ORIGINAL ARTICLE

Overexpression of cellular inhibitor of apoptosis protein 2 is an early event in the progression of pancreatic cancer

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J Clin Pathol 2007;**60**:885–895. doi: 10.1136/jcp.2006.038257

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Accepted 8 June 2006
Published Online First 14 June 2006

Aim: To determine the role of two antiapoptotic proteins of the inhibitor of apoptosis protein family, cellular inhibitor of apoptosis protein 1 (cIAP1) and cellular inhibitor of apoptosis protein 2 (cIAP2), in human pancreatic carcinogenesis.

Methods: mRNA levels were measured in pancreatic tissues and pancreatic cancer cell lines by quantitative reverse transcriptase PCR. Protein expression was assessed in pancreatic cancer cell lines by immunoblotting and in pancreatic tissues by immunohistochemistry, and correlated with pathological and survival data.

Results: cIAP1 expression was constantly high in non-neoplastic pancreatic tissues, in pancreatic intraepithelial neoplasia (PanIN) lesions, as well as in a subset of primary and metastatic pancreatic ductal adenocarcinomas (PDAC), and a preferential cytoplasmatic localisation was observed in the tumour tissues. cIAP1 expression was rare in a cohort of cystic tumours. cIAP2 mRNA levels were significantly higher (2.4 fold) in PDAC than in normal tissues. cIAP2 protein was overexpressed in PDAC, and was detectable in low- and high-grade PanIN lesions. Moreover, cIAP2 was often expressed in pancreatic cystic tumours. cIAP1 and cIAP2 mRNA and protein were detected in all the examined cell lines. Survival analysis revealed a shorter survival in patients with cIAP1/cIAP2-positive tumours.

Conclusions: cIAP1 might contribute to the regulation of the apoptotic process in the normal and in the neoplastic pancreas, depending on its subcellular localisation. Overexpression of cIAP2 is a common and early event in the progression of pancreatic cancer, and could therefore potentially influence the important pathophysiological aspects of PDAC, such as anoikis or chemoresistance.

Inhibition of apoptosis prolongs the survival of cancer cells and facilitates their resistance to chemotherapy and radiotherapy. Pancreatic cancer cells have a variety of mechanisms for escaping apoptotic cell death, which explains their extraordinary radioresistance and chemoresistance. Pancreatic cancer cells are resistant to apoptosis mediated by death receptors of the tumour necrosis factor (TNF) death receptor superfamily, owing to downregulation of the Fas receptor and upregulation of the non-receptor protein tyrosine phosphatase FAP-1 (Fas-associated phosphatase), which blocks the function of Fas.[1] Moreover, pancreatic cancer cells demonstrate overexpression of silencer of death domains, which suppresses TNFα-induced cell death,[2] and are resistant to TNF-related apoptosis-inducing ligand (TRAIL) mediated apoptosis.[3] Additionally, antiapoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-x_L, are overexpressed in pancreatic cancer,[4 5] and the expression of proapoptotic members of the family, such as Bax, is associated with longer survival.[6]

The inhibitor of apoptosis protein (IAP) family of proteins contributes to the chemoresistance of lymphoid and solid malignancies.[7] All IAP family members contain one or more baculovirus *iap* repeats (BIRs), which are relevant for the interaction of IAP proteins with caspases. Some IAP proteins (cIAP1, cIAP2, XIAP and ML-IAP) possess a RING domain at the carboxy terminus that functions as an E3 ubiquitin ligase and mediates the IAP-induced ubiquitination.[8] The antiapoptotic properties of IAPs have been related to the inhibition of caspases and to interaction with the nuclear factor κB pathway. In particular, X-linked inhibitor of apoptosis (XIAP) directly inhibits caspase 3 and 7 and blocks the proteolytic activation of pro-caspase 9, whereas cellular inhibitor of apoptosis protein 1 (cIAP1; HIAP-2/MIHB/BIRC2) and cellular inhibitor of apoptosis protein 2 (cIAP2; HIAP-1/MIHC/BIRC3) bind caspases but are not able to inhibit them.[9] However, cIAP1 and cIAP2 play a role in the inhibition of TNFα-induced apoptosis through a positive feedback with nuclear factor κB and inhibition of caspase 8 activation.[10 11] For cIAP2, a correlation has been reported between overexpression of protein in the tumour tissue and a genomic alteration. In mucosal-associated lymphoid tissue lymphoma, an (11;18) translocation results in the formation of a fusion protein consisting of a RING-deleted form of cIAP2 and the mucosal-associated lymphoid tissue1 protein.[12] Furthermore, *cIAP1* may be the target of the 11q21–q23 amplification, which has been frequently identified in oesophageal squamous cell carcinomas.[13]

The biological relevance of IAP proteins may reside in the inhibition of the induction of apoptosis in epithelial cells on their detachment from the extracellular matrix (anoikis).[14] Cancer cells, including pancreatic cancer cells, are usually resistant to anoikis, owing to different mechanisms, such as the creation of a tumour-supportive microenvironment,[15] and are viable and able to grow in three-dimensional structures, even in the absence of a basal membrane with a normal structure. Recently, it has been demonstrated in intestinal epithelial cells that the *ras* oncogene, which is often mutated in PDAC,[16] suppresses anoikis by the activation of cIAP2 and XIAP.[17]

In this study, we analysed the expression of two members of the IAP family, cIAP1 and cIAP2, in PDAC and in its precursor

Abbreviations: BIR, baculovirus *iap* repeat; cIAP1, cellular inhibitor of apoptosis protein 1; cIAP2, cellular inhibitor of apoptosis protein 2; CP, chronic pancreatitis; EGFR, epidermal growth factor receptor; ESPAC, European Study Group for Pancreatic Cancer; IAP, inhibitor of apoptosis protein; IPMN, intraductal papillary-mucinous neoplasm; PanIN, pancreatic intraepithelial neoplasia; PDAC, pancreatic ductal adenocarcinoma; TNF, tumour necrosis factor; XIAP, X-linked inhibitor of apoptosis

lesions (pancreatic intraepithelial neoplasia (PanIN)), as well as in a panel of pancreatic cystic tumours. We demonstrate that the coexpression of cIAP1 and cIAP2 is common in pancreatic tissues, and that cIAP2 is involved in the progression of pancreatic cancer and might therefore contribute to the deregulation of the apoptotic signals and possibly to the anoikis resistance in PDAC.

MATERIALS AND METHODS

Tissue array

Tissue arrays were constructed using a manual tissue arrayer (Beecher Instruments, Sun Prairie, Wisconsin, USA). They contained 34 samples of primary and 8 samples of metastatic PDAC, none of them derived from or associated with an intraductal papillary-mucinous neoplasm (IPMN), as well as 9 samples of chronic pancreatitis (CP) and 10 from non-inflamed and non-tumorous pancreatic tissue (each sample in triplicate sections in order to assure a reliable immunohistochemical expression profile).[18] In eight patients with primary PDAC, the careful examination of multiple H&E-stained sections obtained from the tumour and the peritumoral pancreatic tissue led to the identification of PanIN lesions of different grade (10 low-grade PanINs and 8 high-grade PanINs), which were then included in the tissue array. In seven patients, core sections from lymph node metastases were also obtained. Pathological staging followed the TNM classification.[19] Grading was assessed according to the World Health Organization criteria.[20] The PanIN lesions were classified according to standardised criteria.[21] Tables 1 and 2 show the demographic and pathological characteristics of the 42 patients with PDAC whose tissues were included in the tissue arrays.

Tissue samples

The tissue samples designated for RNA and protein extraction were obtained from pancreatic resections for PDAC (n = 43) or CP (n = 10) at the University Hospital of Heidelberg, Heidelberg, Germany. Ten normal human pancreas tissue samples were obtained from previously healthy individuals through an organ donor programme (University of Bern, Bern, Switzerland). Tissue samples were taken fresh in the operating room, immediately frozen in liquid nitrogen on surgical removal and kept at −80°C until use.

Formalin-fixed and paraffin-wax-embedded tissue samples from pancreatic cystic tumours—five serous cystadenomas, five IPMNs (one adenoma, one borderline tumour and three carcinomas) and three mucinous cystadenomas—were also included in the analysis.

Tissue sections as well as follow-up data were available for an additional cohort of 33 patients with PDAC. The material available for the histopathological and immunohistochemical analyses was represented by tissue sections obtained from the tumour mass, with only little or no peritumoral pancreatic

Table 1 Demographic and pathological characteristics and cellular inhibitor of apoptosis protein 1/cellular inhibitor of apoptosis protein 2 expression analysis of the pancreatic ductal adenocarcinoma (PDAC) cases included in the tissue arrays (patients with resectable PDAC)

							cIAP1				cIAP2			
									PanIN				PanIN	
Case	Gender	Age (years)	pT	pN	pM	G	Primary	LN	LG	HG	Primary	LN	LG	HG
1	M	58	3	1	0	1	Neg				Pos C			
2	M	70	3	1	0	3	Pos C	Pos C			Pos C	Pos C		
3	M	63	3	1	0	2	Pos C		Neg	Neg	Pos C		Neg	
4	F	58	3	1	0	2	Neg		Neg	Neg	Pos C		Neg	
5	F	70	3	1	0	2	Neg				*			
6	F	73	3	1	0	3	Pos C		Neg		Pos C		Neg	
7	F	57	3	1	0	2	Neg				Neg			
8	M	65	3	1	0	2	Pos C/N				Pos C/N			
9	F	61	3	1	0	2	Pos C			Pos C	Pos N			Pos C
10	M	76	3	1	1 (PER)	2	Pos N				Pos C			
11	M	73	3	1	0	3	Pos C	Pos C	Pos C	Pos C	Pos C	Pos C	Pos C	Pos C
12	F	68	3	1	0	2	Pos C		Pos C	Neg	Pos C		Neg	Neg
13	F	49	3	1	0	2	Neg		Neg		Neg		Pos C	
14	M	38	3	0	0	3	Pos N				Pos C/N			
15	F	66	3	1	1 (LYM)	2	Pos C/N				Pos C/N	Neg		
16	M	65	3	1	0	3	*				Pos C			
17	M	73	3	1	0	2	Pos C/N				Pos C/N	Pos C/N		
18	M	48	3	1	0	3	Pos C	Pos C/N			Pos C	Pos C		
19	M	60	3	1	0	2	Pos N				Pos C			
20	M	75	3	1	0	2	Neg				Neg			
21	F	79	3	1	1 (LIV)	3	Pos C/N				Pos C			
22	M	73	3	1	0	2	Pos C/N	Pos C			Pos C	Pos C		
23	F	57	3	1	0	2	Pos C	Pos C	Pos C		Pos C	Pos C	Neg	
24	M	60	3	1	0	3	Pos C				Pos C/N			
25	M	73	3	0	0	3	Pos C				Pos C			
26	F	67	3	1	0	3	Pos C				Pos C			
27	F	65	3	1	0	2	Pos C				Pos C			
28	M	74	3	1	0	1	Pos C/N				Pos C/N			
29	M	71	3	1	0	3	Pos C/N				Pos C/N			
30	F	73	3	1	0	3	Pos C/N				Pos C/N			
31	M	64	3	1	0	2	Pos C				Pos C			
32	M	65	3	1	0	2	Pos C				Neg			
33	M	63	3	1	0	2	Pos C				Pos C			
34	F	69	3	1	0	3	Pos C				Pos C			

C, cytoplasmic expression; cIAP1, cellular inhibitor of apoptosis protein 1; cIAP2, cellular inhibitor of apoptosis protein 2; F, female; HG, high grade; LG, low grade; LN, lymph node metastasis; M, male; N, nuclear expression; Neg, negative staining in <10% of the cancer cells; PanIN, pancreatic intraepithelial neoplasia; Pos, positive staining in ⩾10% of the cancer cells.
*Two cases could not be evaluated because of insufficient quality of the tissue on the array.

Table 2 Demographic and pathological characteristics and cellular inhibitor of apoptosis protein 1/cellular inhibitor of apoptosis protein 2 expression analysis of the pancreatic ductal adenocarcinoma (PDAC) cases included in the tissue arrays (patients with unresectable PDAC)

Case	Gender	Age (years)	Site of metastases	cIAP1	cIAP2
1	M	76	PER	Pos N	Pos N
2	F	59	LIV	Pos C	Pos C
3	M	55	PER	Pos C	Neg
4	M	62	LIV	Pos C	Pos C/N
5	M	72	LIV	Pos C/N	Pos C
6	M	55	PER	Pos C	Neg
7	M	55	PER	Pos C	Neg
8	M	78	PER	Pos C/N	Pos C

C, cytoplasmic expression; F, female; M, male; N, nuclear expression; neg, staining in <10% of the cancer cells; pos, staining in ⩾10% of the cancer cells.

tissue. PanIN lesions were not identified in these cases. Survival was determined from the date of the initial surgery. No detectable metastases in distal organs were present at the time of surgery. No patient had received chemotherapy and/or radiotherapy before surgery. Most of the patients were inlcuded in adjuvant treatment study protocols (European Study Group for Pancreatic Cancer (ESPAC)-1,[22] ESPAC-3[23] and CapRI[24]). Table 3 shows the demographic and pathological characteristics of this cohort of patients. Follow-up lasted through December 2005, with a median (range) follow-up period of 9 (range 3–42) months.

Cell culture

Pancreatic cancer cell lines were grown routinely in Rosewell Park Memorial Institute medium (ASPC-1, BxPc-3, Capan-1 and T3M4) or Dulbecco's modified Eagle's medium (MiaPaCa-2 and Panc-1 cells), supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Karlsruhe, Germany) and incubated in a 5% CO_2 humidified atmosphere.

Quantitative reverse transcriptase-PCR analysis

All reagents and equipment for mRNA/cDNA preparation were supplied by Roche Applied Science (Mannheim, Germany). mRNA of human pancreatic tissues and cell lines was prepared by automated isolation using the MagNA Pure LC Instrument and Isolation Kit I (for cells) and Kit II (for tissues). cDNA was prepared using the First-strand cDNA Synthesis Kit for RT-PCR (AMV) according to the manufacturer's instructions. Real-time PCR was carried out using the LightCycler FastStart DNA SYBR Green kit. The number of specific transcripts was normalised to housekeeping genes (cyclophilin B and hypoxanthine-guanine phosphoribosyltransferase) as described previously.[25] All primers were from Search LC (Heidelberg, Germany).

Immunoblot analysis

Cells were lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA and 1% sodium dodecyl sulphate. Protein from cell lysates (30 µg) was separated on sodium dodecyl sulphate-polyacrylamide gels, and electroblotted onto nitrocellulose membranes. Membranes were then incubated in blocking solution (5% non-fat milk in 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20; Tris-buffered saline with tween), followed by overnight incubation with anti-cIAP1 (R&D Systems, Minneapolis, Minnesota, USA) or anti-cIAP2 (Abcam Limited, Cambridge, UK) antibodies.

The membranes were then washed in Tris-buffered saline with tween and incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Bioscience Europe, Freiburg, Germany). Detection of antibody was performed with an enhanced chemiluminescence reaction (Amersham Bioscience).

Immunohistochemistry

Tissue sections embedded in paraffin wax were subjected to immunostaining using the streptavidin–peroxidase technique, using diaminobenzidine as a chromogen. For antigen retrieval, the sections were subjected to microwave heating in citrate buffer (pH 6.0). After incubation with polyclonal anti-cIAP1 or anti-cIAP2 antibodies (both at 1:100 dilution), slides were washed in Tris-buffered saline buffer and then the appropriate biotinylated secondary antibody, followed by the streptavidin/peroxidase complex (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA), was added. To ensure antibody specificity, consecutive sections were incubated with isotype-matched control immunoglobulins, and in the absence of the primary antibody. In these cases, no specific immunostaining was detected. An arbitrary cut-off value of 10% of stained cells was introduced to discriminate between positive (⩾10%) and negative (<10%) cases. The site of expression (cytoplasmic/nuclear) was assessed.

Statistical analysis

For statistical analyses, the non-parametric Mann–Whitney U test was used, unless indicated otherwise. Survival analysis was performed according to the Kaplan–Meier method. Significance was defined as $p<0.05$.

RESULTS

cIAP1 and cIAP2 mRNA levels in pancreatic tissues

Quantitative reverse transcriptase-PCR was performed to evaluate the change in cIAP1 and cIAP2 mRNA replicate numbers in normal pancreatic tissue samples (n = 10), CP samples (n = 10) and PDAC samples (n = 43). Tissue samples from normal pancreas had a median (range) replicate number of cIAP1 mRNA of 1762 (0–3048), whereas the median replicate number was 2069 (1340–5860) in CP and 1305 (0–3039) in PDAC. The transcriptional levels of cIAP1 were significantly higher in CP than in PDAC tissues (1.6-fold increase; $p = 0.002$). The median replicate number of cIAP2 mRNA was 424 (162–690) in the normal pancreas, 846 (203–2747) in CP (CP vs normal 1.9-fold increase; $p = 0.02$) and 998 (277–4061) in PDAC (PDAC vs normal 2.4-fold increase; $p<0.001$; fig 1). The transcriptional levels of cIAP2 in CP and PDAC tissues were not significantly different.

cIAP1 and cIAP2 expression in pancreatic cancer cell lines

Measurable mRNA levels of cIAP1 and cIAP2 were detected by quantitative reverse transcriptase-PCR in all examined cell lines. Immunoblot analyses confirmed the expression of the corresponding proteins, as well as the specificity of the antibodies (fig 2).

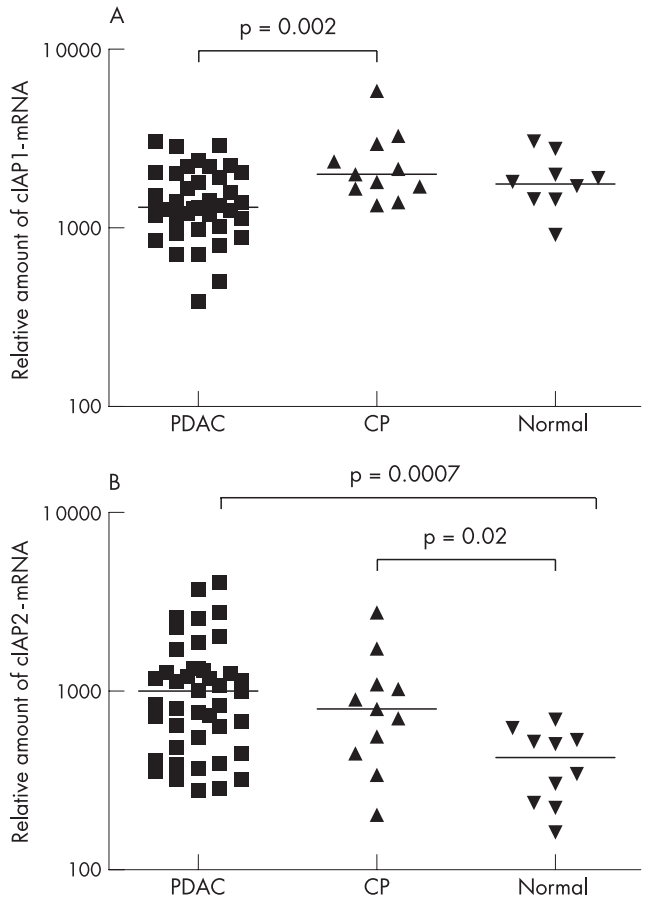

Figure 1 (A) Real-time quantitative reverse transcriptase (RT)-PCR analysis of pancreatic tissues showed significantly higher levels of cellular inhibitor of apoptosis protein 1 (cIAP1) mRNA in chronic pancreatitis (CP) compared with pancreatic ductal adenocarcinoma (PDAC). (B) Real-time quantitative RT-PCR analysis of pancreatic tissues showed significantly higher levels of cellular inhibitor of apoptosis protein 2 (cIAP2) mRNA in PDAC and in CP compared with normal controls. RNA input was normalised to the average expression of the two housekeeping genes: hypoxanthine-guanine phosphoribosyltransferase and cyclophilin B. Horizontal lines represent median expression levels.

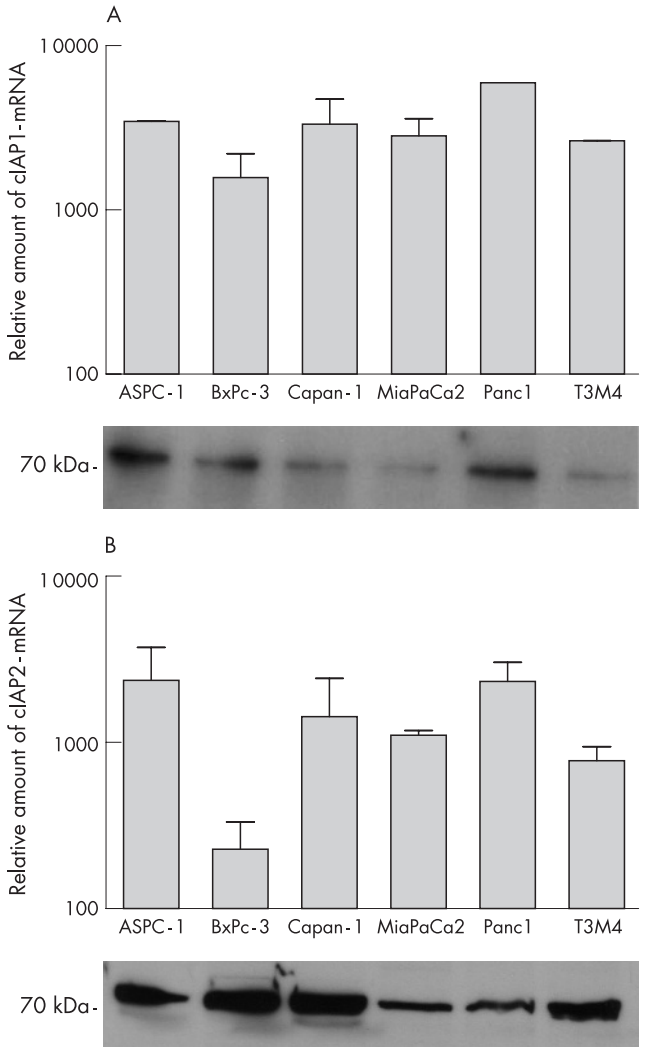

Figure 2 Real-time quantitative reverse transcriptase-PCR analysis of pancreatic cancer cell lines showed measurable levels of cellular inhibitor of apoptosis protein 1 (cIAP1) (A) and cellular inhibitor of apoptosis protein 2 (cIAP2) (B) mRNA in all the tested cell lines. RNA input was normalised to the average expression of the two housekeeping genes, hypoxanthine-guanine phosphoribosyltransferase and cyclophilin B, in three independent experiments (mean (SEM)). Immunoblot analyses of pancreatic cancer cell lysates confirmed the expression of the corresponding protein and the specificity of the antibodies.

cIAP1 and cIAP2 protein expression in pancreatic tissues

Figure 3 summarises the results of the immunohistochemical expression of cIAP1 and cIAP2 in pancreatic tissues. The detailed analysis is reported below.

cIAP1 protein expression in pancreatic tissues

Normal pancreas

In the normal pancreas, 50–100% of the acinar cells consistently showed cytoplasmic and/or nuclear expression of cIAP1. Ductal structures were present in 8 of 10 cases. In five cases, cytoplasmic staining was detected in 30–100% of the ductal epithelia, in two cases nuclear staining was detected in 20–30% of the cells, and in one case the staining was both nuclear and cytoplasmic. The islets of Langerhans always displayed cytoplasmic positivity in all the cells (fig 4B).

Chronic pancreatitis

The CP samples included tubular complexes and islets, surrounded by fibrous tissue. The tubular complexes showed diffuse cytoplasmic expression of cIAP1 in all the cells, whereas the extracellular matrix included positive lymphocytes (fig 4E).

PanIN lesions

For analysis of the expression of cIAP1, 18 lesions from 8 patients were available (10 low-grade and 8 high-grade PanIN); all except 2 associated with cIAP1-positive tumours. In all, 4 out of 10 low-grade PanIN and 4 out of 8 high-grade PanIN expressed cIAP1. cIAP1 expression in the PanIN lesions was always cytoplasmic (fig 4H,K). In two cases, both the primary PDAC and the associated low-grade and high-grade PanIN were negative.

Pancreatic cancer

cIAP1 expression was detected in 27 of 34 cases (79%). Of these, in 16 (59%) cases the expression was weak and localised to the cytoplasm, and in 3 (11%) cases it was weak and localised only to the nucleus. In addition, both cytoplasmic and nuclear expression were found in 8 (30%) cases. In all cases, the majority (>80%) of the tumour cells were stained. There was a concordant pattern of expression between the primary tumour and the respective lymph node metastases in all cases. All the distant metastases displayed weak to moderate expression of

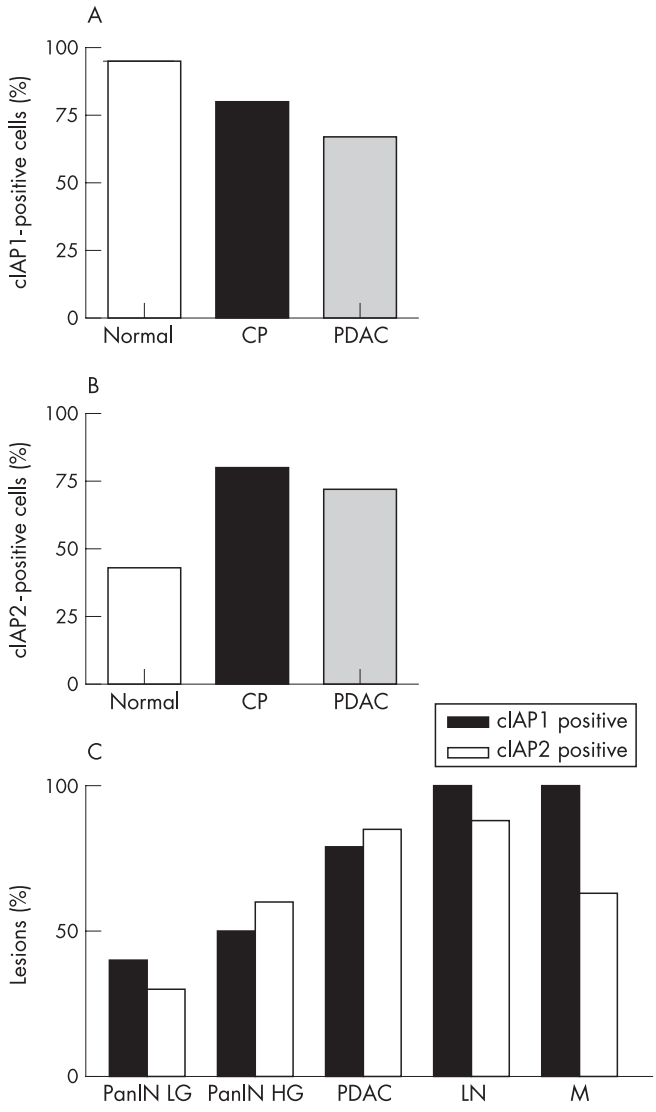

Figure 3 Diagrammatic representation of the results of the immunohistochemical analysis for cellular inhibitor of apoptosis protein 1 (cIAP1; A) and cellular inhibitor of apoptosis protein 2 (cIAP2; B) in pancreatic tissues. The percentage of positive cells in the normal pancreas was obtained by adding the percentage of positivity of acinar, ductal and endocrine cells, independent of the site of expression (ie, nuclear or cytoplasmic), and assuming a distribution of the three compartments in the normal pancreas as follows: acinar cells 84%, ducts 4%, islets 2%, as reported.[26] In the chronic pancreatitis (CP) samples, only two compartments were present and therefore evaluated: tubular complexes and islets. In the cancer samples, the expression of the two proteins was homogeneous, being detected in 80–90% of the tumour cells, so that a mean value of 85% was arbitrarily chosen. The intensity of the staining was not considered for this analysis. (C) Expression of cIAP1 and cIAP2 in the progression of pancreatic cancer. The data are presented as percentage of positive lesions. As described in Results, a total number of 10 low-grade (LG) pancreatic intraepithelial neoplasia (PanIN), 8 high-grade (HG) PanIN, 34 PDAC, 5 lymph node (LN) metastases and 8 distant metastases (M) were available for cIAP1 immunostaining. For cIAP2 immunostaining, the analysis was performed on 10 LG PanIN, 5 HG PanIN, 34 PDAC, 7 LN metastases and 8 M.

c-IAP1 (five cytoplasmic, one nuclear, and two both cytoplasmic and nuclear; fig 4N,Q,T,W).

Cystic tumours

With the exception of one IPMN (borderline) and one serous cystadenoma, none of the cystic tumours included in this analysis showed any expression of cIAP1 (table 4; fig 5B,E,H,K,N).

cIAP2 expression in pancreatic tissues

Normal pancreas

The acinar cell compartment was almost completely negative for cIAP2 (fig 4C), with focal (<2% of the cells) cytoplasmic expression observed only in four cases. The ductal epithelia showed cytoplasmic expression of cIAP2 in five cases (2–100% of the cells). The islets displayed diffuse cytoplasmic staining in all cells.

Chronic pancreatitis

In the CP samples the pattern of expression was identical to that described for cIAP1, with diffuse cytoplasmic expression in the tubular complexes (fig 4F).

PanIN lesions

For the analysis of the expression of cIAP2, 15 lesions from 8 patients were available (10 low grade and 5 high grade). In all, 7 of 10 low-grade PanINs did not show any cIAP2 expression, but their associated PDACs were positive for cIAP2. The three remaining low-grade PanINs were positive for cIAP2 expression; two of them were associated with a cIAP2-positive PDAC, whereas in one case the associated PDAC was negative. Three high-grade PanINs expressed cIAP2, as did their associated PDAC, whereas in the remaining two cases the PanIN lesions were negative and the associated PDAC was positive. Expression of cIAP2 in the PanIN lesions was always cytoplasmic (fig 4I,L).

Pancreatic cancer

Of the 34 PDAC cases included in the tissue array, 29 (85%) were positive for cIAP2. In all, 17 (58%) cases showed weak cytoplasmic expression, 3 (10%) showed moderate cytoplasmic expression and 1 (4%) showed only weak nuclear expression. In addition, both cytoplasmic and nuclear expression were found in 8 (28%) cases. In all cases the majority (>80%) of the tumour cells were stained. There was a concordant pattern of expression between the primary tumour and the respective lymph node metastasis in all but one case. Additionally, 5 of 8 (63%) distant metastases stained positive for cIAP2 (3 cytoplasmic, 1 nuclear and 1 both cytoplasmic and nuclear expression; fig 4O,R,U,X).

Cystic tumours

All the serous cystadenomas, all the IPMNs, as well as one mucinous cystadenoma, showed weak to moderate cytoplasmic staining for cIAP2 (table 4; fig 5C,F,I,L,O).

Survival analysis

The only pathological factor that significantly affected patients' survival was the presence of distant metastases (median survival M1 vs M0: 5 vs 10 months, $p = 0.007$). Moreover, T1–T2 tumours showed a median survival of 12 months compared with 8 months for T3–T4 cases ($p = 0.06$). G1–G2 cases had a median survival of 10 months, compared with 5 months for G3 cases (not significant). Interestingly, a significant correlation was found between the coexpression of cIAP1 and cIAP2 and patients' survival: the cases where only one or none of the proteins was expressed ($n = 16$) had a longer survival (10 vs 7 months, $p = 0.01$) than the cases where the two proteins were coexpressed ($n = 17$). When the two proteins were considered separately, no significant correlation was found between expression and patient survival (fig 6). However, patients with cIAP1-negative or cIAP2-negative tumours survived slightly longer than patients with positive tumours (cIAP1: median survival 10 vs 7.5 months, $p = 0.08$; cIAP2: 9.5 vs 8 months, $p = 0.2$). Table 3 shows the detailed expression analysis of this cohort of patients. Owing to the small number of patients included in this analysis, the cases

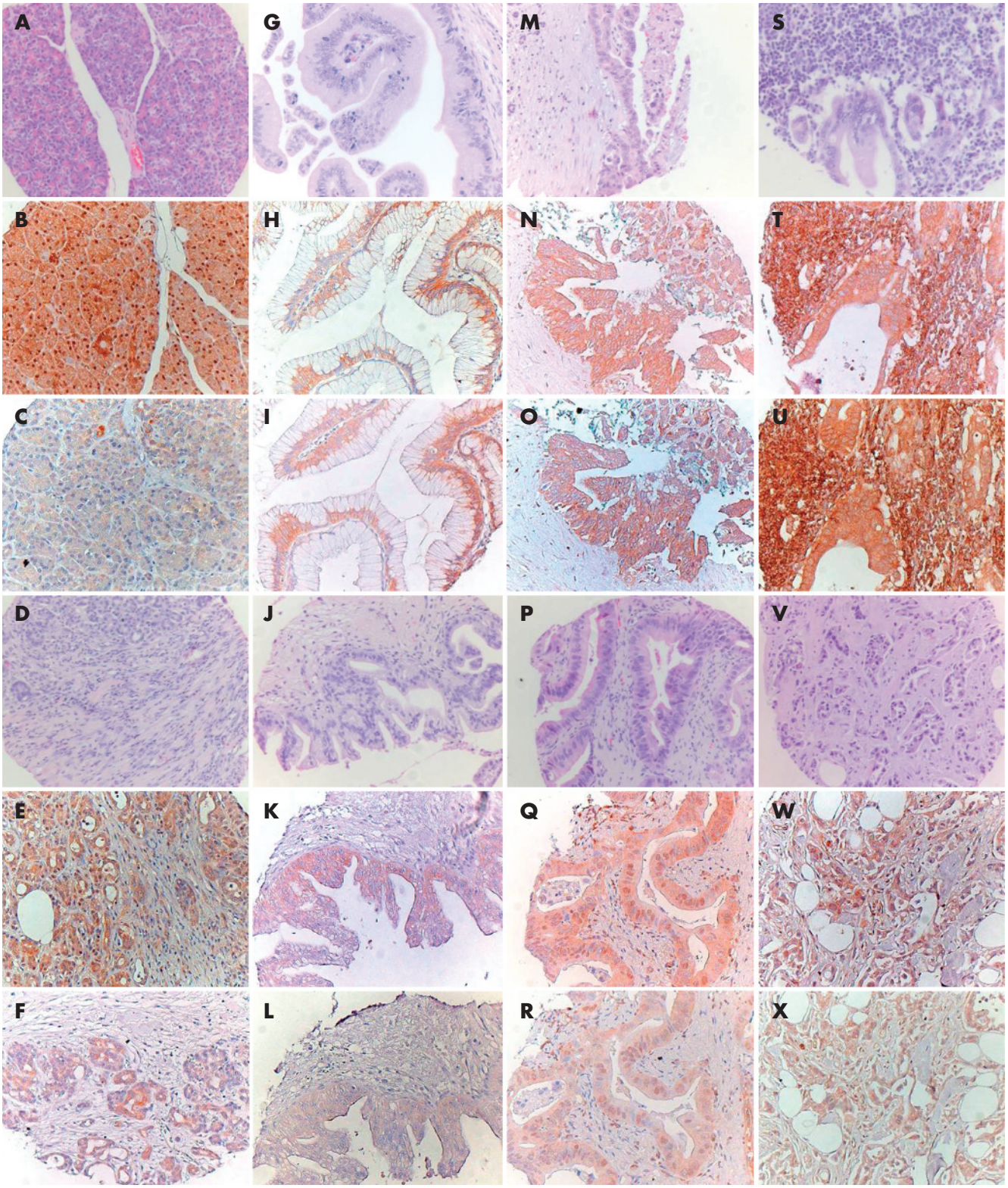

Figure 4 Immunohistochemical analysis of cellular inhibitor of apoptosis protein 1 (cIAP1; B, E, H, K, N, Q, T, W) and cellular inhibitor of apoptosis protein 2 (cIAP2; C, F, I, L, O, R, U, X) expression in pancreatic tissues was performed as described in Materials and methods. The tissues/lesions in the H&E staining and representative patterns of expression are shown for the normal pancreas (A–C), chronic pancreatitis (D–F), low-grade pancreatic intraepithelial neoplasia (PanIN) lesions (G–I), high-grade PanIN lesions (J–L), primary pancreatic ductal adenocarcinoma (PDAC) with cytoplasmic staining (M–O), primary PDAC with cytoplasmic and nuclear staining (P–R), a lymph node metastasis (S–U) and a peritoneal metastasis (V–X). A strong positivity for cIAP1 and cIAP2 was present in the lymphoid tissues, as reported previously.[27 28]

were stratified into positive and negative, and no analysis of subgroups (ie, cytoplasmic vs nuclear expression) was performed. Most of the 33 patients participated in three different adjuvant treatment study protocols (ESPAC-1, ESPAC-3 and CapRI). For this reason, and owing to the small number of patients in each arm, it was not possible to identify any association between cIAP1/cIAP2 expression and the response to adjuvant treatment.

Table 3 Demographic and pathological characteristics and cellular inhibitor of apoptosis protein 1/cellular inhibitor of apoptosis protein 2 expression analysis in a cohort of 33 patients with available follow-up data

Case	Gender	Age (years)	pT	pN	pM	G	cIAP1	Site of expression	cIAP2	Site of expression	Survival (months)
1	F	76	2	0	0	2	Neg		Neg		42
2	M	74	3	0	1	2	Pos	C	Pos	C	5
3	M	68	3	1	0	2	Pos	C	Neg		31
4	M	62	2	1	0	1	Pos	C	Pos	C/N	12
5	M	72	4	0	1	1	Pos	C	Pos	C	3
6	F	66	4	1	0	1	Neg		Neg		10
7	M	58	2	0	0	2	Neg		Neg		7
8	F	77	2	1	0	2	Pos	C	Neg		10
9	M	62	3	1	0	1	Neg		Pos	C	16
10	M	68	4	1	0	2	Pos	C	Neg		10
11	M	70	3	1	0	2	Pos	C	Neg		9
12	M	67	3	1	0	2	Pos	C/N	Neg		14
13	M	76	1	0	0	3	Neg		Pos	C	18
14	F	61	1	0	0	3	Neg		Pos	N	23
15	M	54	3	1	0	3	Pos	C	Pos	C	22
16	F	75	3	1	0	2	Pos	C	Pos	C	10
17	M	74	2	1	0	2	Pos	C	Pos	N	6
18	M	72	3	1	0	2	Pos	C/N	Pos	C/N	8
19	F	59	3	1	0	2	Pos	C	Pos	C/N	7
20	M	68	4	1	0	2	Neg		Pos	N	3
21	F	65	3	1	0	2	Pos	C	Pos	C/N	8
22	M	71	3	0	0	2	Pos	C	Pos	N	12
23	F	66	4	0	0	3	Pos	C	Pos	N	4
24	M	60	3	1	0	2	Pos	C	Neg		13
25	F	66	3	1	0	2	Pos	C	Neg		10
26	F	82	3	0	0	3	Neg		Pos	C/N	5
27	M	60	3	1	0	2	Pos	C	Pos	C	13
28	M	57	3	1	0	3	Pos	C	Pos	N	7
29	F	59	3	1	1	3	Pos	C	Pos	C	5
30	M	76	3	1	0	3	Pos	C/N	Pos	N	5
31	F	44	3	1	0	2	Pos	C	Pos	C	3
32	M	69	3	1	0	3	Pos	C	Pos	C	4
33	M	71	3	1	0	3	Neg		Pos	N	3

C, cytoplasmic expression; cIAP1, cellular inhibitor of apoptosis protein 1; cIAP2, cellular inhibitor of apoptosis protein 2; F, female; M, male; N, nuclear expression; neg, staining in <10% of the cancer cells; pos, staining in ⩾10% of the cancer cells.

DISCUSSION

Pancreatic cancer and the IAP family

Pancreatic cancer is a lethal disease whose incidence and mortality almost coincide.[29] Despite significant improvements in the surgical technique in the past decades, with <5% surgery-associated mortality in high-volume centres,[30] almost all patients die of recurrent disease. The retroperitoneal location of the organ in the vicinity of major vessels and the infiltrative pattern of growth often make complete resection difficult, and an effective regimen of postoperative chemotherapy is required to control local and distant recurrence. Studies aimed at identifying the molecules and mechanisms involved in the resistance of pancreatic cancer cells to apoptotic cell death have revealed the coexistence of multiple antiapoptotic changes.[1] Concerning the role of the IAP family of proteins in suppressing apoptosis and promoting cell survival in PDAC, most of the studies have focused on elucidating the function of survivin, whereas little attention has been given to other members of the IAP family. Survivin is overexpressed in most cases of pancreatic cancer, and its prognostic significance depends on the site of expression (ie, cytoplasmic vs nuclear).[31 32] Silencing of survivin by means of RNA interference leads to reduced radioresistance[33] and growth inhibition of pancreatic cancer cell lines.[34]

The overexpression of two other members of the IAP family, XIAP and cIAP1, has been described in some pancreatic cancer cell lines,[35 36] and a recent report has shown that down-regulation of XIAP leads to enhanced chemosensitivity of the pancreatic cancer cell line SW1990.[37]

In this study, we report for the first time a detailed expression analysis of cIAP1 and cIAP2 in pancreatic tissues.

Localisation of cIAP1 in pancreatic tissues

cIAP1 was widely expressed in the normal pancreas, as well as in CP, in the PanIN lesions and in most of the PDAC samples. Interestingly, nuclear expression of cIAP1 was mainly found in the acinar and ductal compartments of the normal, ''quiescent'', pancreas, where it probably regulates apoptotic signalling and the cell cycle under physiological conditions.[38] Instead, a cytoplasmic redistribution of the protein was observed in the tubular complexes, in the PanIN lesions and in most of the PDACs. This could reflect a phenomenon that has already been described in the HeLa cells, where cIAP1 relocates from the nuclei to the cytoplasm under various apoptotic stimuli, thereby regulating cytosolic caspases and exerting most of its antiapoptotic functions.[38] A similar redistribution has also been described for survivin, whose expression is cell cycle-dependent and mainly restricted to the nuclei of normal cells, whereas in most of the cancer cells it is also present throughout the cytoplasm.[39]

Localisation of cIAP2 in pancreatic tissues

The expression levels of cIAP2 were significantly higher in pancreatic cancer and chronic pancreatitis compared with the normal pancreas, where cIAP2 immunoreactivity was confined to the ductal and islet cells and to a small percentage of the acinar cells. These findings contradict, in part, the results of a recent study in which the expression of cIAP1, cIAP2 and XIAP was analysed in a large panel of normal human tissues, including the pancreas.[27] The authors reported a moderate expression of cIAP2 in the acini and the ducts of the normal pancreas, but did not state the number of tissues examined, so that it seems difficult to draw a general conclusion about cIAP2 expression in the normal pancreas from those results. In the

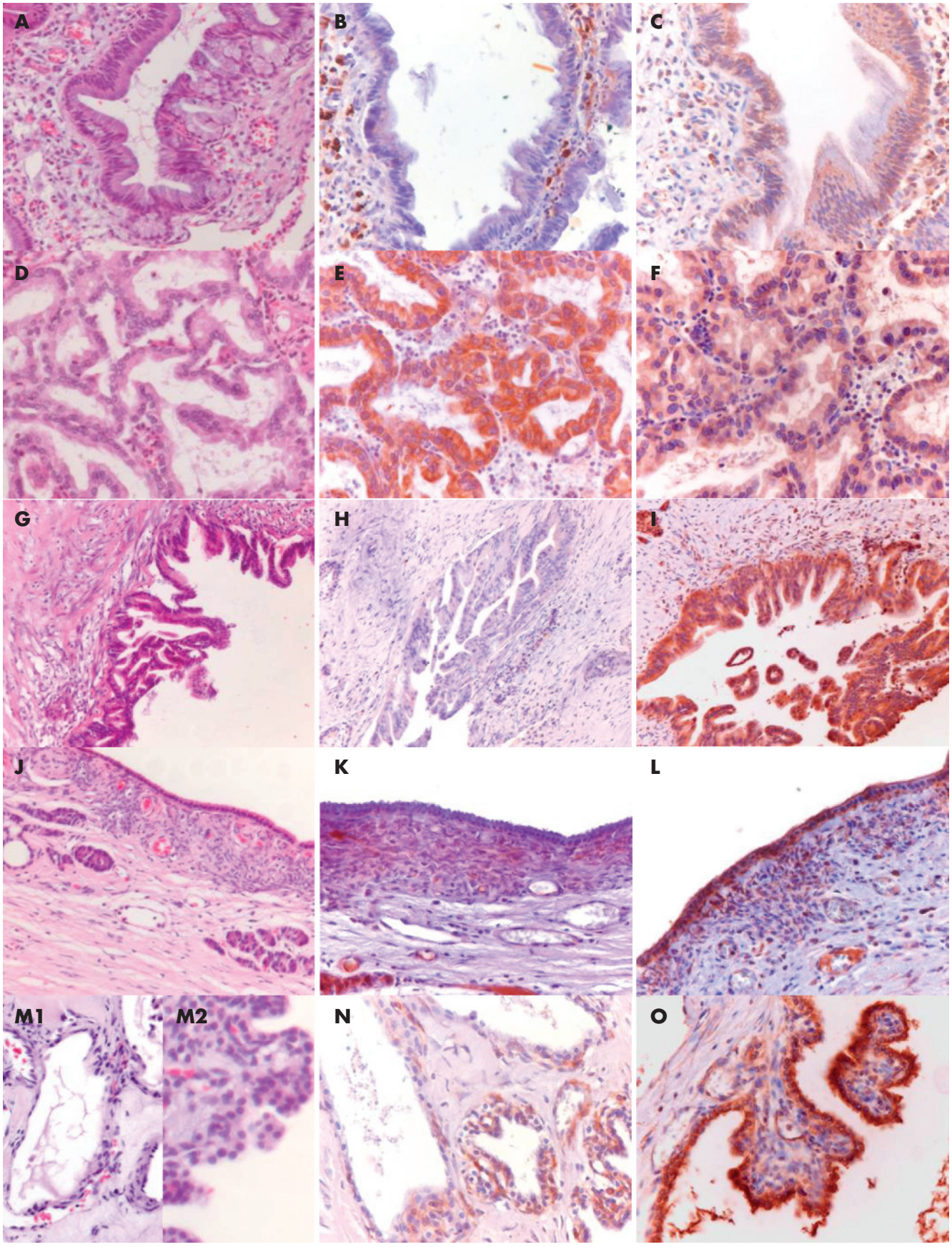

Figure 5 Immunohistochemical analysis of cellular inhibitor of apoptosis protein 1 (cIAP1: B, E, H, K, N) and cellular inhibitor of apoptosis protein 2 (cIAP2: C, F, I, L, O) in a panel of pancreatic cystic tumours revealed that no intraductal papillary-mucinous neoplasm (IPMN) adenomas (B), IPMN carcinomas (H) and mucinous cystadenomas (K) show any expression of cIAP1. In this panel, only one IPMN borderline (E) and one serous cystadenoma (N) were cIAP1-positive. On the contrary, the expression of cIAP2 was common in pancreatic cystic tumours (C, IPMN adenoma; F, IPMN borderline; I, IPMN carcinoma; L, mucinous cystadenoma; O, serous cystadenoma). A, D, G, J and M1–M2 show the corresponding lesions in the H&E staining (A, IPMN adenoma; D, IPMN borderline; G, IPMN carcinoma; J, mucinous cystadenoma; M1 and M2, serous cystadenoma).

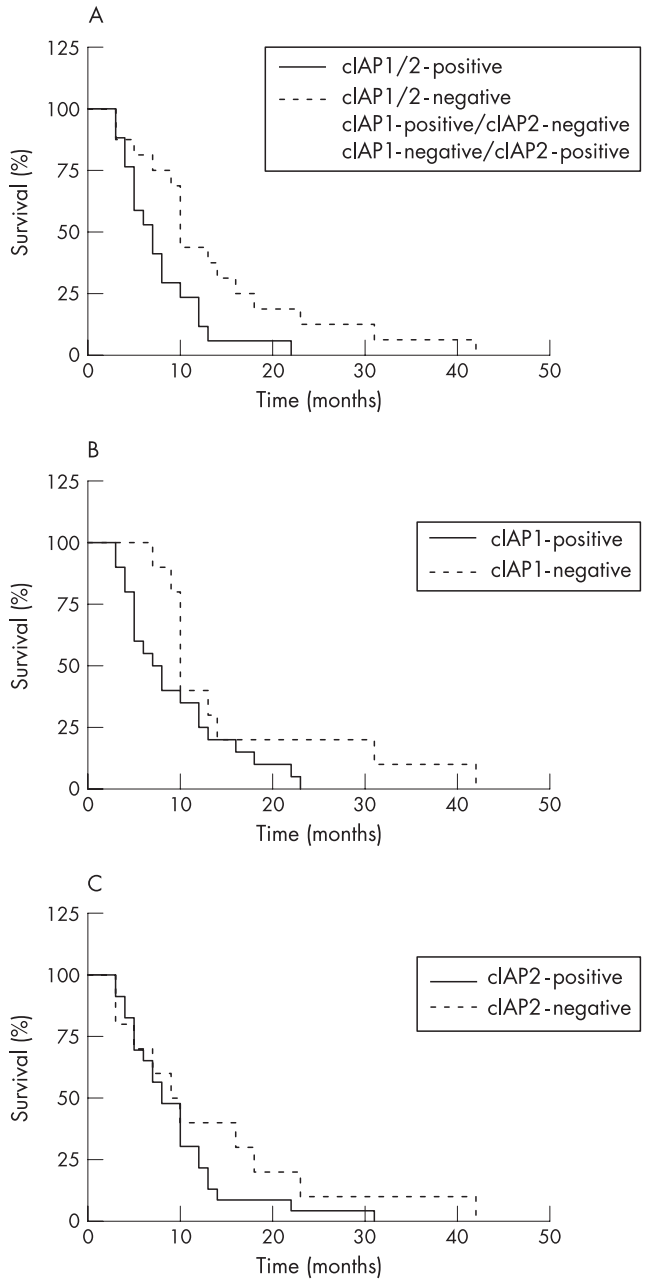

Figure 6 Survival analysis was performed according to the Kaplan–Meier method in a subset of 33 patients with pancreatic ductal adenocarcinoma with available follow-up data, and revealed a shorter median survival (7 vs 10 months) in patients whose tumours coexpressed cellular inhibitor of apoptosis protein 1 (cIAP1) and cellular inhibitor of apoptosis protein 2 (cIAP2), compared with the cases where only one or none of the two proteins was expressed ($p = 0.01$; A). A tendency towards better survival was found in patients with cIAP1-negative tumours ($p = 0.08$; B). Patients with cIAP2-negative tumours survived slightly longer than patients with cIAP2-positive tumours, although the difference was not significant ($p = 0.2$; C).

present study, cIAP2 was expressed in most of the primary PDACs, as well as in lymph node and distant metastases, and, interestingly, in about 30% of low-grade and 50% of high-grade PanIN lesions.

With the exception of a few PDAC cases, cIAP2 expression was cytoplasmic in all phases of pancreatic cancer progression. This is in agreement with other studies investigating the expression of cIAP2 in human cancers,[40–42] and is related to the biological (ie, antiapoptotic) function of the protein. However, cIAP2 has also been detected in the mitochondrial fractions of lung cancer cells[43] and in the cytoplasm and nuclei of HeLa cells,[38] thus suggesting a heterogeneous distribution of cIAP2 into various subcellular compartments–a phenomenon that is common to other IAP proteins (survivin, cIAP1)[38 39] and that could represent a control mechanism on the protein activity.[44]

The overexpression of cIAP2 could also reflect the acquisition of an anoikis-resistant phenotype in PDAC and its precursors, possibly under the control of mutated *ras* and upregulated epidermal growth factor receptor (EGFR). This mechanism has recently been elucidated in intestinal epithelial cells, where *ras*-dependent cIAP2 overexpression requires overproduction of transforming growth factor-α, a ligand for EGFR.[17] This phenomenon is thought to play a role in the growth and invasion of malignant tumours characterised by *ras* mutations and EGFR overexpression, such as lung cancer or pancreatic cancer. *ras* mutations are observed in up to 44% of PanIN1 lesions, in 87% of PanIN2–3 lesions[45] and in up to 100% of PDAC.[16] EGFR overexpression has been reported in a high percentage of PanIN3 and PDAC,[46] including this series, where it was detected in 38% of the cases (not shown), and the activation of the EGFR-signalling pathway has been related to cell dissociation in pancreatic cancer cell lines.[47]

The results of the immunohistochemical analysis also revealed an overexpression of cIAP2 in a wide range of pancreatic cystic tumours, thus implying that an abnormal regulation of the apoptosis—and possibly of anoikis—through IAP-mediated pathways is a common event in pancreatic tumorigenesis.

Role of cIAP1 and cIAP2 in pancreatic cancer progression

The parallel analysis of cIAP1 and cIAP2 expression in PDAC and their associated low-grade and high-grade PanIN lesions in eight cases inlcuded in this study (table 1) allowed us to examine the possible role of the two proteins in the progression of pancreatic cancer. cIAP1 was detected in a variety of non-neoplastic and neoplastic (non-invasive and invasive) pancreatic tissues, so that its expression in the PanIN lesions and in pancreatic cancer cannot be considered as a true ''overexpression'', but rather as the consequence of a different regulation of the protein function, with a change in the site of expression (ie, nuclear vs cytoplasmic), under oncogenic signals. On the other hand, most normal pancreatic tissues were negative or only focally positive for cIAP2, which was then truly overexpressed in the PanIN lesions and in the associated PDAC. This suggests that cIAP2 overexpression is an early event in the multistep pancreatic cancerogenesis, which is then maintained during tumour progression. The expression of cIAP2 in IPMNs, which are also precursors of invasive PDAC, gives further support to this assumption.

In conclusion, we have shown that cIAP1 and cIAP2 proteins are widely coexpressed in a variety of pancreatic tissues. cIAP1 might exert different functions in physiological and/or pathological conditions, depending on its subcellular localisation. cIAP2 is mainly associated with the process of pancreatic tumorigenesis, and its overexpression is an early event in the progression of PDAC. The coexpression of cIAP1 and cIAP2 in the cytoplasm of the cancer cells in the majority of PDAC, a subcellular localisation that is required to exert their anti-apoptotic functions, suggests an important synergistic effect of these two proteins in the inhibition of apoptosis in pancreatic cancer, which potentially influences patients' survival.

ACKNOWLEDGEMENTS

This work was supported by a grant awarded to Irene Esposito by the Medical Faculty of the University of Heidelberg (Juniorantrag 2003). We thank Mounia Chaib-Harrireche and Vesna Vukovic for excellent technical assistance.

Table 4 Expression analysis of cellular inhibitor of apoptosis protein 1 and cellular inhibitor of apoptosis protein 2 in a panel of pancreatic cystic tumours

Tumour histotype		cIAP1-positive cases/total cases (site)	cIAP2-positive cases/total cases (site)
IPMN	Adenoma	0/1	1/1 (C)
	Borderline	1/1 (C)	1/1 (C)
	Carcinoma	0/3	3/3 (C)
Mucinous cystadenoma		0/3	1/3 (C)
Serous cystadenoma		1/5 (C)	5/5 (C)

C, cytoplasmic expression; cIAP1, cellular inhibitor of apoptosis protein 1; cIAP2, cellular inhibitor of apoptosis protein 2; IPMN, intraductal papillary mucinous cystic neoplasm.

Take-home messages

- Cellular inhibitor of apoptosis protein 1 (cIAP1) is widely expressed in neoplastic and non-neoplastic pancreatic tissues and might exert different functions in physiological and/or pathological conditions, depending on its subcellular (ie, nuclear or cytoplasmic) localisation.
- Cellular inhibitor of apoptosis protein 2 (cIAP2) expression increases during pancreatic tumorigenesis, and its overexpression is an early event in the progression of pancreatic ductal adenocarcinoma (PDAC). cIAP2 might contribute to the deregulation of the apoptotic signals and to the anoikis resistance in PDAC.
- cIAP1 and cIAP2 are overexpressed in the majority of PDACs, and their coexpression is predictive of a worse prognosis.

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Competing interests: None.

Ethic approval: All studies were approved by the ethics committees of the University of Bern, Switzerland (No 127/93), and of the University of Heidelberg, Germany (No 301/2001).

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