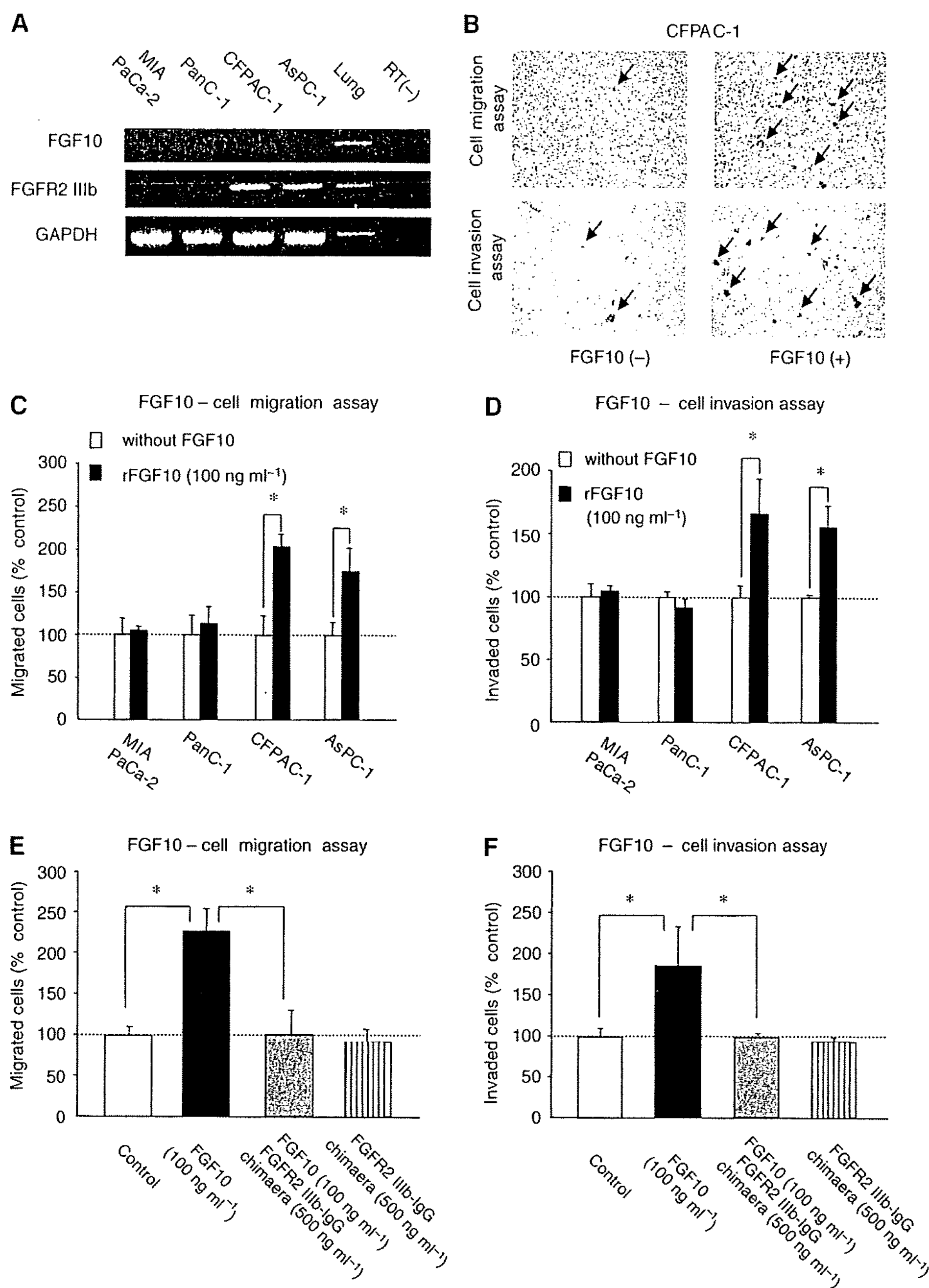

Figure 3 Fibroblast growth factor 10 induces cell migration and invasion in pancreatic cell lines with FGFR2-IIIb expression. (**A**) RT–PCR analysis of FGF10 and FGFR2-IIIb in four pancreatic cell lines and cDNA obtained from normal lung tissue as a positive control. None of the cell lines express FGF10. 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

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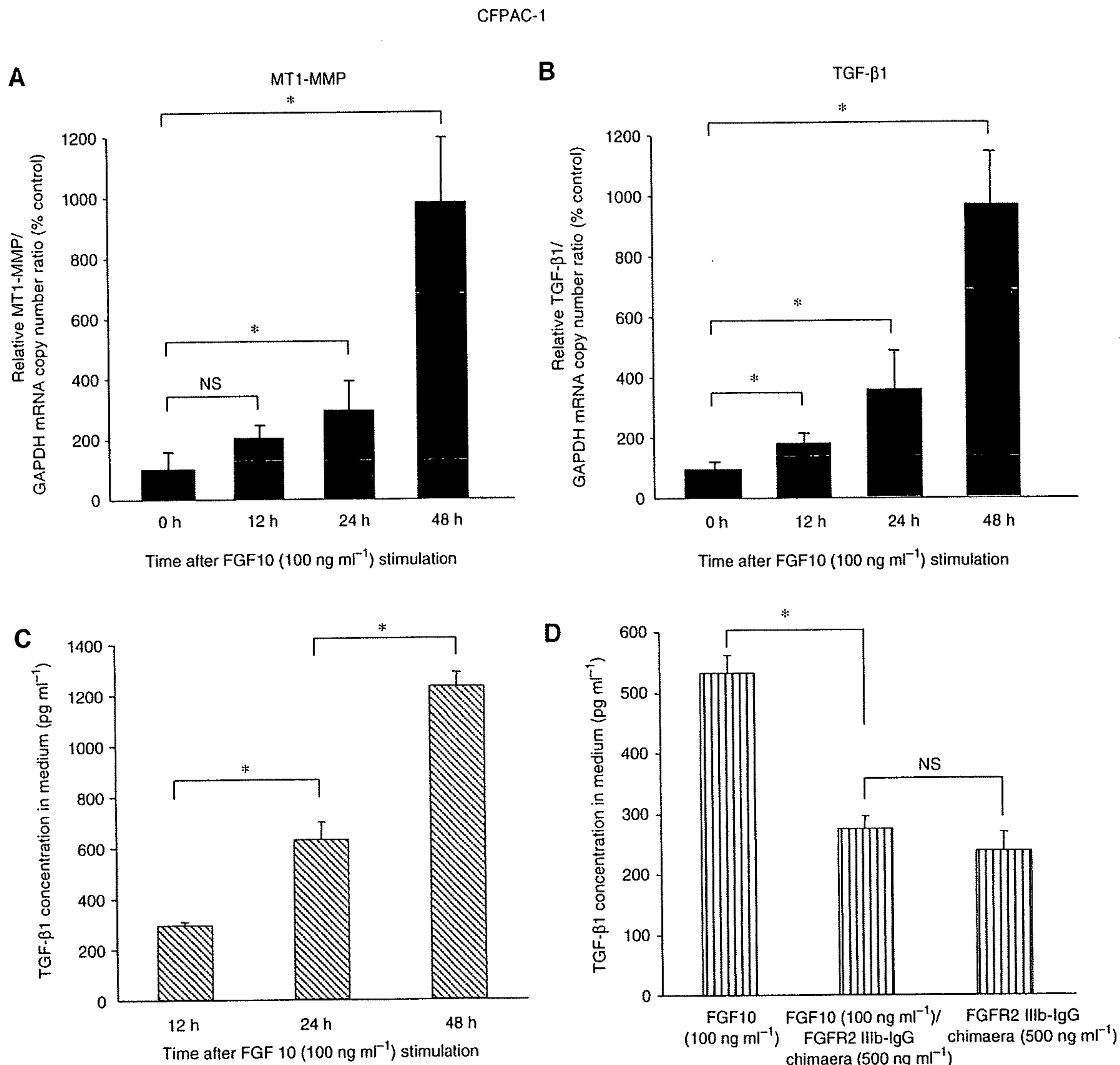

Figure 4 Fibroblast growth factor 10 induces expression of mRNA for MT1-MMP and TGF-β1 in CFPAC-1 cells. The figure shows the relative copy numbers of MT1-MMP (**A**) and TGF-β1 (**B**) mRNA in CFPAC-1 cells cultured with 100 ng ml^{-1} of FGF10 for the indicated times. The MT1-MMP and TGFβ1 mRNA/GAPDH 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.

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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.

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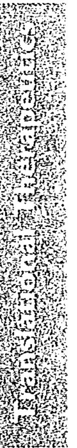

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Heterogeneity and clinical significance of *ETV1* translocations in human prostate cancer

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

Molecular Diagnostics

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

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

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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

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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,

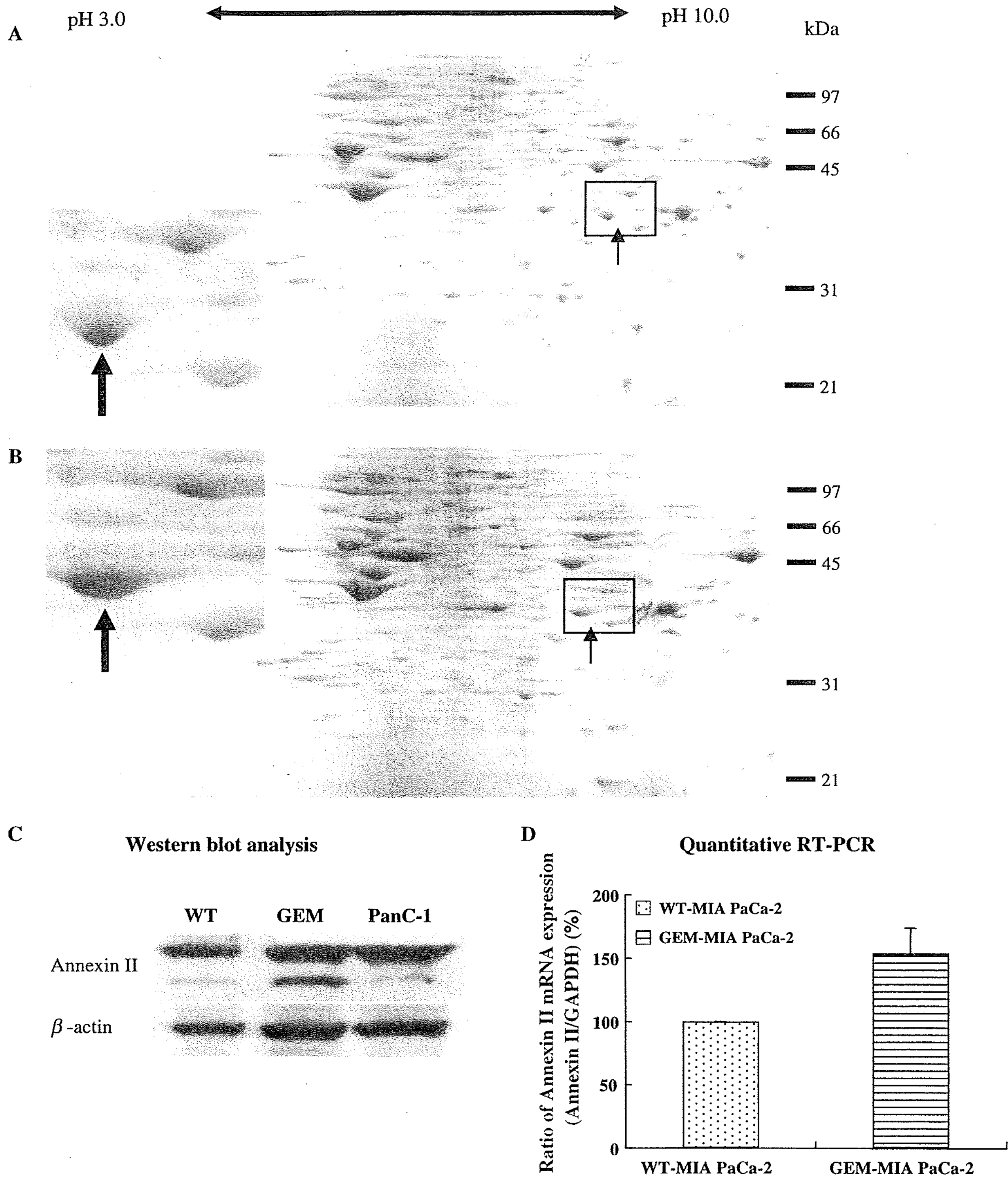

FIG. 1. Coomassie blue-stained agarose two-dimensional gel electrophoresis (2-DE) protein expression profiling of pancreatic cancer cell lines, (**A**) the wild-type MIA PaCa-2 (WT-MIAPaCa-2) cell line and (**B**) the gemcitabine-resistant cell line (GEM-MIAPaCa-2). Arrows with the square in (**A**) and (**B**) indicate the protein spot that was about 36kDa in molecular weight. This spot was excised from gel and subjected to N-terminal amino acid sequence analysis. GEM-MIA PaCa-2 expressed more Annexin II protein (**C**) and mRNA (**D**) than WT-MIA PaCa-2. (**C**) Overexpression of Annexin II in GEM-MIA PaCa-2 by Western blot analysis. Total protein lysates from WT-MIAPaCa-2 (WT) or GEM-MIAPaCa-2 (GEM) were immunoblotted with anti-Annexin II antibody. PanC-1 was used as positive control of Annexin II. (**D**) Quantitative RT-PCR showed higher Annexin II mRNA expression in GEM-MIA PaCa-2 than in WT-MIA PaCa-2.

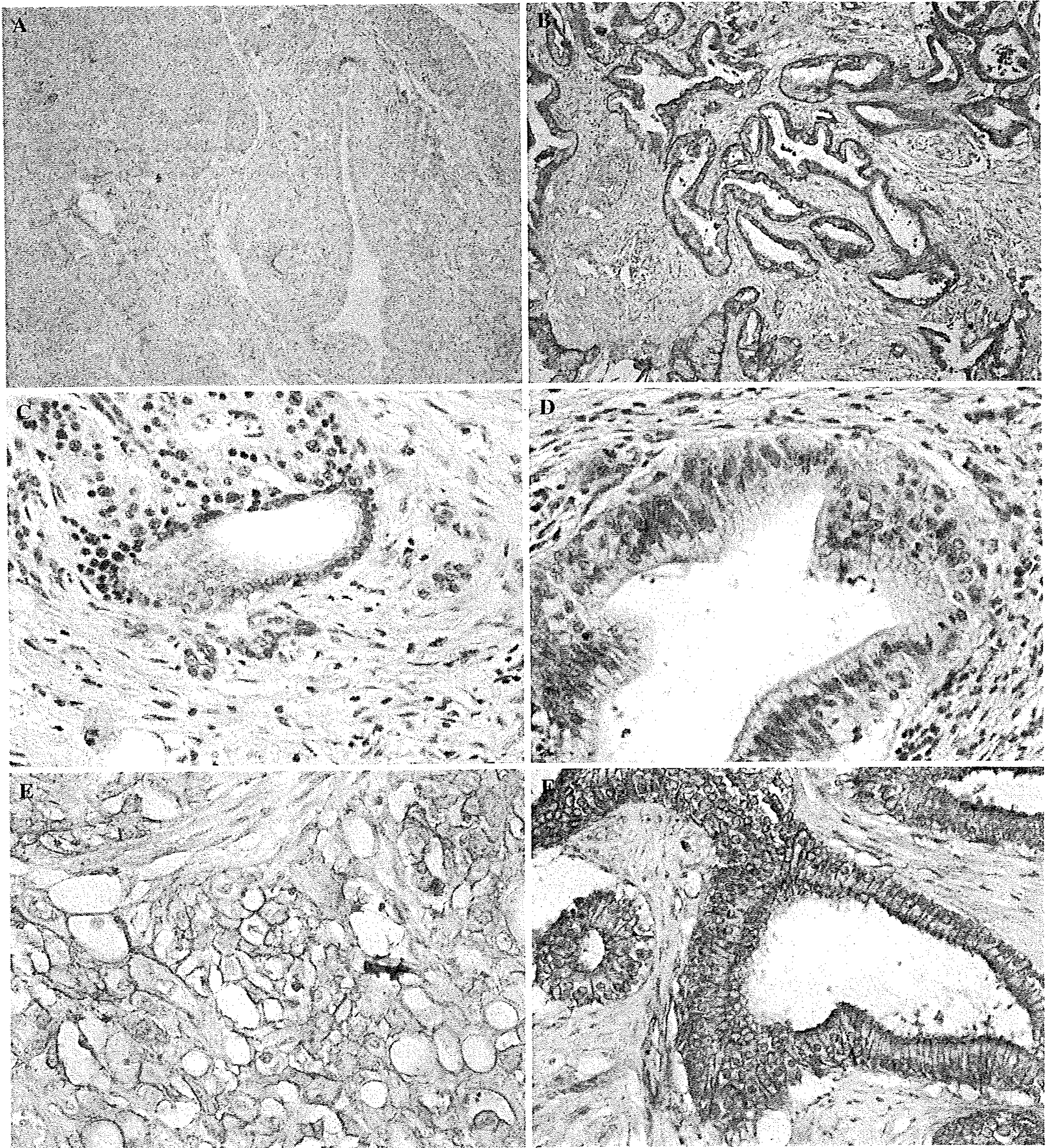

FIG. 2. Immunohistochemistry for Annexin II in normal pancreatic tissue and invasive pancreatic ductal carcinomas. (**A**) Annexin II was weakly expressed in acinar and pancreatic ductal cells in normal pancreas tissue (original magnification 100×). (**B**) Annexin II was strongly overexpressed in the tumor cell surface. The stromal fibrous tissue exhibited little staining for Annexin II in the tumor tissues (100×). (**C–F**) Immunohistochemical staining patterns of Annexin II in resected pancreatic cancer tissues. Representative samples of low expression (**C, D**) and high expression (**E, F**) are indicated, respectively (400×).

acinar and pancreatic ductal cells weakly expressed Annexin II (Fig. 2A). On the other hand, pancreatic cancer cells exhibited significantly greater expression of Annexin II (Fig. 2B). The expression of Annexin II was mainly at the cellular surface of the tumor cells (Fig. 2D–F).

TABLE 1. *Characteristics of 62 pancreatic cancer patients with gemcitabine treatment in IHC analysis*

		Annexin II-IHC staining		
	Total (62)	Low expression (30)	High expression (32)	*P*
Sex				NS
Male	34	16	18	
Female	28	14	14	
Age (years)				NS
Mean	63.4	64.5	62.4	
±SD	±9.2	±7.2	±10.6	
UICC-Stage				NS
IB	2	2	0	
IIA	13	7	6	
IIB	45	21	24	
III	2	0	2	
Histology				NS
Tubular adeno Ca.				
Well	7	4	3	
Moderately	39	20	19	
Poorly	10	4	6	
Invasive Ca. derived from intraductal tumor	3	1	2	
Anaplastic Ca.	2	1	1	
Adenosquamous Ca.	1	0	1	
Total volume of administrating gemcitabine to patients (g)				NS
Mean	26.9	27.7	26.4	
±SD	±16.1	±16.5	±15.8	

UICC, International Union Against Cancer; Ca., carcinoma; NS, not significant.

Annexin II Overexpression in Pancreatic Cancer is Significantly Associated with Rapid Recurrence in Patients Undergoing Adjuvant Chemotherapy with Gemcitabine

Next, we investigated whether the expression level of Annexin II in tumor cells correlated with the clinical outcomes of pancreatic cancer patients, focusing particularly on the resistance against gemcitabine treatment. Sixty-two pancreatic cancer patients, in whom Annexin II expression levels were analyzed by IHC, were divided into two groups according to the immunostaining score (Fig. 2C–F). Thirty-two cases (51.6%) were classified as high expression group, and the remaining 30 cases (48.4%) were classified as low expression group. All patients underwent curative resection of the tumor followed by gemcitabine adjuvant chemotherapy. The clinicopathologic features of both groups are shown in Table 1. Between these two groups, there were no significant correlations in age, sex, UICC classification stage,[19] histological characteristics of the patients, and total volume of gemcitabine administered after surgery.

We investigated whether the expression of Annexin II in pancreatic cancer cells correlated with rapid recurrence after gemcitabine treatment. Kaplan-Meier analysis revealed that disease-free survival was significantly shorter in the patients of the high expression group compared with the patients of the low expression group ($P = .008$, log-rank test) with median disease-free survival times of 7 and 21 months, respectively (Fig. 3A). Notably, there was no significant difference in the volume of gemcitabine administered between the patients of these two groups. In addition, patients with high expression of Annexin II also exhibited shorter overall survival time compared with the low expression group ($P = .033$, log-rank test) with median overall survival times of 19 and 25 months, respectively (Fig. 3B). We next analyzed whether Annexin II expression level was an independent recurrent factor in the patients undergoing gemcitabine adjuvant chemotherapy (Table 2). On univariate analysis, tumor size (≥33 mm vs. <33 mm), UICC classification stage (IIB, III vs. I, IIA), and Annexin II-IHC staining (high vs. low expression) correlated significantly with the disease-free survival time of the patients. Furthermore, among these factors, UICC classification stage and Annexin II-IHC staining (Hazard ratio; 2.728, 95% confidence interval; 1.360–5.474, $P = .0047$) were independent recurrent factors on multivariate analysis. Importantly, the Annexin II-IHC staining level did not correlate with the patient's stage or any other pathological factors.

Inhibition of Annexin II Expression by siRNA Increases the Cytotoxic Efficacy of Gemcitabine to Its Resistant Pancreatic Cancer Cells

The significant correlation of Annexin II-IHC staining with poor clinical outcome of the patients after gemcitabine treatment may indicate the involvement of this protein in the chemotherapeutic efficacy of gemcitabine. For this reason, we next examined whether gene knockdown of this protein would affect the cytotoxicity to pancreatic cancer cells, especially to gemcitabine-resistant cells. To specifically silence the Annexin II gene, GEM-MIA PaCa-2 was transfected with siRNA targeting Annexin II mRNA (Anx2siRNA) or GL2siRNA as a negative control. The suppression of Annexin II protein expression by transfection with Anx2siRNA (40 nM) was confirmed in GEM-MIA PaCa-2 by Western blot analysis (Fig. 4A).

The viability of pancreatic cancer cells was comparatively determined by cell counting after the

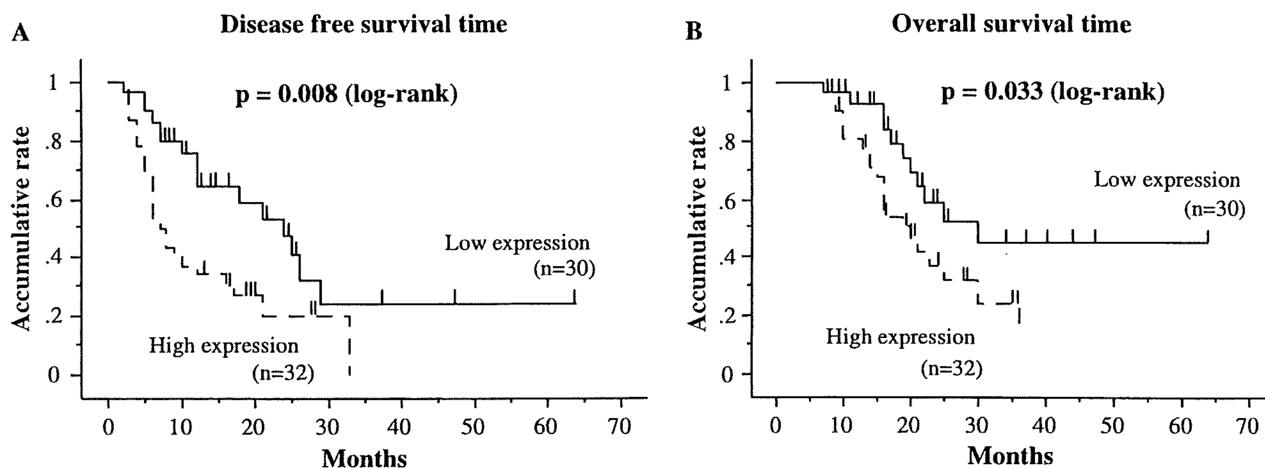

FIG. 3. Annexin II overexpression in pancreatic cancer is significantly associated with rapid recurrence and shorter survival time of patients with adjuvant gemcitabine chemotherapy in the Kaplan-Meier analysis. (**A**) Disease-free survival time was shorter in patients with high expression of Annexin II than in those with low expression (P = .008, log-rank test). (**B**) Patients with high expression of Annexin II also exhibited shorter overall survival time than patients with low expression (P = .033, log-rank test).

TABLE 2. *Recurrent factors of 62 pancreatic cancer patients with gemcitabine treatment in Cox's proportional hazard model*

	Disease-free survival					
	Univariate analysis			Multivariate analysis		
Variables	Hazard ratio	95% confidence interval	*P*	Hazard ratio	95% confidence interval	*P*
Age (>64/≤63 years)	1.027	0.551–1.915	.9332			
Sex (Male/Female)	1.534	0.830–2.836	.1724			
CA19-9 serum level (high/low)	1.356	0.732–2.512	.3330			
Tumor size (33mm; high/low)	1.958	1.055–3.634	**.0331***	1.476	0.782–2.787	.2299
ly (+/−)	1.119	0.463–2.705	.8035			
v (+/−)	0.865	0.451–1.657	.6611			
ne (+/−)	2.480	0.880–6.994	.0859			
N (−/+)	0.468	0.207–1.060	.0688			
Tumor differentiation (well/moderately, poorly)	0.636	0.194–2.088	.4552			
UICC-Stage (IIB, III/IB, IIA)	2.547	1.067–6.079	**.0352***	3.088	1.228–7.767	**.0166***
Annexin II-IHC staining (high/low)	2.278	1.204–4.307	**.0113***	2.728	1.360–5.474	**.0047***
Resection status (R0/1)	0.735	0.326–1.661	.4597			

* Significance value $P < .05$.
Patient stage was determined according to UICC TNM classification ly, lymphatic vessel invasion; v, vascular invasion; ne, neural invasion; N, lymph node status R0, histologically tumor-free surgical margins; R1, macroscopically tumor-free surgical margins.

transfection with Mock, GL2siRNA, and Anx2siRNA. GEM-MIA PaCa-2 cells were transfected with 40 nM siRNA, and total cell number was measured from 1 to 6 days after transfection. The cell proliferation was not affected by gene silencing by transfection of Anx2siRNA, compared with cells treated with GL2siRNA in GEM-MIA PaCa-2 cells (Fig. 4B). Next, we investigated that the efficacy of gemcitabine combination with Annexin II silencing on the cytotoxic ability to cancer cells. The results showed that inhibition of Annexin II expression in GEM-MIA PaCa-2 significantly increased the chemocytotoxic efficacy of gemcitabine to these cells, compared with cells treated with GL2siRNA ($P < .05$; paired t-test) (Fig. 4C).

DISCUSSION

The rapid recurrence of pancreatic cancer after curative surgery is one of the important reasons for the poor prognosis of this disease. However, it has been shown that adjuvant chemotherapy improves the prognosis of this disease; prolonging disease-free survival time.[20,21] In particular, Oettle et al. recently demonstrated the benefits of adjuvant chemotherapy

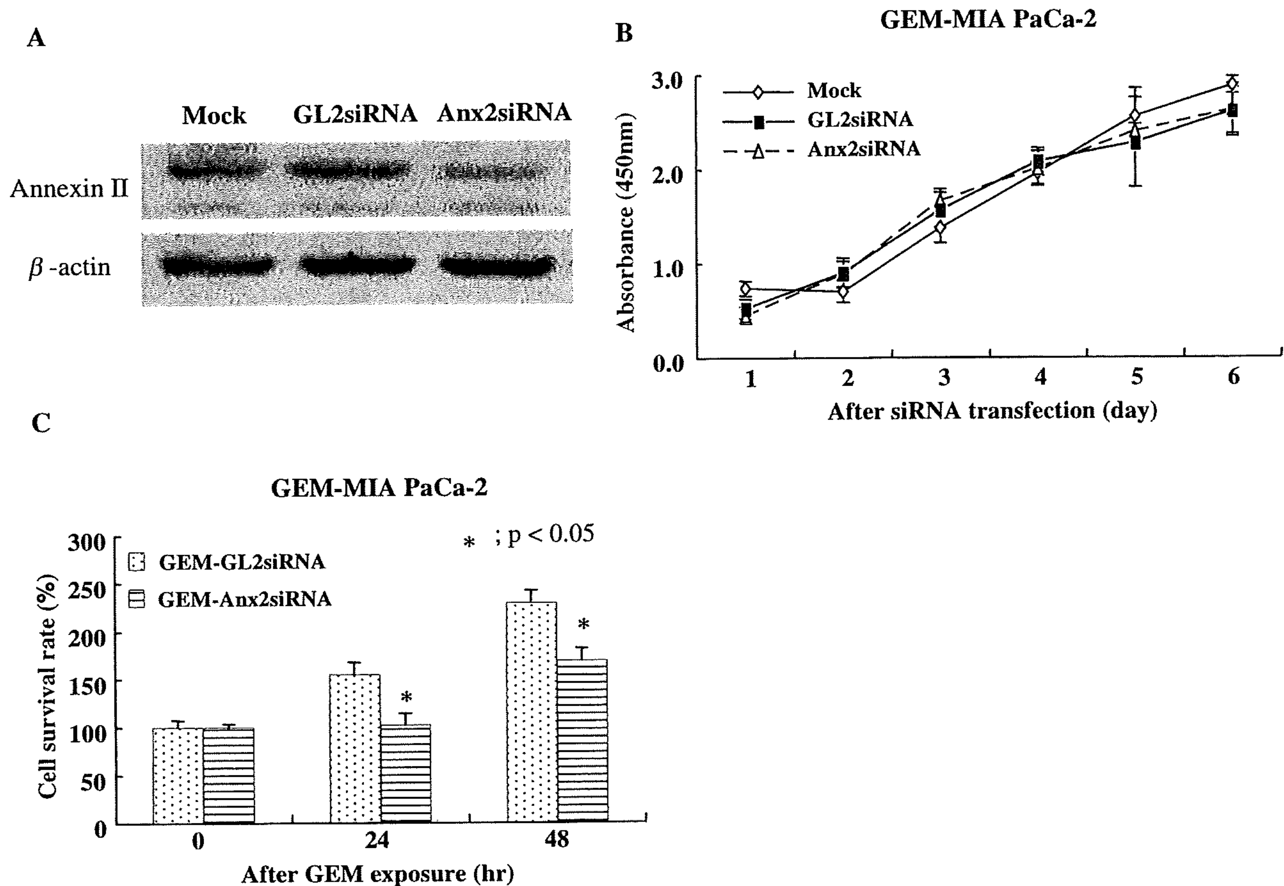

FIG. 4. Suppression of Annexin II expression increases the sensitivity to gemcitabine in gemcitabine-resistant pancreatic cancer cells. (A) Anx2siRNA inhibited Annexin II protein expression in GEM-MIA PaCa-2 in contrast with GL2siRNA. Western blot analysis of Annexin II and β-actin as control. Cells were transfected with no siRNA (Mock), GL2siRNA and Anx2siRNA at 40 nM concentration for 48 h. (B) Inhibition of Annexin II expression by siRNA did not affect the cell proliferation of GEM-MIA PaCa-2. Cells were transfected with Mock, GL2siRNA and Anx2siRNA and cultured for the indicated time. (C) The ratio of viable cells is significantly lower in cells treated with Anx2siRNA than with GL2siRNA after gemcitabine exposure. Cytotoxicity was determined by measuring the residual viable cells after gemcitabine treatment at indicated time after siRNAs transfection. (Differences between GL2siRNA treated cell and Anx2siRNA treated cells were statistically significant. * $P < .05$). Values represent % of control cells treated with GL2siRNA.

using gemcitabine, which is a standard chemotherapeutic reagent for unresectable advanced pancreatic cancer patients.[22] To improve the efficacy of this therapy, it is important to understand the mechanisms of gemcitabine resistance, as this is a major determinant of disease-free and overall survival. In this study, we revealed that Annexin II was one of the gemcitabine-resistant factors in pancreatic cancer. Annexins are a family of phospolipid- and calcium-binding cell surface proteins that were first discovered as major substrates of oncogene and growth factor receptor tyrosine kinases.[23,24] Annexin II, a 36kDa protein, is a coreceptor for tissue-type plasminogen activator (t-PA) and plasminogen in endothelial cells,[25] which forms heterotetrameric complex with Annexin II light chain, a member of the S100 family. It is expressed on the surfaces of epithelial cells of the intestine, lung, liver, and pancreas, and it is also found on the cell surface of vascular endothelial cells, macrophages, and myelomonocytic leukemia cells. Its expression on their cell surface has been implicated in both cell-cell adhesion and plasminogen activation.[26–30]

Annexin II is highly expressed in several malignant tumors, such as brain, colorectal, stomach, lung, breast, liver, and pancreatic tumors.[31–39] Esposito et al. have indicated that the expression of cell surface Annexin II increased the progression from low-grade pancreatic intraepithelial neoplasia (PanIN) lesions to pancreatic cancer.[40] These results suggested that overexpression of Annexin II in pancreas was closely correlated with the processes that lead to malignant differentiation. Using another proteomic approach, Chen et al. identified Annexin II as an overexpressed

protein in pancreatic cancer by isotope-coded affinity tag (ICAT) analysis comparing protein profiling between cancer and normal pancreas and confirmed this by tissue array IHC.[41] These results suggest that Annexin II is involved in the development and progression of pancreatic cancer and may become a candidate target protein for pancreatic cancer therapy.

In this study, using gemcitabine-resistant cell lines, which we previously established,[14] and agarose 2-DE analysis, we demonstrated for the first time that Annexin II overexpression was associated with rapid recurrence in patients undergoing adjuvant therapy with gemcitabine after surgery. One of the advantages of this technique is its ability to distinguish intact isomeric forms or post-translationally modified forms of a protein. These different isoforms or post-translationally modified forms of Annexin II may exhibit different biological functions. In this study, Western blot analysis showed at least two bands. Although these two bands both showed increased band intensity in GEM-MIA PaCa-2 cells compared with that of WT-MIA PaCa-2, the differences of the intensities between these two cells were not equivalent in these bands. It may suggest that these two bands may indicate the existence of isomeric forms or post-translationally modified forms that have different biological functions. Further study will be needed to examine this hypothesis. We also confirmed that mRNA expression of Annexin II was increased in gemcitabine-resistant cells compared with its parental cells.

Patients with high levels of expression of Annexin II-IHC staining exhibited rapid recurrence. Considering that Annexin II expression in pancreatic cancer did not correlate with the patient's stage, histological factors, or total volume of gemcitabine administered, overexpression of Annexin II may induce drug resistance against gemcitabine. Based on these results, Annexin II was associated with malignant behavior of pancreatic cancer and was considered as a potential factor providing gemcitabine-resistance. The estimation of Annexin II expression level in resected cancerous tissues may predict rapid recurrence after gemcitabine adjuvant chemotherapy and enable us to select suitable candidate patients for this adjuvant therapy. As a result, it may provide clinical benefits in selecting patients in whom unnecessary gemcitabine-based chemotherapy could be omitted and to attempt other chemotherapeutic agents, besides gemcitabine.

Annexin II in pancreatic cancer cells is expressed on the cell surface, and anti-Annexin II antibody specifically inhibits binding of plasminogen and tissue plasminogen activator (t-PA) to endothelial cells.[25] Furthermore, Diaz et al. indicated that t-PA, which is overexpressed in pancreatic cancer cells, promotes invasion, tumor growth, and angiogenesis,[42] and binds specifically to Annexin II on the extracellular membrane of pancreatic cancer cells, leading to activation of local plasmin production and tumor cell invasion.[43] Ortiz et al. also demonstrated that two membrane receptors, EGFR and Annexin II, which were overexpressed in pancreatic cancer, were involved in the transduction of tPA signaling in pancreatic tumors.[15] Annexin II may induce malignant potential in pancreatic cancer cell by affecting tPA signaling. On the other hand, the mechanism by which Annexin II induces chemoresistance in cancer cells are not clear. However, supporting our data, Chuthapisith et al. showed that the protein expression of Annexin II (Annexin A2) was increased in several types of chemoresistant cells in breast cancer cell line.[44] Furthermore, Huang et al. showed that Annexin II negatively regulated p53 induced apoptosis in lung cancer.[45] Considering that many chemoreagents affect cell-cycle regulation and apoptosis through p53, Annexin II may induce chemoresistance by regulating p53. We are now trying to reveal the mechanisms by which Annexin II induces gemcitabine resistance.

We show as the first study that inhibition of Annexin II expression in gemcitabine-resistant pancreatic cancer cells increases the cytotoxic efficacy of gemcitabine. It may be possible that the specific inhibitor of Annexin II, similar to the EGFR tyrosine kinase inhibitor, increases the cytotoxic efficacy of gemcitabine and is applied to the development of a new therapy in combination with gemcitabine against pancreatic cancer. Further examinations are needed to confirm this.

In conclusion, we identified Annexin II as a novel candidate gemcitabine-resistance factor in pancreatic cancer. The evaluation of Annexin II in pancreatic cancer patients would be helpful in selecting patients who might not respond to gemcitabine-based chemotherapy beforehand, and this would contribute to improve the quality of life of pancreatic cancer patients by avoiding unsuitable chemotherapy. Furthermore, Annexin II may be an attractive target molecule for treatment of pancreatic cancer to increase the efficacy of gemcitabine-based chemotherapy. Further analysis will be needed to reveal details of the mechanism whereby Annexin II regulates cancer progression in pancreatic cancer cells under exposure to gemcitabine.

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ORIGINAL ARTICLE

Apolipoprotein C-1 maintains cell survival by preventing from apoptosis in pancreatic cancer cells

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Pancreatic cancer still remains one of the most lethal diseases and establishment of new therapy is needed. The purpose of this study is to find novel factors involved in pancreatic cancer progression by proteomic approach. We compared pre- and postoperative serum protein profiling obtained from pancreatic cancer patients who had curative pancreatectomy using surface-enhanced laser desorption ionization time-of-flight mass spectrometry. The peak intensity levels of both 6630 and 6420 Da were significantly higher in the preoperative serum than in the postoperative serum ($P < 0.002$). Sequential amino acid analysis identified these proteins to be apolipoprotein C-1 (ApoC-1). The high level of ApoC-1 in preoperative serum significantly correlated with poor prognosis. Furthermore, ApoC-1 was abundantly expressed in pancreas neoplastic epithelium, and was detected in the culture medium of the pancreatic cancer cell line *in vitro*, which suggests that cancer cells secrete ApoC-1. Inhibition of ApoC-1 expression by short interfering RNA suppressed cell proliferation and induced apoptosis of pancreatic cancer cells. The specific expression of ApoC-1 and its role in preventing from spontaneous apoptosis in pancreatic cancer cells suggest that ApoC-1 contributes to the aggressiveness of pancreatic cancer and will be useful as a new therapeutic target.
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Keywords: apolipoprotein C-1; pancreatic cancer; SELDI-TOF MS; serum marker; apoptosis

Introduction

Pancreatic cancer continues to be one of the most lethal malignancies, with a 5-year survival rate of only 4–5% (Jemal *et al.*, 2006). Recent advances in the clinical management of this disease, especially new chemotherapeutic reagents, such as gemcitabine (Burris *et al.*, 1997), have improved patient outcome. In addition, recent progress in molecular cancer biology has led to the development of new molecular targeting therapies for pancreatic cancer; some of these new drugs, such as erlotinib, an epidermal growth factor receptor inhibitor (Moore *et al.*, 2007), have already shown clinical benefits. However, the efficacy of these new therapies has not been sufficient enough, with only a few months' improvement in median survival time, and additional molecular targets are urgently needed.

So far, genetic studies have identified the signature molecular profiles of this malignancy, consisting of mutation in KRAS, CDKN2A, TP53 and SMAD4/DPC4 (Bardeesy and Depinho, 2002). These genetic mutations have been revealed by classical methods of molecular biology, such as genetic analysis of familial cancer syndrome and systematic scan of a loss of heterozygosity in patients with this disease. Recently, development of new technologies has enabled the mass analysis of genetic and proteomic profiles in cancer biology. Among these technologies, proteomic approaches are used to identify new cancer biomarkers. The technique has advantages for detecting differences in protein profiling including post-translational modifications. Although standard methods for protein profiling, such as two-dimensional gel electrophoresis (2DE), have been successfully used to identify new proteins involved in cancer development (Tomonaga *et al.*, 2004), these methods have several limitations. Hydrophobic and low molecular weight proteins resolve poorly, and proteins present in low concentrations often cannot be detected. Surface-enhanced laser desorption and ionization time-of-flight mass spectrometry (SELDI-TOF MS) combined with the ProteinChip array provides a potentially powerful tool for overcoming these limitations. This procedure results in the identification of protein profiles composed of isolated or clustered peaks that differ according to molecular weight. Thus, different pathological conditions can be identified with high sensitivity and significant reproducibility. This approach has been used to identify specific and sensitive molecular markers (Nomura *et al.*, 2004) in patients with prostatic, pancreatic, liver, colorectal and ovarian malignancies (Xiao *et al.*, 2001; Petricoin *et al.*,

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2002; Rosty et al., 2002; Melle et al., 2005; Paradis et al., 2005).

In this study, we sought to identify a molecule that may be involved in pancreatic cancer development or progression using this proteomic approach. Specifically, we used paired pre- and postoperative serum samples obtained from the same patients to exclude individual differences. We found successfully a serum protein, which was upregulated in preoperative serum of pancreatic cancer patients. We also identified an unexpected biological role of this protein in pancreatic cancer cells. Our results indicate that the use of proteomic approach will lead to new insights in research dealing with cancer biology through detection of new biomarkers and elucidation of the molecular mechanisms of cancer.

Results

Serum protein profiling associated with pancreatic cancer based on SELDI-TOF MS analysis

To identify new serum protein characteristics in pancreatic cancer patients, we compared the protein profiling of serum collected from the same patients before and after curative resection to exclude individual differences in serum protein expression. Using SELDI-TOF MS analysis and ProteinChip arrays, serum protein profiling of pre- and postoperative sera were compared in 20 pancreatic cancer patients who had curative resection (Table 1). The Biomarker Wizard function of the ProteinChip software identified clusters of 85 peaks, which had different expression levels in pre- and postoperative sera of pancreatic cancer patients. Proteins with 6630 and 6420 Da m/z peaks in the urea buffer at pH 6.5 were candidates for pancreatic cancer-specific serum proteins (Figure 1a). A SELDI profile of 6630 Da showed a higher intensity in the preoperative serum than in the postoperative serum of 15 patients (75%); the average of 6630 Da peak normalized intensities of the preoperative serum of all 20 patients (mean ± s.d.; 2.80 ± 1.95) was significantly higher than that of the postoperative serum (1.65 ± 1.61; $P<0.002$; Student's paired t-test; Figure 1b). Peak intensities at 6420 Da also showed a significantly higher average peak intensity in the preoperative serum of 20 patients (1.46 ± 1.10) than in their postoperative serum (0.92 ± 0.78; $P<0.002$; Student's paired t-test; data not

Table 1 Characteristics of pancreatic cancer patients in SELDI-TOF MS analysis

	20 patients of test set				*69 patients of validation set*			
		ApoC-1 PI				*ApoC-1 PI*		
	Total	*LL*	*HL*	P	*Total*	*LL*	*HL*	P
Sex				NS				NS
M	12	6	6		43	22	21	
F	8	4	4		26	12	14	
Age				NS				NS
Mean ± s.d.	59.9 ± 9.0	58.9 ± 9.9	60.8 ± 7.9		62.9 ± 9.2	62.1 ± 8.4	63.8 ± 10.0	
Jaundice				NS				NS
(T-bil > 2.0 mg per dilution)								
+	3	2	1		19	8	11	
−	17	8	9		50	26	24	
Diabetes mellitus				NS				NS
(FBS > 130 mg per dilution)								
+	4	2	2		25	13	12	
−	16	8	8		44	21	23	
Stage				NS				NS
IA	2	2	0		2	2	0	
IB	0	0	0		1	1	0	
IIA	5	3	2		14	7	7	
IIB	13	5	8		51	24	27	
III	0	0	0		1	0	1	
Histology				NS				NS
Tubular adenocarcinoma								
Well	0	0	0		7	3	4	
Mod	13	6	7		41	21	20	
Poor	5	2	3		13	4	9	
Anaplastic ca.	2	2	0		6	5	1	
Adenosquamous ca.	0	0	0		2	1	1	

Abbreviations: APOC-1, apolipoprotein C-1; ca., carcinoma; FBS, fasting blood sugar; HL, high level; LL, low level; Mod, moderately differentiated; NS, not significant; PI, peak intensity; Poor, poorly differentiated; T-bil, total bilirubin; Well, well differentiated. Patient stage was determined according to the UICC TNM classification.

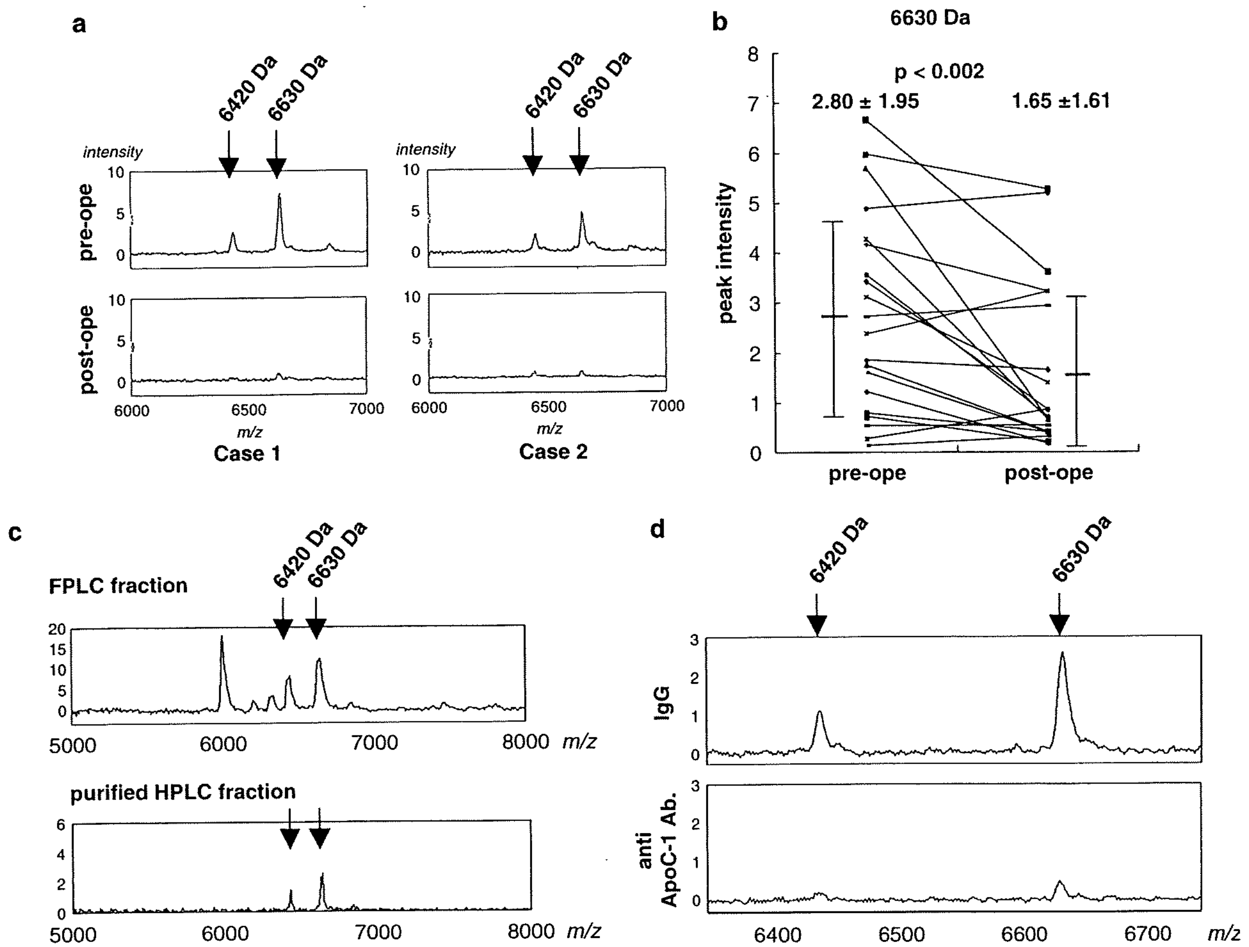

Figure 1 SELDI-TOF MS analysis of serum obtained from pancreatic cancer patients. Peak intensities of 6630 and 6420 Da proteins were reduced after curative surgery. (**a**) Representative spectra of SELDI-TOF MS analysis using WCX2 array. Upper and lower panels show a portion of the 6630 and 6420 Da protein profiles in pre- (upper panel) and postoperative (lower panel) serum, respectively. (**b**) Comparison between peak intensities of 6630 Da protein in the pre- (mean ± s.d., 2.80 ± 1.95) and postoperative (1.65 ± 1.61) sera of 20 pancreatic cancer cases by ProteinChip analysis. Purification and identification of 6630 and 6420 Da proteins. (**c**) Crude serum subjected to ion-exchange fractionation by fast protein liquid chromatography (FPLC) under optimal conditions was monitored on the NP20 ProteinChip array (upper panel). The purified fraction subjected to the second HPLC was monitored on the Gold Chip array (lower panel). (**d**) The identified proteins were confirmed to be apolipoprotein C-1 (ApoC-1) on immunodepletion assay. Two peaks were present in samples reacted with control mouse immunoglobulin G (IgG; upper panel). They were clearly decreased when beads treated with monoclonal anti-human ApoC-1 antibody were used (lower panel).

shown). We selected these two peaks for further analysis.

Identification of 6630 and 6420 Da proteins as apolipoprotein C-1

We next tried to purify and identify these proteins. The optimized purification conditions were directly transferred to fractionations using fast protein liquid chromatography (FPLC). The eluate was applied to reverse phase columns for further separation from other proteins, and the fractionation was carried out with a stepwise gradient, using two-dimensional high performance liquid chromatography (HPLC). Using these procedures, the target 6630 and 6420 Da proteins were successfully purified (Figure 1c).

N-terminal amino-acid sequence analysis of the purified proteins revealed that they were apolipoprotein C-1 (ApoC-1; Lauer *et al.*, 1988). This analysis was carried out with completely identifying the first 15 amino-acid sequences on the N terminus. We obtained two sequences, which were identical except for two additional amino acids on the N terminus. ApoC-1 is known to have molecular sizes of 6630 and 6420 Da (Wroblewski *et al.*, 2006), which may represent these two amino-acids truncation. The immunodepletion assay confirmed that these peaks were ApoC-1; the reaction of serum with control mouse immunoglobulin G (IgG) did not affect the 6630 and 6420 Da peaks of the ProteinChip array, whereas both peaks were clearly decreased when the anti-ApoC-1 antibody was used (Figure 1d). In addition, western blot analysis of