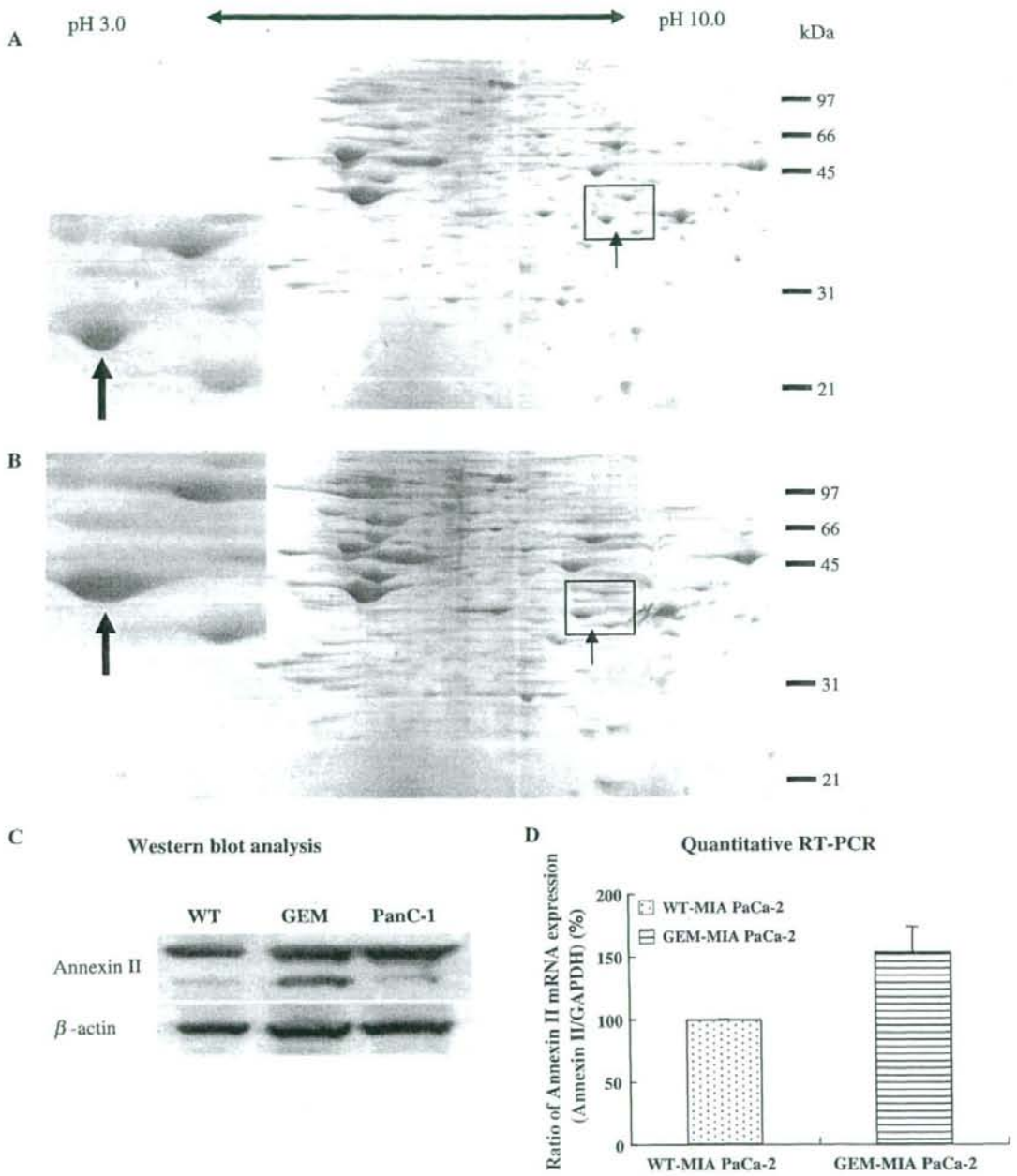

FIG. 1. Coomassie blue-stained agarose two-dimensional gel electrophoresis (2-DE) protein expression profiling of pancreatic cancer cell lines, (**A**) the wild-type MIA PaCa-2 (WT-MIAPaCa-2) cell line and (**B**) the gemcitabine-resistant cell line (GEM-MIAPaCa-2). Arrows with the square in (**A**) 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.

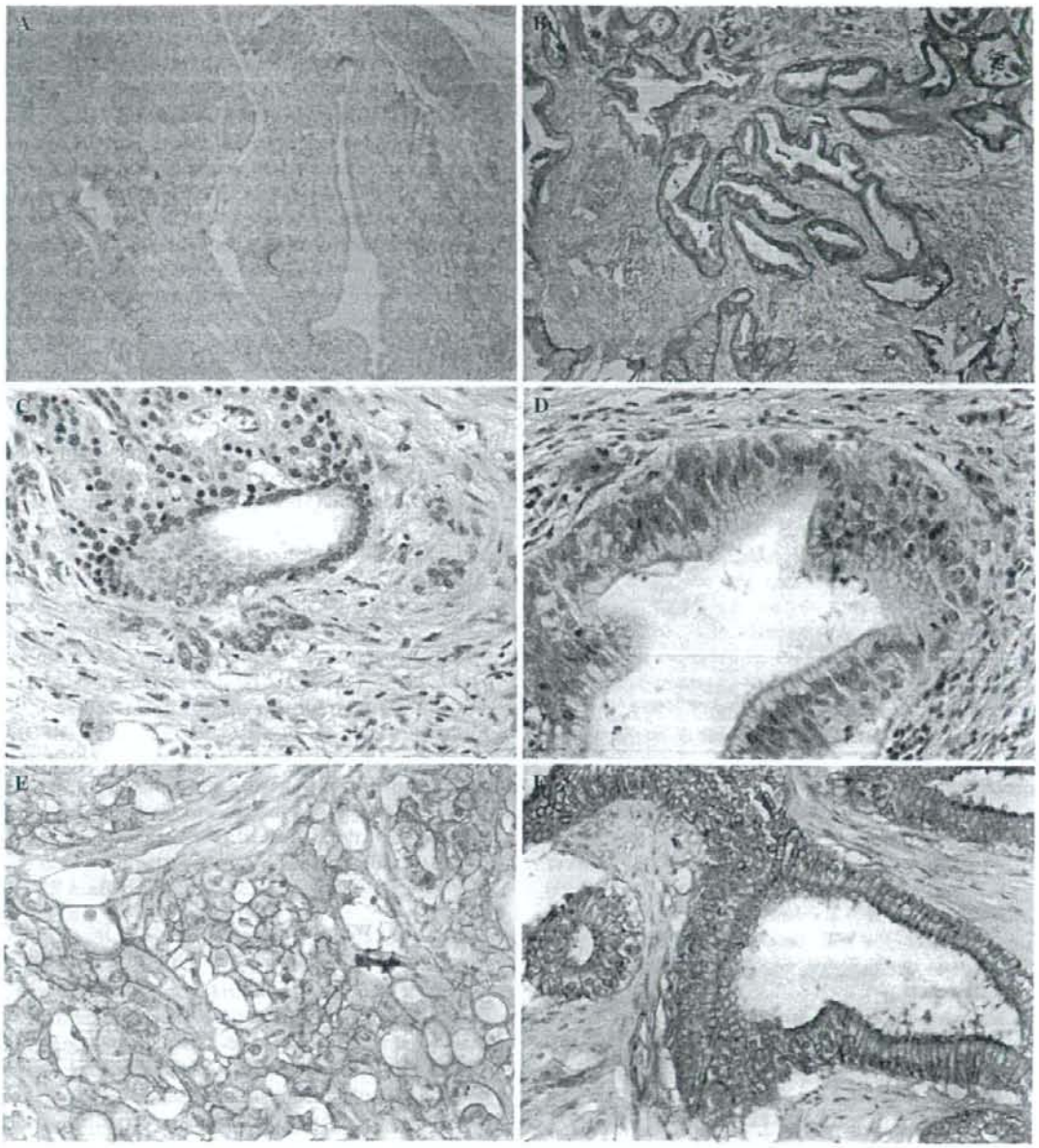

FIG. 2. Immunohistochemistry for Annexin II in normal pancreatic tissue and invasive pancreatic ductal carcinomas. (**A**) Annexin II was weakly expressed in acinar and pancreatic ductal cells in normal pancreas tissue (original magnification 100×). (**B**) Annexin II was strongly overexpressed in the 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

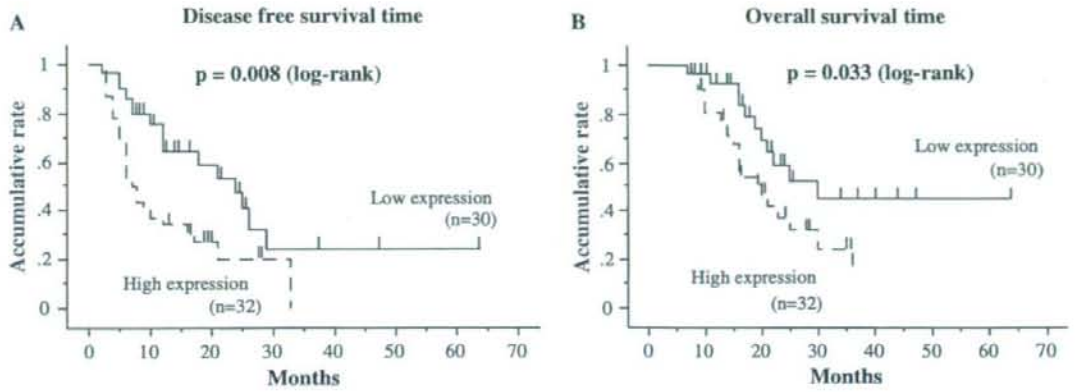

FIG. 3. Annexin II overexpression in pancreatic cancer is significantly associated with rapid recurrence and shorter survival time of patients with adjuvant gemcitabine chemotherapy in the Kaplan-Meier analysis. (A) Disease-free survival time was shorter in patients with high expression of Annexin II 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

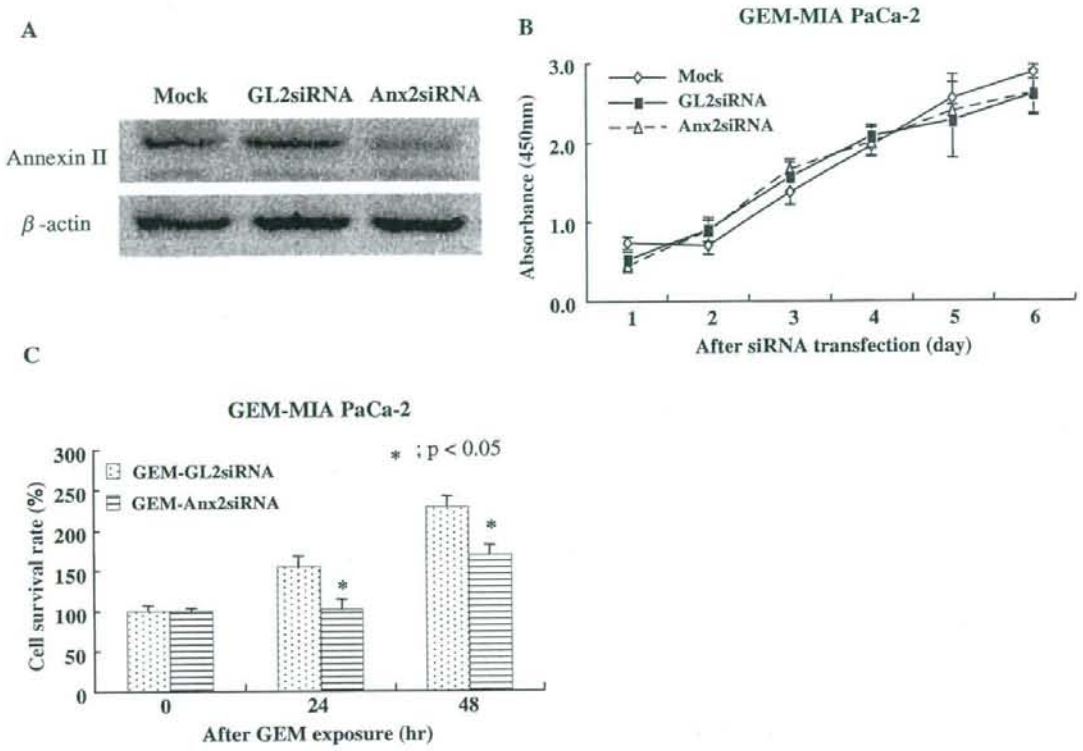

FIG. 4. Suppression of Annexin II expression increases the sensitivity to gemcitabine in gemcitabine-resistant pancreatic cancer cells. **(A)** Anx2siRNA inhibited Annexin II protein expression in GEM-MIA PaCa-2 in contrast with GL2siRNA. Western blot analysis of Annexin II and β-actin as control. Cells 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

S Takano[1,2], H Yoshitomi[1], A Togawa[1], K Sogawa[2], T Shida[1], F Kimura[1], H Shimizu[1], T Tomonaga[2], F Nomura[2] and M Miyazaki[1]

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

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 chemotherapeautic 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.*,

Correspondence: Dr H Yoshitomi, Department of General Surgery, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan.
E-mail: yoshitomi@faculty.chiba-u.jp
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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 (T-bil > 2.0 mg per dilution)				NS				NS
+	3	2	1		19	8	11	
−	17	8	9		50	26	24	
Diabetes mellitus (FBS > 130 mg per dilution)				NS				NS
+	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.

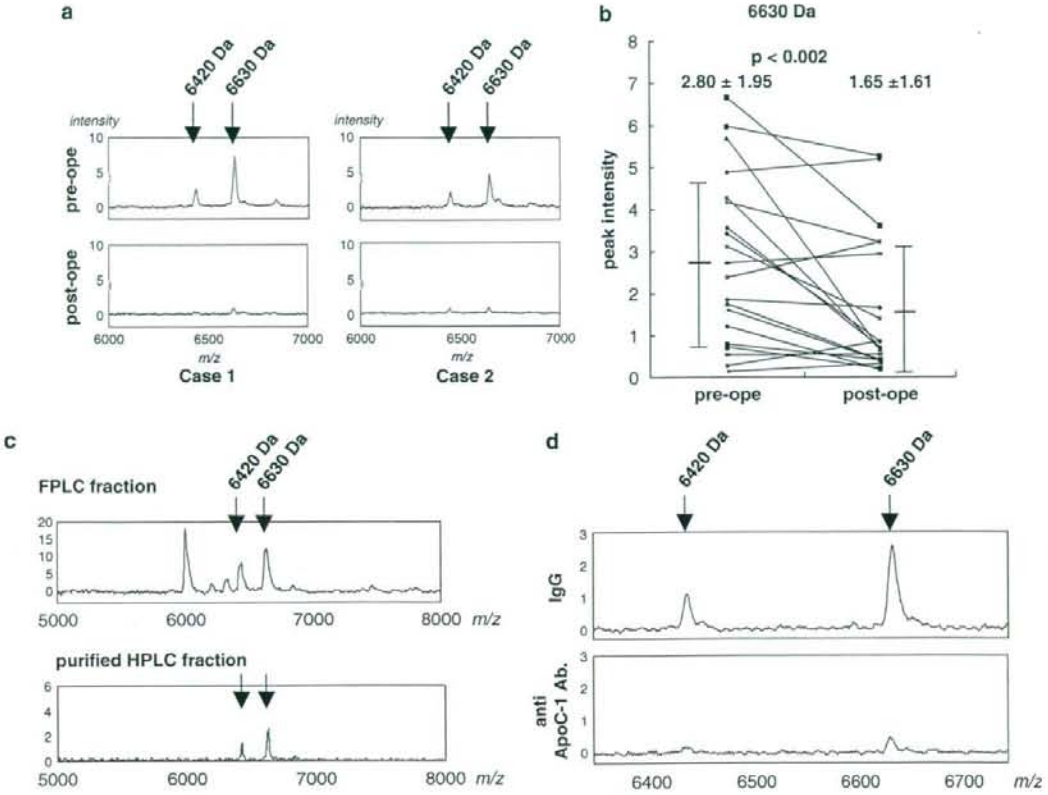

Figure 1 SELDI-TOF MS analysis of serum obtained from pancreatic cancer patients. Peak intensities of 6630 and 6420 Da proteins were reduced after curative surgery. (**a**) Representative spectra of SELDI-TOF MS analysis using WCX2 array. Upper and lower panels show a portion of the 6630 and 6420 Da 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

the HPLC final fraction also showed that the band reacted with anti-human ApoC-1 antibody (data not shown).

A high serum ApoC-1 level is associated with a poor prognosis in pancreatic cancer patients

To examine the clinical significance of the ApoC-1 serum level, we assessed whether the ApoC-1 peak intensity level in the serum was clinically relevant in pancreatic cancer patients. Twenty patients were divided into two groups of 10 patients each based on the median value of the sum of 6630 and 6420 Da peak intensity levels of their preoperative serum: the low-level group (LL) had levels below the median value, and the high-level group (HL) had levels above the median value. The Kaplan–Meier analysis showed that the LL group patients had a significantly longer overall survival time than the HL group patients ($P=0.005$, log-rank test; Figure 2a). To confirm this result, we analysed the peak intensity levels of the 6630 and 6420 Da peptide in the preoperative serum samples from an independent group of 69 pancreatic cancer patients who had surgical resection of cancer (Table 1). The 69 patients were also divided into two groups based on the median value of the sum of 6630 and 6420 Da peak intensity preoperative serum levels. In this 69 patients group, the overall survival time was also longer in LL group patients than in HL group patients ($P=0.023$, log-rank test; Figure 2b).

We next compared the usefulness of the serum ApoC-1 peak intensity levels as a prognostic marker for pancreatic cancer with serum level of carbohydrate tumor-associated antigen 19-9 (CA19-9), which is most popular serum marker for pancreatic cancer. Again, these 69 patients were divided into two groups based on the median value of preoperative serum CA19-9 levels (median values: 175 U ml^{-1}; HL group, patients with CA19-9 level higher than 175 U ml^{-1}; LL group, patients with CA19-9 level lower than 175 U ml^{-1}). However, there was no statistically significant difference of the overall survival time between these two groups ($P=0.090$, log-rank test; Figure 2c). We also carried out the receiver-operator-characteristics (ROC) analysis between patients with survival time more and less than 2 years. The respective area under the ROC curve (AUC) was 0.66 for ApoC-1 peak intensity and 0.60 for CA19-9 level (Figure 2d). These results indicated that serum ApoC-1 peak intensity had the better ability as a prognostic marker than serum CA19-9 level.

On univariate analysis, tumor size ($\geqslant 30$ mm vs <30 mm), the existence of lymph node metastasis, UICC classification stage (IIB, III vs I, IIA), and serum ApoC-1 peak intensity level (HL vs LL) were correlated with overall survival time. Furthermore, among these factors, only serum ApoC-1 peak intensity level was an independent prognostic factor on multivariate analysis (hazard ratio; 2.160, 95% confidence interval; 1.084–4.302, $P=0.0285$; Table 2).

Abundant expression of ApoC-1 in pancreatic cancer tissues

The decreased serum ApoC-1 level, which was found after curative surgery, suggested that ApoC-1 was overexpressed in cancerous tissue. To confirm this, we analysed the expression of ApoC-1 in pancreatic cancer tissues. Reverse transcription (RT)–PCR revealed that the expression level of ApoC-1 mRNA was much higher in pancreatic cancer tissues than in adjacent normal pancreatic tissue (Figure 3a). Quantitative RT–PCR using samples of resected pancreatic tissue obtained from 16 patients confirmed this finding; a significantly higher ApoC-1 mRNA level was found in pancreatic cancer tissues than in adjacent normal pancreatic tissue (11.49 ± 15.91- and 0.21 ± 0.18-fold ApoC-1/glyceraldehyde-3-phosphate dehydrogenase mRNA copy number, respectively, $P<0.0001$; Mann–Whitney U-test; Figure 3b).

Western blot analysis also confirmed that ApoC-1 protein was expressed in pancreatic cancer tissues. The cancerous tissues of the four cases that were examined were positive for ApoC-1 protein with a 6.6 Da band; in contrast, a same size band could not be found in adjacent normal pancreatic tissues (Figure 3c).

ApoC-1 is expressed in neoplastic epithelium of pancreatic cancer

To examine the localization of ApoC-1 in pancreatic cancer tissues, we carried out immunohistochemical staining for ApoC-1 in 66 invasive pancreatic ductal carcinoma tissues and in adjacent normal pancreatic tissues. In Figure 4a, hepatocytes from normal liver tissue, which are known to express ApoC-1 (Schaefer *et al*., 1978; Lauer *et al*., 1988), are clearly stained with anti-ApoC-1 antibody. As previously reported (Lauer *et al*., 1988), ApoC-1 expression was negative in pancreatic ductal cells that were located in adjacent normal pancreatic tissue (Figure 4b). On the other hand, ApoC-1 expression was found in carcinoma cells of 48 of 66 invasive pancreatic ductal carcinoma cases (72.7%; Figures 4c–f). The ApoC-1 expression was localized in the neoplastic epithelial cells and was not found in the stromal cells surrounding the ductal carcinoma cells. Moderately differentiated adenocarcinoma (Figures 4c and d) and poorly differentiated adenocarcinoma (Figures 4e and f) were almost equally stained with ApoC-1 antibody.

Furthermore, we analysed the correlation of ApoC-1 expression in carcinoma cells with serum ApoC-1 peak intensity level of SELDI in 66 pancreatic cancer patients. Interestingly, positive staining of ApoC-1 significantly correlated with high serum ApoC-1 peak intensity level ($P=0.036$; Mann–Whitney U-test).

ApoC-1 is secreted from pancreatic cancer cells

Next, we investigated whether ApoC-1 was also expressed in pancreatic cancer cell lines. In all four pancreatic cancer cell lines (MIA PaCa II, PanC-1, CFPAC-1 and AsPC-1), ApoC-1 expression was confirmed by RT–PCR (Figure 5a) and western blot

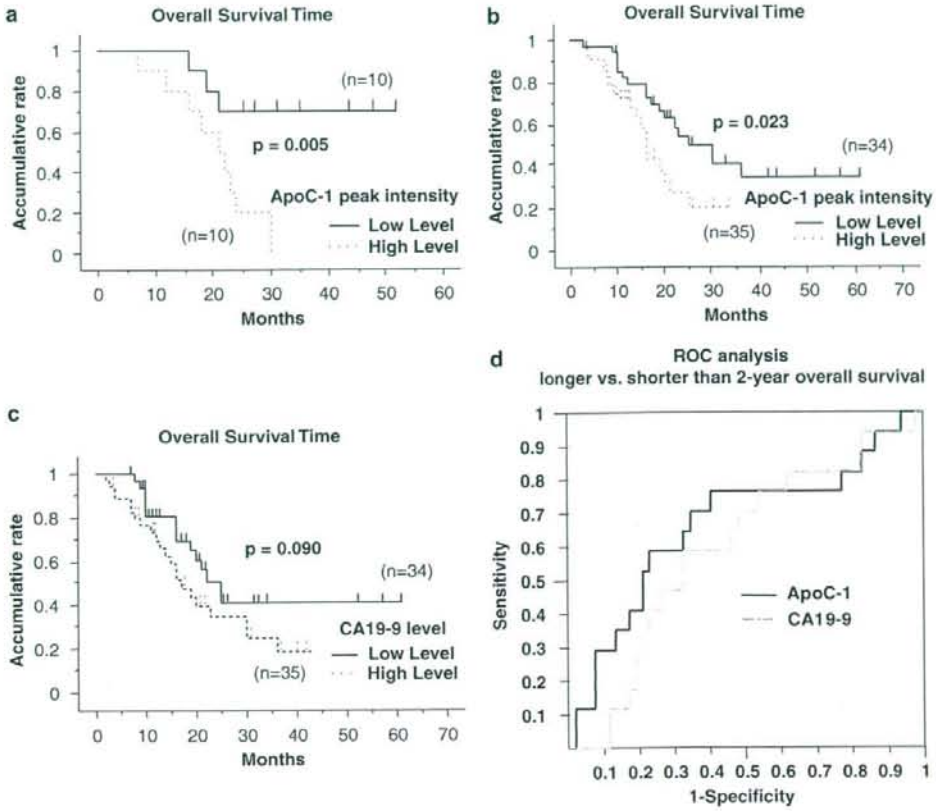

Figure 2 The Kaplan–Meier and receiver-operator-characteristics (ROC) analysis revealed that the peak intensity of apolipoprotein C-1 (ApoC-1) in the preoperative serum correlates with the overall survival of pancreatic cancer patients. (**a**) A total of 20 patients were divided into two groups ($n = 10$ for each group) based on the median value of their preoperative ApoC-1 peak intensity serum level (6630 + 6420 Da peak; LL, low-level group (peak intensity < median value); HL, high-level group (peak intensity > median value)). HL patients had a significantly shorter overall survival time than LL patients. (**b**) An independent group of 69 pancreatic cancer patients was divided into two groups based on their ApoC-1 peak intensity preoperative serum levels (6630 + 6420 Da peak; LL; $n = 34$, HL; $n = 35$, for each group). HL patients had a significantly shorter overall survival time than LL patients. (**c**) These 69 pancreatic cancer patients were divided into two groups based on their CA19-9 preoperative serum levels (cutoff level; 175 U ml^{-1} (median value of these patients), LL; $n = 34$, HL; $n = 35$, for each group). There was no significant difference in overall survival time between their two groups. (**d**) The ROC analyses were carried out for preoperative ApoC-1 peak intensity and CA19-9 serum levels between patients with longer and shorter than 2-year survival time. The respective AUCs were 0.66 for ApoC-1 peak intensity and 0.60 for CA19-9 level.

Table 2 Prognostic factors of 69 pancreatic cancer patients in Cox's proportional hazards model

Overall survival	*Univariate analysis*			*Multivariate analysis*		
Variables	*Hazard ratio*	*95% CI*	P	*Hazard ratio*	*95% CI*	P
Age (> 64 years per ≤ 63 years)	1.251	0.654 ~ 2.394	0.4989			
Sex (F/M)	0.814	0.413 ~ 1.606	0.5526			
ApoC-1 peak intensity (H/L)	2.117	1.081 ~ 4.143	0.0286*	2.160	1.084 ~ 4.302	0.0285*
CA19-9 level (H/L)	1.739	0.901 ~ 3.358	0.0993			
N (+/–)	2.611	1.087 ~ 6.273	0.0318*			
Tumor size (≥ 30 mm per < 30 mm)	2.137	1.093 ~ 4.177	0.0264*			
UICC-stage (IIB, III/I, IIA)	2.871	1.117 ~ 7.377	0.0285*			

Abbreviations: F, female; M, male;*Significant value.

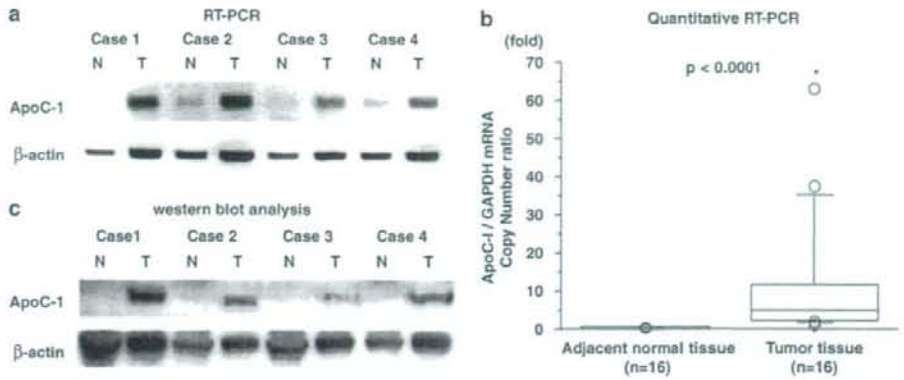

Figure 3 Pancreatic cancer tissues expressed more apolipoprotein C-1 (ApoC-1) mRNA and protein than adjacent normal pancreatic tissues. (**a**) Reverse transcription (RT)–PCR showed stronger ApoC-1 mRNA expression in pancreatic cancer tissues (T) than in adjacent normal pancreatic tissues (N) obtained from four patients. (**b**) Quantitative RT–PCR showed significantly higher ApoC-1 mRNA expression in pancreatic cancer tissues ($n = 16$) than in adjacent normal pancreatic tissues ($n = 16$). (**c**) Western blot analysis of ApoC-1. Western blot analysis showed abundant ApoC-1 protein expression in pancreatic cancer tissues (T), but not in adjacent normal pancreatic tissues (N) in all four cases examined.

analysis (Figure 5b). Furthermore, we investigated the possibility that ApoC-1 is secreted from pancreatic cancer cells. Western blot analysis showed that ApoC-1 protein with a 6.6 Da band was present in the medium with cultured MIA PaCa II cells, but not in the medium with no cultured cells (Figure 5c). These results indicated that the MIA PaCa II cells secreted ApoC-1.

Inhibition of ApoC-1 expression by siRNA suppresses cell proliferation of pancreatic cancer cells by inducing apoptotic cell death

The strong correlation of ApoC-1 serum level with poor clinical outcome, accompanied by expression in tumor cells, may indicate the involvement of this protein in cancer progression. For this reason, we next examined whether gene knockdown of this protein would affect the proliferation of pancreatic cancer cells. To specifically silence the ApoC-1 gene, two pancreatic cancer cell lines (MIA PaCa II and AsPC-1) were transfected with short interfering RNA (siRNA) targeting ApoC-1 mRNA (ApoC-1 siRNA1 and -2) or GL2siRNA as a negative control. The suppression of ApoC-1 mRNA level by transfection with 20 nM ApoC-1 siRNA1 and -2 were confirmed in both MIA PaCa II and AsPC-1 (Figure 5d). ApoC-1 protein levels were successfully reduced with ApoC-1 siRNA1 at 5–200 nM concentration 48 h after transfection, as confirmed by western blot analysis (Figure 5e). ApoC-1 siRNA2 also showed similar effects in reducing ApoC-1 protein expression level in MIA PaCa II (Figure 5f; 20 nM concentration of each siRNA).

Pancreatic cancer cells proliferation was comparatively determined by cell counting after the transfection with Mock, GL2siRNA, ApoC-1 siRNA1 and -2. MIA PaCa II and AsPC-1 cells were transfected with 20 nM siRNA, and total cell proliferation was counted 1–4 days after transfection. Cell proliferation was significantly suppressed by transfection with ApoC-1 siRNA1 and -2, compared with cells treated with GL2siRNA in both cell lines (Figure 6a). Interestingly, Trypan blue staining showed that the percentage of dead cells was significantly increased from 2 days after transfection with two ApoC-1 siRNA1 and -2, compared with cells transfected with GL2siRNA (Figure 6b). We also investigated the effect of ApoC-1 silencing on the invasion ability of cancer cells. The cell invasion ability was not affected by gene silencing by transfection of ApoC-1 siRNA1 and -2, compared with cells treated with GL2siRNA in both cell lines (see Supplementary data).

These results indicate that inhibition of ApoC-1 expression may induce apoptotic cell death in pancreatic cancer cells. To examine this, we investigated whether the rate of apoptotic cell death was increased by treatment with ApoC-1 siRNA in these pancreatic cancer cells. ApoC-1 siRNA1 treatment obviously increased the number of apoptotic cells, which were stained purple red, compared with cells treated with control GL2siRNA (Figure 7a). The proportion of apoptotic cells was significantly higher in MIA PaCa II cells treated with ApoC-1 siRNA1 than GL2siRNA at 24 h after transfection ($P<0.002$; Student's paired t-test; Figure 7b). We also investigated whether the inhibition of ApoC-1 led to activate the procaspase-3 in MIA PaCa II cells. As shown in Figure 7c, the western blot analysis showed that procaspase-3 was more cleaved to activate forms by transfection with both ApoC-1 siRNA1 and -2, compared with GL2siRNA, in MIA PaCa II cells. As well, the caspase-3 activity was significantly higher in MIA PaCa II cells treated with ApoC-1 siRNA1 than GL2siRNA at 24 h after transfection ($P<0.02$; Student's paired t-test; Figure 7d). These results confirmed that the inhibition of ApoC-1 expression induced apoptosis in pancreatic cancer cells.

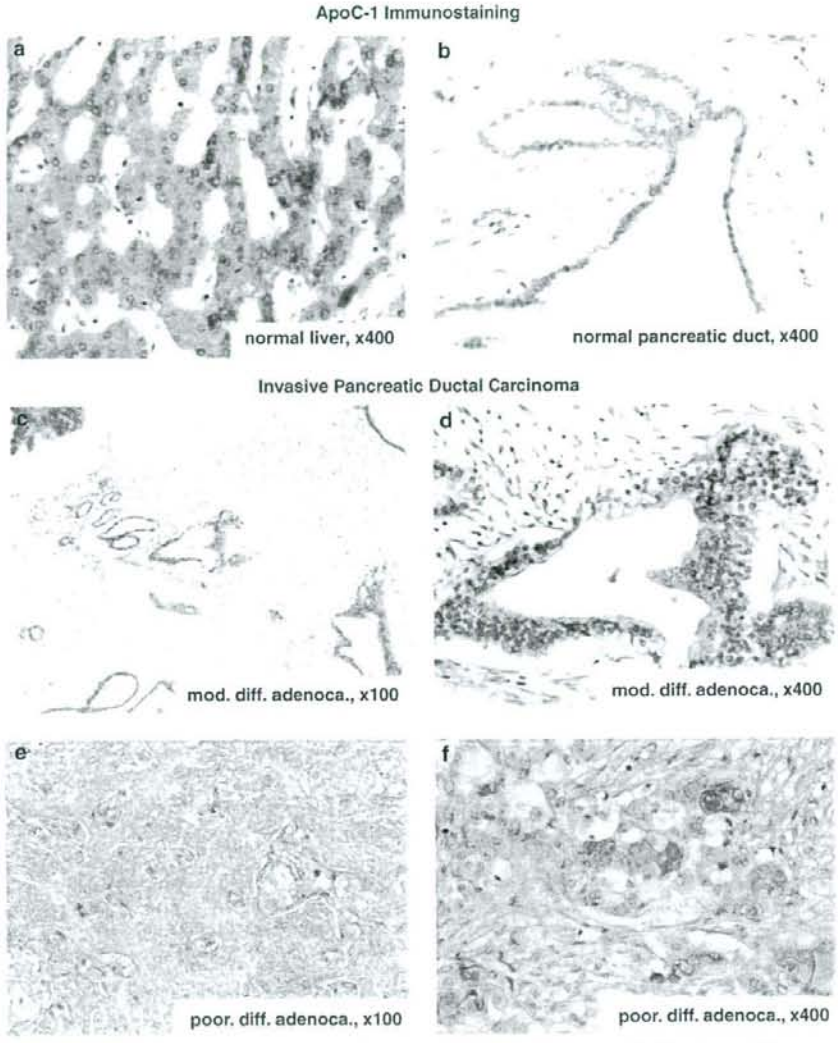

Figure 4 Immunostaining of apolipoprotein C-1 (ApoC-1) showed that ApoC-1 is specifically expressed in pancreatic cancer epithelium. (**a**) ApoC-1 staining in hepatocytes as a positive control (× 400). (**b**) Normal pancreatic ductal cells do not express ApoC-1 (× 400). (**c**–**f**) Pancreatic invasive ductal carcinoma cells express ApoC-1. Moderately differentiated adenocarcinoma (**c**, × 100; **d**, × 400) and poorly differentiated adenocarcinoma (**e**, × 100; **f**, × 400). Note that ApoC-1 expression is limited to the cancer epithelium, but is not found in stromal cells surrounding the cancer.

To analyse the effects of ApoC-1 gene knockdown in individual cells, immunofluorescence studies were carried out in MIA PaCa II cells treated with GL2siRNA (Figures 7e–g) and ApoC-1 siRNA (Figures 7h–j). Although the abundant expression of ApoC-1 could be found in cells treated with GL2siRNA (Figures 7e–g, red staining), many cells displayed only faint or no fluorostaining with anti-ApoC-1 antibody due to ApoC-1 siRNA treatment. These cells with faint ApoC-1 staining also showed typical apoptotic features, including dense chromatin condensation with 4,6-diamidino-2-phenylindole (DAPI) staining (Figure 7i, arrow; magnified figure is shown in Figure 7l), whereas the GL2siRNA treatment cells that still expressed high amount of ApoC-1 did not show the features (Figure 7f; magnified figure is shown in Figure 7k). These data also revealed that the inhibition of ApoC-1 resulted in apoptotic cell death in pancreatic cancer cells.

Discussion

To identify molecules related to pancreatic cancer progression, we used a new strategy based on the proteomic approach. We identified serum proteins that

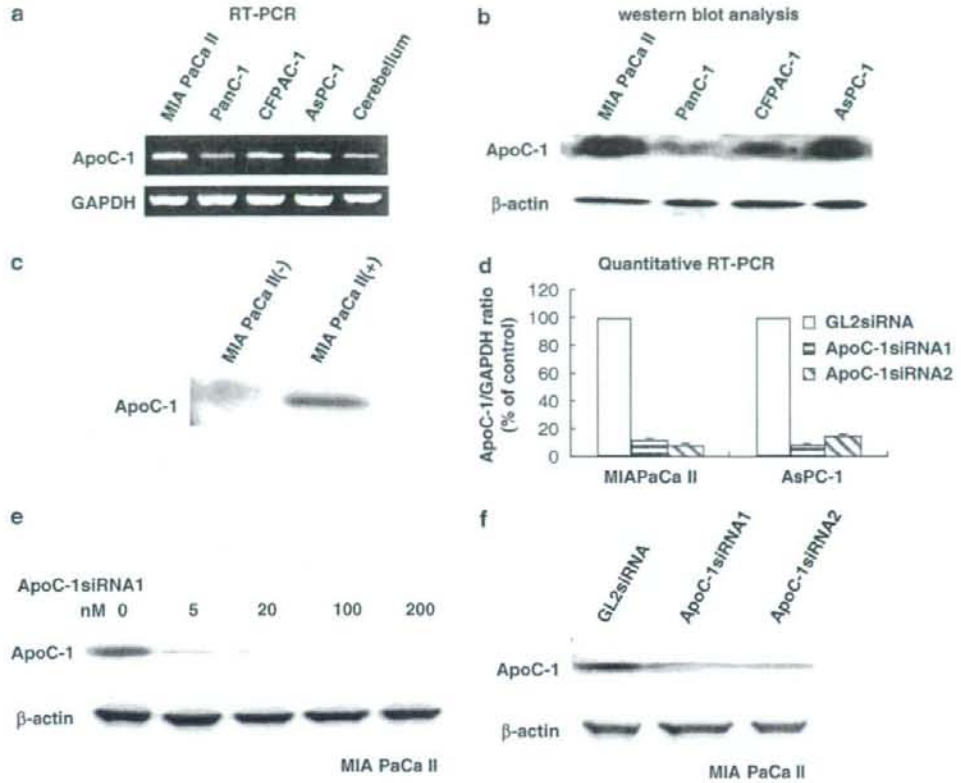

Figure 5 Apolipoprotein C-1 (ApoC-1) is expressed in pancreatic cancer cell lines and its expression is inhibited by short interfering RNA (siRNA) treatment. (**a** and **b**) ApoC-1 is expressed in all four pancreatic cancer cell lines (MIA PaCa II, PanC-1, CFPAC-1 and AsPC-1) analysed by reverse transcription (RT)–PCR (**a**) and western blot (**b**). (**c**) The supernatant of the medium in which no cells were cultured was used as a negative control (left lane). The supernatant of the medium in which MIA PaCa II cells were cultured expressed ApoC-1 protein with a 6.6 Da band (right lane). (**d**) ApoC-1 siRNA1 and -2 inhibit ApoC-1 mRNA expression in MIA PaCa II and AsPC-1. Cells are transfected with ApoC-1 siRNA1 and -2 at 20 nM for 24 h and mRNA levels normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA copy number are detected by quantitative RT–PCR. (**e**) ApoC-1 siRNA1 specifically inhibits ApoC-1 protein expression in MIA PaCa II in a dose-dependent manner. Western blot analysis of ApoC-1 and β-actin. Cells were transfected with ApoC-1 siRNA1 at the indicated concentration for 48 h. (**f**) Both ApoC-1 siRNA1 and -2 inhibit ApoC-1 protein expression in MIA PaCa II in contrast with GL2siRNA. Cells are transfected with siRNAs at 20 nM for 48 h.

were highly expressed in patients with pancreatic cancer using SELDI-TOF MS. The unique strength of SELDI-TOF MS is its ability to analyse proteins from a variety of crude samples with minimal sample consumption; this enables high-throughput analysis (Xiao *et al.*, 2005). In addition, SELDI-TOF MS has an advantage in resolving hydrophobic and low molecular weight proteins, as compared to conventional 2DE analyses. Thus, SELDI-TOF MS has been used extensively in cancer research and has led to the discovery of better serum markers for many cancers. When analysing human samples, it is important to analyse many samples to diminish individual background differences in protein expression. To minimize this, we compared serum samples obtained from the same patient before and after curative surgery. Using this strategy, we successfully identified proteins whose serum expression levels were reduced after curative surgery, based on the analysis of samples from a limited number of patients. Protein purification and amino-acid sequence analysis identified the proteins to be ApoC-1. Surprisingly, the SELDI peak intensity level of ApoC-1 in the preoperative serum was significantly correlated with patients' overall survival. This result was confirmed by a validation study involving 69 serum samples from an independent group of pancreatic cancer patients.

Serum CA19-9 level has been used as marker for the pancreatic cancer (Rhodes, 1999). Recently Ferrone *et al.* (2006) showed that perioperative CA19-9 levels predicted survival in patients with curative resection of pancreatic cancer. However, in our study, preoperative ApoC-1 peak intensity level of SELDI is better prognostic serum factor than serum CA19-9 levels in both analysis of Kaplan–Meier method and ROC curve.

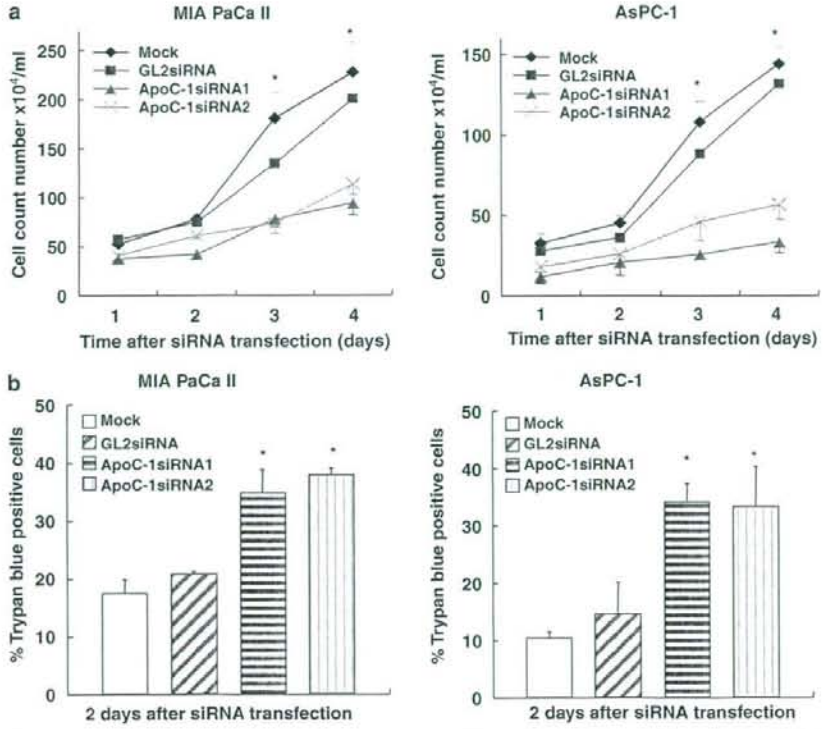

Figure 6 Inhibition of apolipoprotein C-1 (ApoC-1) suppresses cell proliferation and increases cell death. (**a**) Inhibition of ApoC-1 expression by ApoC-1 short interfering RNAs (siRNAs) significantly suppresses proliferation of pancreatic cancer cell lines (MIA PaCa II and AsPC-1). Cells were transfected with Mock, GL2siRNA, ApoC-1 siRNA1 and -2 and cultured for the indicated time (differences between GL2siRNA- and ApoC-1 siRNAs-treated cells; $^*P<0.05$). (**b**) ApoC-1 siRNAs transfection increased the ratio of dead cells in MIA PaCa II and AsPC-1. Cell death was determined by the Trypan blue exclusion test at 2 days after siRNAs transfection. ApoC-1 siRNA transfected cells show significantly higher proportion of dead cells than GL2siRNA-transfected cells ($^*P<0.05$).

This finding indicates the usefulness of the ApoC-1 serum level as a potentially prognostic marker for pancreatic cancer. In support of this, using serum protein profiling, several groups have recently reported increased serum levels of other apolipoproteins in patients with several types of cancer (Yu *et al*., 2005; Goncalves *et al*., 2006). On the other hand, Ehmann *et al*. (2007) recently found that serum levels of different apolipoproteins (ApoA-1 and -2) were decreased in pancreatic cancer patients compared with healthy volunteers. These facts may indicate that several apolipoproteins have different roles in pancreatic cancer development. Further analysis of the other peaks that were differentially expressed in the pre- and post-operative sera of pancreatic cancer patients will provide new and important information.

This is the first study that has found that ApoC-1 is highly expressed in pancreatic cancer cells but is faintly expressed in normal pancreatic ductal and stromal cells that surround cancerous cells. These findings are supported by studies indicating that ApoC-1 mRNA was highly expressed in pancreatic cancer tissues based on the serial analysis of gene expression analysis (Ryu *et al*., 2001; Iacobuzio-Donahue *et al*., 2002). We also found that ApoC-1 was expressed in the supernatant of medium used to culture pancreatic cancer cells. Based on these results, we consider that serum ApoC-1 protein is derived from cancer cells; ApoC-1 is overexpressed in the neoplastic epithelium of pancreatic cancer and is secreted into the blood, which results in elevated serum ApoC-1 levels. This is also supported by the correlation between ApoC-1 expression in cancer cells and high serum peak intensity in preoperative serum.

The correlation of ApoC-1 levels with overall survival in pancreatic cancer, together with its specific expression in cancer cells, may indicate that this protein is involved in cancer progression. This encouraged us to analyse whether ApoC-1 involves in cancer cell proliferation. Using siRNA, we showed that the inhibition of ApoC-1 expression suppressed cell proliferation of pancreatic cancer cell lines. Moreover, we found that this reduced cell proliferation was due to the increased rate of apoptotic cell death. These facts suggest that expression

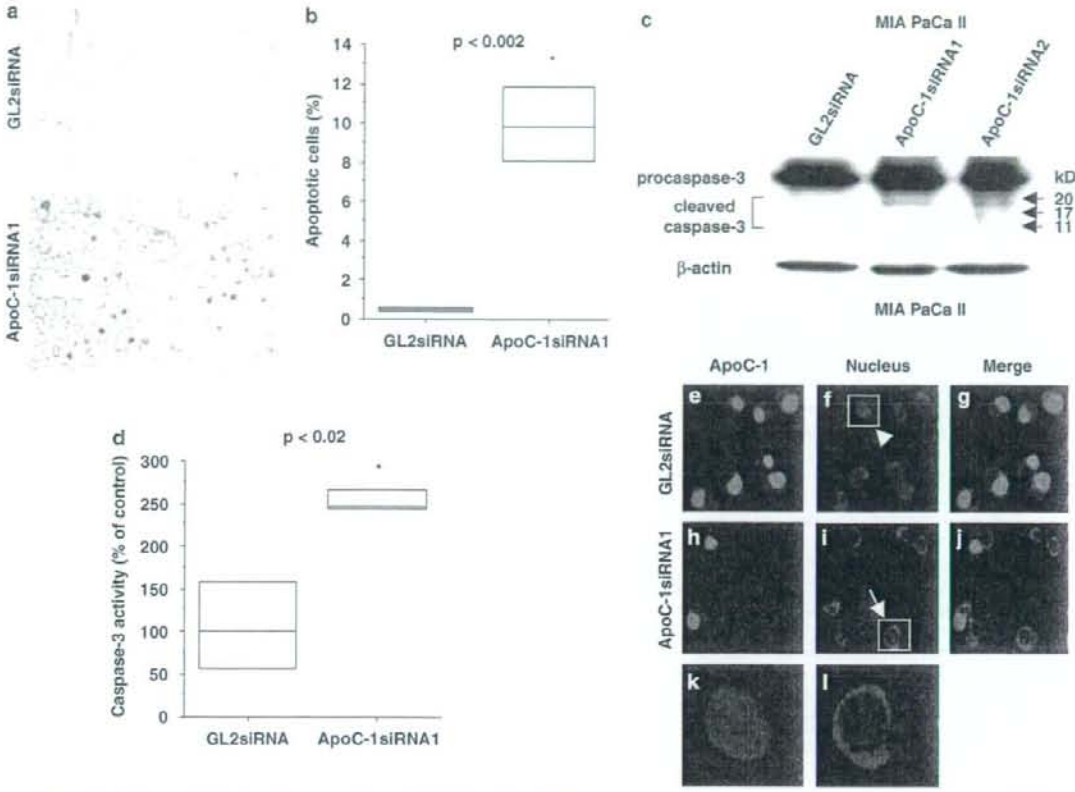

Figure 7 Apolipoprotein C-1 (ApoC-1) short interfering RNA (siRNA) treatment induces apoptotic cell death in MIA PaCa II. (**a**) APOPercentage assay is carried out at 24 h after siRNA transfection and representative results of GL2siRNA and ApoC-1 siRNA1-transfected cells are shown. Apoptotic cells are stained purple red. (**b**) The ratio of apoptotic cells is significantly higher in cells treated with ApoC-1 siRNA1 than with GL2siRNA. (**c**, **d**) ApoC-1 siRNAs induce the activation of procaspase-3 in MIA PaCa II. (**c**) Western blot analysis showed that the inhibition of ApoC-1 expression increases the cleaved caspase-3 (20, 17 and 11 Da; active form) compared to control (cells treated with GL2 siRNA). (**d**) Effect of the inhibition of ApoC-1 on caspase-3 activity in MIA PaCa II. Values represent percentage of control cells treated with GL2siRNA. ApoC-1 siRNA1-transfected cells show significantly higher proportion of caspase-3 activity than GL2siRNA-transfected cells at 24 h after transfection. (**e–l**) Immunofluorescence studies of ApoC-1 in MIA PaCa II cells. Cells treated with GL2 siRNA (**e–g**) and ApoC-1 siRNA1 (**h–j**) are shown. ApoC-1 staining with red color in the cytoplasm (**e**, **h**), nucleus with blue-stained by DAPI (**f**, **i**), and merge features (**g**, **j**). The nuclear morphology displayed apoptotic cell death with chromatin condensation by ApoC-1 siRNA treatment (arrow in (**i**); magnified picture showed in (**l**)), compared nonapoptotic cell treated with GL2 siRNA (arrow head in (**f**); magnified picture showed in (**k**)).

of ApoC-1 (secreted by autocrine manner) is essential for cancer cell survival by preventing from apoptosis, contributing to the malignant phenotype of pancreatic cancer. Supporting this, silencing expression of the apolipoprotein J gene, another apolipoprotein, in osteosarcoma and prostate cancer cells induced a significant reduction of cellular growth and high rates of spontaneous endogenous apoptosis (Trougakos *et al.*, 2004). Chen *et al.* (2005) also showed that the inhibition of apolipoprotein E, which is genetically linked closely with ApoC-1 (Lauer *et al.*, 1988), in ovarian cancer cells led to G2 cell-cycle arrest and apoptosis. In addition, RELN pathway through signaling via the VLDL receptor, to which ApoC-1 is known to bind, influences cell motility in pancreatic cancer (Sato *et al.*, 2006). It is very tempting to speculate that the inhibition of ApoC-1 expression in pancreatic cancer suppresses the tumor progression *in vivo*, and siRNA oligonucleotides against ApoC-1 may prove valuable agents for antipancreatic cancer therapy.

In conclusion, we found that serum levels of ApoC-1, which appears to be secreted by cancer cells, can predict the prognosis of pancreatic cancer patients. We also found an unexpected role of ApoC-1 in regulating cancer cell proliferation by avoiding spontaneous apoptotic cell death. Further research to determine the molecular mechanisms whether ApoC-1 inhibits apoptosis in cancer cells is warranted and will likely lead to the discovery of new therapies for pancreatic cancer using ApoC-1 as a therapeutic target.

Materials and methods

Patient samples and cell lines

To identify novel serum markers, serum samples were collected pre- and postoperatively (3–4 weeks after surgery, when the serum levels of C-reactive protein returned to the normal range) from 20 pancreatic cancer patients who had curative surgery. For the validation study, blood samples were obtained preoperatively from 69 patients diagnosed with primary invasive pancreatic ductal carcinoma who had surgery in the Department of General Surgery, Chiba University Hospital, Chiba, Japan, from June 2001 to April 2006. All blood samples were processed according to a standardized protocol, and serum samples were immediately frozen in aliquots at −80 °C until the proteomic study was done. In all patients, the diagnoses of carcinoma were confirmed histologically. Patient characteristics are summarized in Table 1 (Sobin and Wittekind, 2002). None of the patients received any additional therapies, such as radiation or chemotherapy, pre- or postoperatively, until serum samples were collected. The excised pancreatic tissue samples were placed in liquid nitrogen and stored at −80 °C until use. The ethics committee of our institute approved the protocol. Written informed consent was obtained from all patients. The four human pancreatic cancer cell lines, MIA PaCa II and PanC-1 (American Type Culture Collection, Manassas, VA, USA), CFPAC-1 and AsPC-1 (DS Pharma Biomedical Co., Ltd., Japan) that were used in this study were cultured in the appropriate medium and incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

SELDI-TOF MS analysis

To discover the candidate protein, an aliquot of the stored 20-paired pre- and postoperative serum samples was used for SELDI-TOF MS analysis with a weak cationic exchanger 2 (WCX2; Ciphergen Biosystems, Fremont, CA, USA; see Supplementary methods for detail). Each analysis was carried out in duplicate. Peak detection was carried out using ProteinChip Software 3.1 (Ciphergen).

For the validation study, the peak intensity of 6630 and 6420 Da proteins was measured using the SELDI-TOF MS analysis on CM10 ProteinChip arrays (Ciphergen) in 69 preoperative sera of pancreatic cancer patients; the measurements were done in duplicate under the same urea buffer condition as the WCX2 experiment. To reduce the coefficient of variation for peak intensities, we used a robot, Biomek 3000 Laboratory Automation Workstation (Beckman Coulter Inc., Fullerton, CA, USA) in this validation study. Mass accuracy was calibrated externally with an all-in-one-peptide molecular mass standard (Ciphergen).

Isolation and identification of the target proteins

The candidate proteins were purified, isolated and identified (see Supplementary methods for detail). After purification, the target protein was identified by N-terminal amino-acid sequence analysis. The immunodepletion assay and western blot were carried out for the confirmation of the identified protein.

RT–PCR and quantitative RT–PCR

Total RNA was extracted with the RNeasy Mini Kit (Qiagen Tokyo, Japan) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA with the T-Primed First-Strand Kit for RT–PCR (Amersham Biosciences, Buckinghamshire, UK). Human brain (cerebellum) total RNA (BD Bioscience Clontech, Takaka Bio company, Shiga, Japan) was used as positive control of ApoC-1 (Lauer *et al.*, 1988). Quantitative RT–PCR was carried out as previously described (Mitsuhashi *et al.*, 2003) using LightCycler with LightCycler-Fast Start DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany; see Supplementary Methods for detail).

Western blot analysis

The extracted proteins from frozen tissue samples, the cultured cells and the supernatant of the cultured cells were subjected to western blot analysis using mouse anti-human ApoC-1 monoclonal antibody (CHEMICON International, Temecula, CA, USA) and rabbit anti-human procaspase-3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; see Supplementary Methods for detail).

Immunohistochemistry

Paraffin-embedded tissues were cut in 4-μm-thick serial sections and were deparaffinized. These slides were placed in citric acid buffer (10 mmol l^{-1}, pH 6.0) with 0.2% Tween 20 and boiled in a microwave oven (2 × 6 min) to retrieve the antigen. The slides were then rinsed and blocked in 10% H_2O_2 solution with methanol for 10 min. Next, they were incubated with mouse anti-human ApoC-1 monoclonal antibody (CHEMICON International) at 1:200 dilution overnight at 4 °C. They were then rinsed in phosphate-buffered saline (PBS), and incubated for 60 min with secondary antibody labeled with streptoavidin-biotin-peroxidase (DAKO LSAB2 kit, DakoCytomation, Glostrup, Denmark). The bound complex was visualized using diaminobenzidine liquid chromogen and counterstained with hematoxylin. Mouse-monoclonal IgG2a (X0943, DAKO) was used as a negative control at an optimal dilution.

Gene knockdown using siRNA

Short interfering RNA (Hannon and Rossi, 2004) that specifically targeted ApoC-1 mRNA was used to reduce ApoC-1 expression. The target sequences for ApoC-1 RNA interference were as follows; ApoC-1 siRNA1: 5′-CTGGAG GACAAGGCTCGGGAA-3′, ApoC-1 siRNA2: 5′-CTGAAG GAGTTTGGAAACACA-3′. Double-stranded synthetic siRNA1 and 2, and luciferase (GL2) siRNA as a negative control, were purchased from Qiagen. *In vitro* transfection was carried out using Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions (see Supplementary Methods for detail).

Cell proliferation assay and Trypan blue exclusion test

A total of 20×10^4 MIA PaCa II and AsPC-1 human pancreatic cancer cell lines in six-well plates, were cultured in Dulbecco's modified Eagle medium (DMEM; Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% heat-inactivate fetal bovine serum (FBS), and were cultured in RPMI 1640 (Gibco BRL, Grand Island, NY, USA) with 20% FBS, respectively, incubated in a humidified atmosphere containing 5% CO_2 at 37 °C for 24 h. After washing with PBS, these cells were transfected with siRNA (20 nM final concentration). Both attached and floating cells were corrected with trypsinization. After staining with Trypan blue, the total cell number and the number of Trypan blue positive cells were counted on days 1–4 after transfection. All of these experiments were carried out in triplicate for three times, independently. The medium was changed every 2 days.

Invasion assay

A total of 1×10^5 MIA PaCa II or AsPC-1 cells in Opti-MEM I Reduced Serum Medium were plated onto BD BioCoat

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)