Does β-Catenin Cross-Regulate NFκB Signalling in Pancreatic Cancer and Chronic Pancreatitis?

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Key Words
β-Catenin · NFκB · Chronic pancreatitis · Pancreatic cancer

Abstract
Background: It is not clear by which mechanism nuclear factor-kappaB (NFκB) induces cell proliferation and escapes from the apoptotic pathway in pancreatic carcinogenesis. This study aimed to investigate β-catenin and NFκB 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 (TNFα), β-catenin and NFκB, Western blot for TNFα, NFκB, β-catenin, c-Myc and FasL, co-immunoprecipitation for β-catenin with NFκB and RT-PCR for cyclin D1, c-Myc and Fas.

Results: TNFα and NFκB 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 NFκB 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 NFκB in pancreatic cancer and a non-significant interaction in chronic pancreatitis.

Conclusions: β-Catenin might cross-regulate NFκB by interrupting the balance of cell death and cell survival via recruiting NFκB into the cell survival pathway alone in most cases of pancreatic cancer and some cases of chronic pancreatitis.

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 (TNFα) and nuclear factor-kappaB (NFκB) are known to be the key mediators of the inflammatory process in chronic pancreatitis [9]. TNF initiates its signalling by binding to and trimerising TNFRI. TNFRI 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 NFXB signalling [10]. This interaction activates the IKK complex [11], the inhibitor of NFXB (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 IκBα [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 NFXB 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]. NFXB 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 NFXB 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 patient 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>
<thead>
<tr>
<th>Table 1. Baseline characteristics of patients and controls</th>
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<tr>
<td>Characteristic</td>
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<td>----------------</td>
</tr>
<tr>
<td>Total number</td>
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<tr>
<td>Males/females</td>
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<tr>
<td>Mean age, years</td>
</tr>
<tr>
<td>Smokersa, n</td>
</tr>
<tr>
<td>Alcohol consumptionb, n</td>
</tr>
<tr>
<td>Pancreatic duct calculi, n</td>
</tr>
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<td>Diabeticsc, n</td>
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</table>

* Patients who consumed ≥1 pack of cigarettes (>20 mg of nicotine) a day.
* Patients who drank ≥3 alcoholic drinks (>1 litre of beer, >450 ml of red wine or >150 ml of liquor) a day.
* Patients who 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-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.
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 μg) 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 electrophorler. Blots were incubated with mouse monoclonal anti-TNFα, rabbit polyclonal anti-β-catenin, rabbit polyclonal anti-NFκB 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 correspond- ing 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 NFκB
To determine whether the interaction of β-catenin with NFκB plays a role in the mechanism of carcinogenesis, co-immunoprecipitation of NFκB with β-catenin was carried out. Pancreatic tissue lysates (100 μl) were immunoprecipitated with 1 μl of rabbit polyclonal NFκB in a shaker overnight at 4°C. Protein A sepharose beads (20 μl; 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 μl homogenising buffer, re-suspended in 30 μl of sam- ple 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.

TNFα Expression and Localization
The inflammatory marker TNFα 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 TNFα 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% (35/126) 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 supplemental table 3.

Western blot results also showed significantly higher expression of β-catenin (molecular weight ~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 the pancreatic cancer samples and in 54.62% (59/108) of the chronic pancreatitis samples (p < 0.001) and in 55.5% (60/108) of chronic pancreatitis samples (p < 0.001).

NFκB Localization and Expression
High cytoplasmic stability of NFκB was displayed in 53.70% (58/108) of the chronic pancreatitis samples and in 36.23% (25/69) of the pancreatic cancer samples . In
46.29% (50/108) of the chronic pancreatitis samples and 63.76% (44/69) of the pancreatic cancer samples, NFκB was localized in the nucleus (fig. 2a–c; online suppl. table 4). The semi-quantitative results of NFκB 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 NFκB (molecular weight \( \sim 65 \) kDa) in the samples of chronic pancreatitis and pancreatic cancer (fig. 2d, f). 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.

Interaction of β-Catenin with NFκB

A 95-kDa band of β-catenin-NFκB 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 NFκB 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-
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 \( (\text{fig. 3a, b; table 2}) \).

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 \( (\text{online suppl. fig. 2e, f; table 2}) \).

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...
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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-NFκB interaction and down-regulation of the FasL gene (fig. 3 c, 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 TNFα and NFκB in chronic pancreatitis and pancreatic cancer. In pancreatic cancer, inflammatory mediators, including TNFα and NFκB, are known to facilitate the growth of tumour cells and metastasis [5]. 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 gene [16]. It has been reported that c-Myc plays an important role in the proliferation of cells. We observed that NFκB expression was increased 2.4-fold in chronic pancreatitis and pancreatic cancer samples compared to normal pancreas samples. NFκB 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.

Table 2. Association of β-catenin-NFκB interaction with c-Myc and Fas expression

<table>
<thead>
<tr>
<th>Expression intensity</th>
<th>β-Catenin-NFκB interaction</th>
<th>Mean ± SD</th>
<th>t value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Myc positive</td>
<td>2.61±0.49</td>
<td>19.342</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>1.15±0.51</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fas positive</td>
<td>0.51±0.61</td>
<td>31.691</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>2.84±0.42</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Mean ± SD was used to study this association. p < 0.05 was statistically significant.

**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.
targets. Fas and Fasl expression are under the control of various transcription factors, including NFκB. It has been reported that TNFα combined with IFN-α accelerates NFκB-mediated apoptosis by enhancing Fas expression in RPMI4788 human colon cancer cells. Luo et al. [27] clearly demonstrated the dual role of NFκB 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.

GSK3β has been shown to be required for NFκB activation [29]. As β-catenin is a major substrate of GSK3β, 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 GSK3β in pancreatic cancer samples (unpublished data). In this study, we found that β-catenin was in strong association with NFκB 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 NFκB-β-catenin interaction. Interestingly, the samples which showed the interaction of β-catenin with NFκB 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 NFκB 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 NFκB 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 NFκB signalling [30]. Several studies have found that β-catenin can physically complex with NFκB, resulting in a reduction of NFκB 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 NFκB by interrupting the balance maintained during cell death and cell survival, via the recruiting of NFκB 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.

Acknowledgments

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Disclosure Statement

The authors declare no conflict of interest.

References


