Does β -Catenin Cross-Regulate NF κ B Signalling in Pancreatic Cancer and Chronic Pancreatitis?

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Key Words

β -Catenin · NF κ B · Chronic pancreatitis · Pancreatic cancer

Abstract

Background: It is not clear by which mechanism nuclear factor-kappaB (NF κ B) induces cell proliferation and escapes from the apoptotic pathway in pancreatic carcinogenesis. This study aimed to investigate β -catenin and NF κ B signalling in chronic pancreatitis and pancreatic cancer. **Materials and Methods:** On tissue samples of chronic pancreatitis and pancreatic cancer, we performed immunohistochemistry for detecting the expression of tumour necrosis factor alpha (TNF α), β -catenin and NF κ B, Western blot for TNF α , NF κ B, β -catenin, c-Myc and FasL, co-immunoprecipitation for β -catenin with NF κ B and RT-PCR for cyclin D1, c-Myc and Fas. **Results:** TNF α and NF κ B expression was increased in chronic pancreatitis and pancreatic cancer. β -Catenin and cyclin D1 expression was intense in pancreatic cancer and moderate in chronic pancreatitis. Of the NF κ B response elements, the expression of pro-proliferative c-Myc is intense in pancreatic cancer and moderate in chronic pancreatitis, but the pro-apoptotic factors, Fas and FasL were down-regulated in pancreatic cancer and moderately expressed in chronic pancreatitis. The co-immunoprecipitation results showed a significant interaction of β -catenin with NF κ B in pancreatic cancer and a non-significant interaction in chronic pancreatitis. **Conclusions:** β -Catenin might cross-regulate NF κ B by inter-

rupting the balance of cell death and cell survival via recruiting NF κ B into the cell survival pathway alone in most cases of pancreatic cancer and some cases of chronic pancreatitis.

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Introduction

Pancreatic cancer is a serious, life-threatening clinical problem of the world in terms of diagnosis and management [1]. The link between cancer and inflammation has been recognised for decades [2]. Cancer is frequently the outcome of chronic inflammatory disease in different tissues and organs, and a few studies have assessed the critical role of the inflammatory component in pancreatic cancer development [3–5]. Studies have shown that chronic pancreatitis is the most consistent risk factor for pancreatic cancer, and several confirm the relationship between them [6, 7]. Pancreatic cancer causes a desmoplastic reaction and duct obstruction that result in distal pancreatic atrophy and fibrosis resembling chronic pancreatitis [8].

Tumour necrosis factor alpha (TNF α) and nuclear factor-kappaB (NF κ B) are known to be the key mediators of the inflammatory process in chronic pancreatitis [9]. TNF initiates its signalling by binding to and trimerising TNFR1. TNFR1 interacts directly with its signal transducer, the TNFR1-associated death domain protein (TRADD), which, in turn, interacts with TNF receptor-

associated factor 2 (TRAF2) and receptor-interacting protein (RIP) to activate NFκB signalling [10]. This interaction activates the IKK complex [11], the inhibitor of NFκB (IκB) kinase (IKK) which consists of IKKα, IKKβ and IKKγ [12]. Activated IKK phosphorylates IκB [13] which inhibits the translocation of NFκB at specific serine residues, and then initiates the ubiquitination of IκB [14]. NFκB is released after IκB is degraded, and promptly translocates to the nucleus, binds to the κB site on DNA and initiates the transcription of target genes including Fas, FasL, c-Myc and IκBα [15].

The transcriptional factor NFκB has been reported to have pro- and anti-apoptotic effects, depending on the stimulus. It promotes cell-cycle progression by regulating the expression of the c-Myc and cyclin D1 genes [16]. NFκB has been implicated as a transcriptional activator of pro-apoptotic genes, such as Fas and FasL [17], and is required for p53-mediated apoptosis [18].

β-Catenin is a major component of Wnt signalling and has a role in cell proliferation and cell adhesion. De-regulation of β-catenin expression has been described in pancreatic neoplasia [19]. β-Catenin has two basic physiological roles inside the cells: cell-to-cell adhesion by association with E-cadherin, α-catenin or γ-catenin [20], and serving as an important mediator in the Wnt/β-catenin-signalling transduction pathway to regulate cell growth and cell differentiation [21, 22]. Several studies have suggested that the functional or stabilised β-catenin promotes cell survival, which is probably one of the underlying mechanisms for cancer development [23]. This study was aimed to study β-catenin and NFκB signalling in samples of pancreatic cancer and chronic pancreatitis.

Materials and Methods

This study was conducted on patients at the Stanley Medical College and Hospital, Chennai, India. Samples were collected under the approved protocol by the Institutional Ethical Review Board. The study population consisted of 69 patients with pancreatic cancer, 108 with chronic pancreatitis and 27 with a normal pancreas. Patients who had undergone chemotherapy or radiotherapy as well as post-operative cases (surgery for chronic pancreatitis or carcinoma of the pancreas) were excluded from the study. Patients with contrast-enhanced CT evidence of chronic pancreatitis and histologically proven adenocarcinoma of the pancreas were included in the study. Normal pancreatic tissue samples were collected from patients undergoing palliative surgery for advanced hepatocellular, gastric or colon cancer. The baseline characteristics of the patients and control group are given in table 1. Formalin-fixed and paraffin-embedded tissues were used for microscopic examination. Histological examinations were performed on hematoxylin and eosin-stained sections.

Table 1. Baseline characteristics of patients and controls

Characteristic	Chronic pancreatitis group	Pancreatic cancer group	Controls
Total number	108	69	27
Males:females	62:46	39:30	15:12
Mean age, years	38	62	48
Smokers ^a , n	31	31	0
Alcohol consumption ^b , n	55	32	0
Pancreatic duct calculi, n	108	11	0
Diabetics ^c , n	49	53	0

^a Patients who consumed >1 pack of cigarettes (>20 mg of nicotine) a day.

^b Patients who drank ≥3 alcoholic drinks (>1 litre of beer, >450 ml of red wine or >150 ml of liquor) a day.

^c Patients who had had type 2 diabetes for >5 years.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on 4-μm, paraffin-embedded pancreatic sections on poly-L-lysine-coated glass slides. The tissue sections were de-paraffinized by placing the slides in an oven at 65°C for 10 min and then 2 times in xylene for 10 min each. The slides were hydrated in a graded ethanol series for 10 min each. The sections were subsequently soaked in 1× PBS for 10 min and in 0.3% H₂O₂ for 30 min to block endogenous peroxidase activity. Sections were pressure-cooked in 10 mM sodium citrate buffer (pH 6) for 5 min. After washing with 1× PBS, they were blocked with 3% BSA for about 1 h at room temperature. The slides were washed in 1× PBS and incubated with primary antibodies [mouse monoclonal anti-TNFα (dilution 1:200), rabbit polyclonal anti-NFκB p65 (1:100) and rabbit polyclonal anti-β-catenin (1:200); Santa Cruz Biotechnology, Santa Cruz, Calif., USA] overnight at 4°C. The next day, the slides were washed with 1× PBS to remove any unbound primary antibodies and were incubated with the corresponding HRP-conjugated secondary antibodies at room temperature for 1 h. The slides were washed again with 1× PBS and the colour reaction was developed by incubating for 10 min in a solution containing 0.05% 3,3'-diaminobenzidine tetrachloride and 0.03% H₂O₂ in 1× PBS. The tissue sections were then counter-stained with hematoxylin. They were then dehydrated in a series of graded ethanol and cleared 2 times in xylene. Finally, they were mounted in DPX. Negative controls include incubating the tissue sections in the absence of the primary antibodies to confirm the specificity of the antibodies.

Stain intensity was graded on a scale of 0–3 (0 = non-staining, 1 = shallow brown, 2 = brown, 3 = dark brown). Positive cells were then graded on a scale of 0–3 (0 = no staining, 1 = <25% staining cells, 2 = 25–50% staining cells, 3 = >50% staining cells). Samples were then categorized by summation as: – (0–1), + (1–2), ++ (2–3) and +++ (3–4). Any staining with a score of ≥2 (+) was considered as positive expression and a score of 3 (+) was considered as over-expression. The slides were examined under Axioscop 2 plus, a Carl Zeiss microscope, and the cells were scored in 5 different fields. The mean value of the 5 areas was taken as representative of the whole section.

RT-PCR

c-Myc is a downstream gene of NF κ B and plays a significant role in cell proliferation. Cyclin D1 is a downstream gene of both β -catenin and NF κ B and also plays a significant role in cell proliferation. Fas is a downstream gene of NF κ B and is important in apoptosis. The expression intensity of these genes was analysed by RT-PCR. Pancreatic tissue (100 mg) was completely homogenised and total RNA was extracted using TRIZOL according to the manufacturer's instructions. Total RNA (1 μ g) was preheated for 5 min at 65°C and reverse-transcribed for 50 min at 42°C in a 20- μ l reaction mixture. The reverse transcription reaction was terminated by heating for 15 min at 65°C. The PCR reaction was performed using 1 μ l of cDNA with gene-specific primers for cyclin D1, c-Myc, Fas and β -actin in a 20- μ l reaction volume. The forward and reverse primers were as follows: β -actin, 5'-GGCATCGTCATGGACTCCG-3' and 5'-GCTGGAAGGTGGACAGCGA-3'; cyclin D1, 5'-CTGGAGCCCGTGAAAAAGAGC-3' and 5'-CTGGAGAGGAAGCGTGTGAGG-3'; c-Myc, 5'-TGGTCTTCCCCTACCCTCTCAAC-3' and 5'-GATCCAGACTCTGACCTTTTGCC-3'; Fas, 5'-CAGAACTTGAAGGCCTGCATC-3' and 5'-TCTGTTCTGCTGTGTCTTGGAC-3'. The PCR was performed with a Techgene thermal cycler (TECHNE, Duxford, UK). The β -actin was used as a positive control. Products were analysed using 2% agarose gel electrophoresis. The results were analysed by densitometry analysis with ImageJ software.

Western Blot

Human pancreatic tissue (10 mg) was homogenized in 1 ml of lysis buffer and the protein concentration was determined by Lowry's method [24]. Protein (20 μ g) was separated on 10% (w/v) polyacrylamide gels and transferred to a PVDF membrane (Hybond P, Amersham) by means of a semi-dry, multi-gel electroblotter. Blots were incubated with mouse monoclonal anti-TNF α , rabbit polyclonal anti- β -catenin, rabbit polyclonal anti-NF κ B p65, rabbit polyclonal anti-c-Myc and rabbit polyclonal anti-Fas-L in a dilution of 1:5,000 (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) for 4 h. After incubation with the corresponding secondary antibody conjugated with HRP, the bands were detected with an ECL Plus detection kit (Amersham). Band intensity was quantified with ImageJ software and normalised with β -actin.

Co-Immunoprecipitation of β -Catenin with NF κ B

To determine whether the interaction of β -catenin with NF κ B plays a role in the mechanism of carcinogenesis, co-immunoprecipitation of NF κ B with β -catenin was carried out. Pancreatic tissue lysates (100 μ l) were immunoprecipitated with 1 μ l of rabbit polyclonal NF κ B in a shaker overnight at 4°C. Protein A sepharose beads (20 μ l; Sigma) were added to the immunocomplex and incubated for 30 min. The immunocomplex along with the sepharose beads was retrieved by centrifuging at 12,000 rpm for 1 min at 4°C. The pellets were washed 5 times, with 100 μ l homogenising buffer, re-suspended in 30 μ l of sample buffer, vortexed briefly, boiled for 10 min at 95–100°C and separated on 10% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane, immunoblotted with rabbit polyclonal anti- β -catenin and detected with an ECL Plus kit (Amersham) according to the manufacturer's protocol. A sample was processed with PBS instead of tissue lysate and this served as negative control.

Statistics

SPSS version 15 was used for the statistical analysis. The data were expressed as mean $\pm$ SD. A non-parametric test, the Kruskal-Wallis test, was used for ordered categorical data versus continuous data. $p < 0.05$ was considered to be statistically significant. ImageJ software was used to analyse the relative expression of all Western blots and RT-PCR results.

Results

TNF α Expression and Localization

The inflammatory marker TNF α was highly localized around the cell membranes in 53.62% (37/69) of the pancreatic cancer samples and in 54.62% (59/108) of the chronic pancreatitis samples. It was significantly higher in the samples of chronic pancreatitis ($p < 0.001$) and pancreatic cancer ($p < 0.001$) than in the normal pancreas samples (online suppl. table 1, online suppl. fig. 1a–c; for all online suppl. material, see www.karger.com/doi/10.1159/000369887). The Western blott results also showed significantly higher expression of TNF α in the samples of chronic pancreatitis ($p < 0.001$) and pancreatic cancer ($p < 0.001$; online suppl. fig. 1d–f).

β -Catenin Localization and Expression

β -Catenin was highly localized around the cell membranes in 53.7% (58/108) of chronic pancreatitis samples and in 21.7% (15/69) of the pancreatic cancer samples; 22.22% (24/108) of the chronic pancreatitis and 27.5% (19/69) of the pancreatic cancer samples, respectively, showed high cytoplasmic stability of β -catenin. In 24.07% (26/108) of the chronic pancreatitis samples and 50.7% (35/69) of the pancreatic cancer samples, respectively, β -catenin was highly localized in the nucleus (online suppl. table 2; fig. 1a–c). The semi-quantitative results of β -catenin IHC for the normal pancreas, chronic pancreatitis and pancreatic cancer are given in online supplementary table 3.

Western blot results also showed significantly higher expression of β -catenin (molecular weight $\sim$ 95 kDa) in the samples of chronic pancreatitis and pancreatic cancer (fig. 1d–f). Total β -catenin expression was significantly high in 66.6% (46/69) of pancreatic cancer samples ($p < 0.001$) and in 55.5% (60/108) of chronic pancreatitis samples ($p < 0.001$).

NF κ B Localization and Expression

High cytoplasmic stability of NF κ B was displayed in 53.70% (58/108) of the chronic pancreatitis samples and in 36.23% (25/69) of the pancreatic cancer samples. In

Fig. 1. a–c IHC analysis of β -catenin in a normal pancreas (N), chronic pancreatitis (CP) and pancreatic cancer (PC). **a** Normal pancreatic tissue showing β -catenin expression. **b** Chronic pancreatitis tissue showing moderate expression of β -catenin. **c** Pancreatic cancer tissue showing high expression of β -catenin. Arrows indicate β -catenin expression. $\times 20$. **d** Tissue lysates were analysed by immunoblotting with anti- β -catenin antibody (lane N, lane CP and lane PC). The expression intensity of β -catenin (95 kDa) was moderate in chronic pancreatitis and strong in pancreatic cancer. **e** β -Actin was used as loading control for all the Western blots (lane N, lane CP and lane PC). **f** Graph showing the relative expression of β -catenin.

46.29% (50/108) of the chronic pancreatitis samples and 63.76% (44/69) of the pancreatic cancer samples, NF κ B was localized in the nucleus (fig. 2a–c; online suppl. table 4). The semi-quantitative results of NF κ B IHC for the normal pancreas, chronic pancreatitis and pancreatic cancer are given in online supplementary table 5.

Western blot results also showed significantly higher expression of NF κ B (molecular weight ~65 kDa) in the samples of chronic pancreatitis and pancreatic cancer (fig. 2d, f). NF κ B was up-regulated in 41/69 (59.42%) of the pancreatic cancer samples ($p < 0.001$) and in 61/108 (56.48%) of the chronic pancreatitis samples ($p < 0.001$).

Interaction of β -Catenin with NF κ B

A 95-kDa band of β -catenin-NF κ B interaction complex was clearly observed in 57.97% (40/69) of the pancreatic cancer samples and in 22.22% (24/108) of the chronic pancreatitis samples. The results showed that

β -catenin was significantly associated with NF κ B in the pancreatic cancer samples ($p < 0.001$). Interestingly, this interaction was not significant in the samples of chronic pancreatitis ($p > 0.05$; fig. 2e).

Expression of Cyclin D1 Gene by RT-PCR

In 57.97% (40/69) of the pancreatic cancer samples and in 56.48% (61/108) of the chronic pancreatitis samples, there was high expression of cyclin D1 mRNA. It was significantly higher ($p < 0.001$ in both) than in the normal pancreatic tissue samples (online suppl. fig. 2a, b).

Expression of c-Myc Gene by RT-PCR

There was increased mRNA expression of c-Myc in 55.07% (38/69) of the pancreatic cancer samples ($p < 0.001$) and in 24.07% (26/108) of the chronic pancreatitis samples ($p > 0.05$). c-Myc mRNA expression was signifi-

Fig. 2. a–c IHC analysis of NFκB in a normal pancreas (N), chronic pancreatitis (CP) and pancreatic cancer (PC). **a** Normal pancreatic tissue showing NFκB expression. **b** Chronic pancreatitis tissue showing moderate expression of NFκB. **c** Pancreatic cancer tissue showing high expression of NFκB. Arrows indicate NFκB expression. $\times 20$. **d** Tissue lysates were analysed by immunoblotting with anti-NFκB antibody (lane N, lane CP and lane PC). The expression of NFκB (65 kDa) was seen in all the stages with strong intensity in chronic pancreatitis and pancreatic cancer. **e** Interaction of NFκB with β -catenin was analysed by immunoprecipitation. Tissue lysates from all the 3 stages were immunoprecipitated with NFκB antibody and blotted with anti β -catenin antibody. Bands corresponding to β -catenin-NFκB interaction/complex were detected as high in pancreatic cancer and mild in chronic pancreatitis. The normal pancreas did not show any interaction. Bands were not detected in PBS (lane S). **f** Graph showing the relative expression of NFκB.

cantly increased in about half of the samples of pancreatic cancer and in a few of chronic pancreatitis when compared to the normal pancreatic tissue samples (online suppl. fig. 2c, d).

Western Blot of c-Myc

c-Myc expression was significantly increased in the pancreatic cancer samples ($p < 0.001$), but the number of chronic pancreatitis samples showing high expression of c-Myc was non-significant ($p > 0.05$) compared to the normal pancreatic tissue samples. Interestingly, the samples which showed the interaction of β -catenin with NFκB also showed up-regulation of the c-Myc gene; 57.97% (40/69) of the pancreatic cancer samples, 22.22% (24/108) of the chronic pancreatitis samples and 11.11% (3/27) of the normal samples showed both β -catenin-NFκB interaction and high expression of the c-Myc gene (fig. 3a, b; table 2).

Expression of Fas Gene by RT-PCR

There was low mRNA expression of Fas in 57.97% (40/69) of the pancreatic cancer samples and in 22.2% (24/108) of the chronic pancreatitis samples. Down-regulation of Fas mRNA was significant in the pancreatic cancer samples ($p < 0.001$) and non-significant in the chronic pancreatitis samples ($p > 0.05$) when compared to the normal pancreatic tissue samples, with 57.97% (40/69) of the pancreatic cancer samples, 22.22% (24/108) of the chronic pancreatitis samples and 11.11% (3/27) of the normal pancreatic tissue samples showing both β -catenin-NFκB interaction and low expression of Fas mRNA (online suppl. fig. 2e, f; table 2).

Expression of FasL by Western Blot

FasL expression was significantly lower in the pancreatic cancer samples ($p < 0.001$), but the number of chronic pancreatitis samples showing low expression of FasL

Fig. 3. a Tissue lysates were analysed by immunoblotting with anti-c-Myc antibody. The expression intensity of c-Myc (62 kDa) was moderate in chronic pancreatitis and strong in pancreatic cancer. **b** Graph showing relative expression of c-Myc. **c** Tissue lysates were analysed by immunoblotting with anti-FasL antibody. The expression intensity of FasL (48 kDa) was strong in the normal pancreas, moderate in chronic pancreatitis and mild in pancreatic cancer. **d** Graph showing relative expression of FasL. CP = Chronic pancreatitis; N = normal; PC = pancreatic cancer.

Table 2. Association of β -catenin-NF κ B interaction with c-Myc and Fas expression

Expression intensity	β -Catenin-NF κ B interaction	Mean $\pm$ SD	t value	p value
c-Myc	positive	2.61 $\pm$ 0.49	19.342	<0.001
	negative	1.15 $\pm$ 0.51		
Fas	positive	0.51 $\pm$ 0.61	31.691	<0.001
	negative	2.84 $\pm$ 0.42		

Mean $\pm$ SD was used to study this association. $p < 0.05$ was statistically significant.

was non-significant ($p > 0.05$), with 57.97% (40/69) of the pancreatic cancer samples and 21.29% (23/108) of the chronic pancreatitis samples showing both β -catenin-NF κ B interaction and down-regulation of the FasL gene (fig. 3c, d).

Discussion

This study confirms the up-regulation of β -catenin, a component of the Wnt pathway. It was noticed that the normal pancreas and chronic pancreatitis samples showed the usual membranous expression of β -catenin in about half of the cases. Few of the chronic pancreatitis samples and most of the pancreatic cancer samples showed cytoplasmic stability and nuclear localization of

β -catenin. Here, β -catenin may act as an oncogene. β -Catenin is the foremost upstream regulator of cyclin D1. The mRNA expression of cyclin D1 was increased 3.2-fold in the pancreatic cancer samples compared to the normal pancreatic tissue samples. Progression through the G1 checkpoint of the mammalian cell cycle is regulated by the activity of D-type cyclins and their partners, the cyclin-dependent kinases, CDK-4 and CDK-6. Of the 3 D-type cyclins, cyclin D1 has been convincingly implicated as an oncogene in human tumorigenesis [25]. The proto-oncogenic function of cyclin D1 has been recognised as the role it plays in promoting cell cycle progression. Cyclin D1 is a key cell-cycle regulator of the G1-S phase progression [26]. Over-expression of cyclin D1 is observed in a wide variety of malignancies [25].

Our results document an up-regulation of the inflammatory mediators TNF α and NF κ B in chronic pancreatitis and pancreatic cancer. In pancreatic cancer, inflammatory mediators, including TNF α and NF κ B, are known to facilitate the growth of tumour cells and metastasis [5]. NF κ B has been reported to have pro- and anti-apoptotic effects, depending on the stimulus. It promotes cell-cycle progression by regulating the expression of the c-Myc gene [16]. It has been reported that c-Myc plays an important role in the proliferation of cells. We observed that NF κ B expression was increased 2.4-fold in chronic pancreatitis and pancreatic cancer samples compared to in normal pancreas samples. NF κ B has been implicated as a transcriptional activator of pro-apoptotic genes, such as Fas and FasL [17]. Activated T cells express FasL as a major cytotoxic effector through which these cells kill their

targets. Fas and FasL expression are under the control of various transcription factors, including NF κ B. It has been reported that TNF α combined with IFN- α accelerates NF κ B-mediated apoptosis by enhancing Fas expression in RPMI4788 human colon cancer cells. Luo et al. [27] clearly demonstrated the dual role of NF κ B in cell proliferation and cell death. Studies have suggested that resistance to apoptosis with a loss of Fas function may play an important role in the pathogenesis of several malignancies, including colon and pancreatic cancer [28]. Our study also showed the low expression of Fas and FasL in most of the pancreatic cancer samples and a few of the chronic pancreatitis samples.

GSK3 β has been shown to be required for NF κ B activation [29]. As β -catenin is a major substrate of GSK3 β , this raises the interesting possibility that β -catenin might serve as a mediator for the cross-regulation between these two pathways. We have already found the low expression of GSK3 β in pancreatic cancer samples (unpublished data). In this study, we found that β -catenin was in strong association with NF κ B in the pancreatic cancer samples, i.e. 4.5-fold higher than that found in normal pancreatic tissue samples. Chronic pancreatitis samples showed a non-significant NF κ B- β -catenin interaction. Interestingly, the samples which showed the interaction of β -catenin with NF κ B also showed high expression of the c-Myc gene and low expression of the Fas gene. Thus, we found that β -catenin physically interacted with NF κ B and inhibited its apoptotic activity in about half of the pancreatic cancer samples and a few of the chronic pancreatitis samples. In addition, suppression of the apoptotic activity of NF κ B and its target gene expression can be found in cancer samples expressing high levels of β -catenin. Importantly, β -catenin was found to inhibit

Fas expression through NF κ B signalling [30]. Several studies have found that β -catenin can physically complex with NF κ B, resulting in a reduction of NF κ B DNA-binding, transactivation activity, possibly thereby activating the transcription of only a subset of its target gene expression [31–33].

This study found out that β -catenin might cross-regulate NF κ B by interrupting the balance maintained during cell death and cell survival, via the recruiting of NF κ B only into the cell survival pathway in most cases of pancreatic cancer and in a few cases of chronic pancreatitis. Chronic pancreatitis is the most consistent risk factor for pancreatic cancer, so even though only a few of the chronic pancreatitis samples showed the above expression, these patients may develop later progression of the disease to pancreatic cancer. Further studies are needed.

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Disclosure Statement

The authors declare no conflict of interest.

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