surgery to the time of recurrent disease; recurrence was confirmed by radiograms (CT scan, PET, etc.). No patient was lost during follow-up.

Protein Extraction

Subconfluent WT-MIA PaCa-2 and GEM-MIA PaCa-2 cells were washed twice with PBS and detached from the dishes. Pelleted cells were lysed in buffer [250mM Tris HCl, (pH 6.8), 40% glycerol, 5% SDS, 5% BPB and containing 5% β-ME] and incubated for 5 min at 100°C, following centrifugation at 15,000 rpm for 15 min at 4°C. The supernatant was subjected to the agarose 2-DE and Western blot analysis.

Agarose 2-DE

Agarose gels were prepared as described previously.[11] Protein extracts (500 μg) from WT-MIA PaCa-2 and GEM-MIA PaCa-2 cells were applied to the agarose isoelectric focusing gel and one-dimensional isoelectric focusing was initially conducted at 4°C, followed by fixation in 10% trichloroacetic acid and 5% sulfosalicylic acid at room temperature. After washing with deionized water for 1 hour, the agarose gel was then transferred to a 12% polyacrylamide gel, and two-dimensional SDS-PAGE was performed. The two-dimensional gel was incubated in 30% methanol and 10% acetic acid overnight and was then stained with Coomassie PhastGel Blue R (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Each gel was scanned using Epson ES 2000 (Nagano, Japan), and the strength of their spots was compared manually. The protein spots were excised from the gel; in-gel tryptic digestion was performed and the protein was identified, as described previously.[12]

Western Blot

The supernatant proteins (10 μg per sample) were separated by electrophoresis on 10–20% gradient gels (PerfectNT Gel, DRC, Tokyo, Japan) and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). After blocking with 0.5% skim milk in PBS, the membranes were reacted with rabbit anti-human Annexin II (H-50) polyclonal antibody (Santa Cruz Biotechnology, Inc., CA, USA; diluted 1:300), and with goat anti-β-actin antibody (Santa Cruz Biotechnology, Inc., CA, USA; diluted 1:500) in blocking buffer. Goat anti-rabbit IgG horseradish peroxidase (diluted 1:3000), and rabbit anti-goat IgG horseradish peroxidase (Cappel, West Chester, PA, USA; diluted 1:500) in blocking buffer were used as secondary antibodies. Antigens on the membrane were detected using enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The intensity of each band was measured using NIH image, and the relative Annexin II protein levels normalized to β-actin were calculated. PanC-1, a pancreatic cancer cell line, was used as positive control of Annexin II.[15]

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted and subjected to quantitative RT-PCR as previously described.[16] PCR was done with the following primer sets; Annexin II: forward 5′-CAGCCTTATCTGGCCACCTG-3′, reverse 5′-CCAGCGTCATAGAGAGATCCCG-3′, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward 5′-ACCCAGAAGACTGTGG ATGG-3′, reverse 5′-TTCTAGACGGCAGGTCAG GT-3′. The conditions for quantitative RT-PCR were as follows. For Annexin II, the initial denaturation was at 95°C for 10 min, followed by 45 cycles denaturation at 95°C for 10 s, annealing at 62°C for 5 s, extension at 72°C for 7 s; for GAPDH, annealing at 57°C for 5 s, extension at 72°C for 9 s. Annexin II mRNA levels were determined as the absolute copy number normalized against that of GAPDH mRNA.

Immunohistochemistry

Pancreatic tumor and adjacent normal tissues were cut in 4-μm-thick serial sections and were deparaffinized. Slides were autoclaved in 500 mL of citric acid buffer (10 mmol/L pH 6.0) with 0.2% Tween20 at 120°C for 10 min for antigen retrieval. Immunohistochemical staining was performed using the labeled streptavidin-biotin-peroxidase method (Dako LSAB2 kit, Dako, Japan) according to the manufacturer's protocol. All slides were incubated with the primary antibody, anti-Annexin II polyclonal antibody (Santa Cruz Biotechnology, Inc., CA, USA; 1:200 dilution), in a humidified chamber for 2 h at room temperature. Counterstaining was performed with hematoxylin before dehydration and mounting.

The staining patterns were scored as follows: Low expression: 0–30% of the tumor cells with positive staining; High expression: more than 30% of the tumor cells with positive staining. American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 (HER2) Testing in Breast Cancer,[17] was, in part, referenced on the thresholds for scoring of Annexin II-IHC staining. Scoring was conducted by two of the authors, independently (ST and TS).

Gene Knockdown Using Short Interfering RNA

Short interfering RNA (siRNA) that specifically targeted Annexin II mRNA was used to reduce Annexin II expression. The target sequences for Annexin II RNA interference were described previously[15] [Annexin II (Anx2) siRNA: 5′-CTGGAG-GACAAG GCTCGGGAA-3′]. Double-stranded synthetic Anx2siRNA and Luciferase (GL2) siRNA

as a negative control were purchased from QIAGEN, Tokyo, Japan. In vitro transfection was performed using Lipofectamine 2000 reagent (Invitrogen Life Technologies, CA, USA) as previously described.[16] Briefly, 24 h before transfection, a total of 2×10^5 cells were plated in six-well plates and were cultured in medium without antibiotics. After incubation with siRNA in Opti-MEM I Reduced Serum Medium (GIBCO) for 4 h, the medium was changed into medium with fetal bovine serum.

Cell Viability and Cytotoxicity Assay

A total of 4×10^4 GEM-MIA PaCa-2 cells in appropriate medium were plated in 96-well plates and incubated for 24 h. After being transfected with each siRNA (40 nM final concentration), cells were incubated for 6 days, the relative number of viable cells was quantified by colorimetric cell proliferation assay using the Cell Counting Kit-8 (DOJINDO, Kumamoto, Japan) according to the manufacture's instructions. Absorbance was measured on a Bio-Rad Microplate Reader at 450 nm wavelength. All of these experiments were performed in quadruplicate for three times, independently.

For the gemcitabine cytotoxicity assay, 4×10^4 cells/mL (GEM-MIA PaCa-2) were cultured in 96-well plates in 100 μL culture medium for 24 h, and then the cultured cells were transfected with each siRNA (40 nM final concentration). Forty-eight hours later, gemcitabine was added into culture medium to a final concentration of 100 ng/mL. The medium was changed to fresh at 24 h. Cell viability was measured by using Cell Counting Kit-8 at 0, 24, and 48 h after gemcitabine exposure.

Statistical Analysis

For correlations of Annexin II staining to clinicopathological variables and evaluation of the cell viability and cytotoxicity, *t*-test was used for parametric measurements. Disease-free survival and overall survival time were calculated according to the Kaplan-Meier method and compared by the log-rank test based on the pattern of Annexin II-IHC staining. Univariate analysis was performed for the correlation between disease-free survival time and various clinical characteristics included patient age, sex, preoperative serum level of tumor marker, Carbohydrate Antigen 19-9 (CA19-9), and various pathological characteristics,[18] as shown in Table 2 (see "Results" section), and the strength of Annexin II-IHC staining. Cox's proportional hazards model was used for univariate and multivariate analysis. Statistical significance was assumed when $P < .05$ was obtained.

RESULTS

Protein Profiling by Agarose 2-DE Analysis Revealed Annexin II was Overexpressed in GEM-MIA PaCa-2

To identify proteins that are differentially expressed between the gemcitabine-resistant cell line and its wild type, protein profiling of WT-MIA PaCa-2 and GEM-MIA PaCa-2 were compared by agarose 2-DE analysis. The agarose 2-DE protein patterns of both WT-MIA PaCa-2 and GEM-MIA PaCa-2 were visualized by Coomassie blue staining (Fig. 1A–B). The candidate spots that were reproducibly expressed in triplicate experiments were selected in this study. Analysis of protein expression in these two cell lines revealed that several protein spots were upregulated in GEM-MIA PaCa-2 compared with in WT-MIA PaCa-2. These spots were excised from the gel and subjected to N-terminal amino acid sequence analysis. One of three candidate proteins, the ~36 kDa protein, was identified as Annexin II (squared in Fig. 1A–B).

The overexpression of Annexin II, shown by the agarose 2-DE analysis, was confirmed by Western blot analysis. It is known that Annexin II is detected with multiple bands in Western blot analysis and may be caused by cleavage and/or post-translational modification.[12] We also found that immunoblotting of cellular lysates from these cell lines with anti-Annexin II antibody revealed two bands nearby 36kDa (Fig. 1C). The lower and upper band intensities were approximately 2.9 fold and 1.3 fold higher in GEM-MIA PaCa-2 than those of WT-MIA PaCa-2, respectively. The total amount intensity of these two bands was approximately 1.8 fold higher in GEM-MIA PaCa-2 than those of WT-MIA PaCa-2 (Fig. 1C). Furthermore, quantitative RT-PCR revealed that the expression level of Annexin II mRNA was approximately 1.6 fold higher in GEM-MIA PaCa-2 than in WT-MIA PaCa-2 (Fig. 1D).

Annexin II is Overexpressed in Pancreatic Cancer by IHC

Based on these in vitro results, we examined whether Annexin II was expressed in the pancreatic cancer tissues by IHC. We performed IHC of Annexin II in 62 samples of pancreatic cancer tissues and adjacent normal tissues. In normal pancreatic tissue,