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Moderate Activation of the Apoptosis Inhibitor bcl-xL Worsens the Prognosis in Pancreatic Cancer

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Objective

To analyze the expression of the antiapoptotic gene bcl-xL in human pancreatic cancer and to correlate the results with clinical patient parameters.

Summary Background Data

Bcl-xL belongs to the bcl-2-related gene family and acts as a broad antiapoptotic factor to extend both normal and tumor cell survival. Recent findings indicate that tumor cell death induced by chemotherapy and radiotherapy is mediated by the activation of apoptosis. The fact that pancreatic cancer has an extremely malignant potential and that it is resistant to most anticancer treatment modalities suggests that mechanisms are activated that increase the viability of pancreatic cancer cells.

Methods

Seventy-four pancreatic cancer tissue samples were obtained from 32 female and 42 male patients undergoing surgery for exocrine pancreatic cancer. Normal human pancreatic tissue samples were available from 11 organ donors and 4 patients without pancreatic disease. The levels of bcl-xL mRNA expression were analyzed by Northern blot analysis. The exact site of bcl-xL mRNA transcription was determined by nonradioactive *in situ* hybridization. In addition, immunohistochemistry using specific polyclonal antibodies was used to localize the protein.

Results

Northern blot analysis indicated that, in comparison with the normal pancreas, bcl-xL mRNA was markedly overexpressed in 54% of the pancreatic cancer samples. Densitometric analysis revealed that pancreatic adenocarcinomas exhibited a mean 3.4-fold increase ($p < 0.01$) in bcl-xL mRNA levels in comparison with normal controls. With *in situ* hybridization, bcl-xL mRNA was found to be highly expressed in the cancer cells of tumor samples that exhibited increased mRNA expression by Northern blot analysis. Immunohistochemical analysis revealed bcl-x immunostaining in 88% of the cancer samples. Correlation of the molecular data with clinical patient parameters revealed that patients whose tumors exhibited no, faint, or weak bcl-xL expression lived significantly longer after tumor resection (median 12 months) than patients whose tumors exhibited moderate bcl-xL mRNA expression (median 5 months) ($p < 0.05$). However, 5 patients whose tumors exhibited intense bcl-xL mRNA expression tended to live longer (median 14 months).

Conclusion

Enhanced expression of the antiapoptotic gene bcl-xL in pancreatic cancer and its association with shorter patient survival suggests that this factor may enhance the viability of pancreatic cancer cells *in vivo*. Inhibition of apoptotic pathways might be one of the reasons why pancreatic cancer shows only limited sensitivity to anticancer treatment.

Although pancreatic cancer has an incidence of about 10 cases/100,000 persons, it is still the fourth or fifth leading cause of cancer-related death in Western industrialized countries.[1] Most cases are diagnosed in advanced tumor stages, and therefore only a few patients qualify for tumor resection.[2–4] In most patients the tumor has already metastasized at the time of diagnosis into lymph nodes or distant organs, or the primary tumor has infiltrated the large retroperitoneal vessels, making tumor resection impossible without major vessel resection and substitution.

In addition to the extensive progression in local tumor

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growth and the early formation of metastases,[5] pancreatic cancers are unresponsive to most standard oncologic therapies, including chemotherapy, radiotherapy, immunotherapy, and/or antihormonal treatment.[3,5–9] The reason for pancreatic cancer cells' insensitivity to standard anticancer treatment is poorly understood.

Therapy with cytotoxic anticancer drugs and radiation induces cancer cell death by activating apoptotic pathways.[10,11] However, the activation of antiapoptotic factors inhibits the cell death initiated by ionizing radiation and anticancer drugs, resulting in the failure of treatment response.[12,13] These findings underline the importance of the fine balance between different apoptotic pathways in chemotherapy and/or radiotherapy. Moreover, it may be possible that changes in apoptosis-promoting or apoptosis-inhibiting parameters contribute to the unresponsiveness of pancreatic cancer cells to these treatment modalities. A deeper knowledge of these relations could give rise to new therapies.

Apoptosis is a central regulator of tissue homeostasis. It contributes to the elimination of damaged cells in normal tissues and provides the balance of the appropriate cell number under the circumstances of physiologic cell proliferation and tissue repair.[14,15] Further, the spontaneous appearance of malignant tumors in transgenic mice overexpressing the apoptosis inhibitor bcl-2 underlines the potential importance of these pathways in cancer development and progression.[16,17]

The bcl-2 family is the best characterized group of apoptosis-mediating factors, which include bcl-2, mcl-1, bcl-x, bax, bak, and several others.[18] Although its members share close structural homologies, their biologic functions differentiate into apoptosis-promoting (bax, bak, bcl-xS) or apoptosis-inhibiting (bcl-2, mcl-1, bcl-xL) properties.[16] Bcl-2 was the first gene in this apoptotic family to be discovered, and knowledge of its properties is expanding rapidly.[18,19] Bcl-2, located at chromosome 18q21, consists of three exons separated by large introns.[20] It functions as an antiapoptotic factor by inhibiting different cell death mechanisms.[10,12,21,22] Bcl-x is a bcl-2-related gene that can function as a bcl-2-independent regulator of apoptosis.[23] Two distinct bcl-x subforms have been identified in humans. Bcl-xL, the longer form, contains an open reading frame encoding a 241-amino acid protein of 21 kDa and includes the BH1 (bcl-2 homology 1) and BH2 (bcl-2 homology 2) domains.[18] It is 44% identical to bcl-2 and functions like bcl-2 as a blocker of apoptosis. Bcl-xS, the shorter form, encodes a 178-amino acid protein of 19 kDa. The carboxy-terminal 63 amino acids encoding the BH1 and BH2 domains are deleted, and in contrast to bcl-xL it functions as an apoptosis promoter.[18,23]

The functions of apoptosis-influencing genes in the pathogenesis and progression of human pancreatic cancer are not known, but activation and downregulation of apoptosis-blocking or -stimulating genes may influence cancer cell viability, cancer cell sensitivity to anticancer drugs and radiation, and subsequently tumor progression. In the present study we analyzed the expression and distribution of bcl-xL in 74 human exocrine pancreatic cancers and correlated the molecular and immunohistochemical results with clinicopathologic parameters.

PATIENTS AND METHODS

Normal pancreatic tissues were obtained from 11 previously healthy organ donors (2 women, 9 men) and from 4 patients (2 women, 2 men) in whom pancreatic tissue was resected as an extension to another intraabdominal resection. The median age of the patients in the control group was 44 years (range 24 to 71 years). Pancreatic cancer tissues were obtained from 74 patients (32 women, 42 men) undergoing surgery for exocrine pancreatic cancer. Patients with cancer of the papilla of Vater, distal bile duct, juxtapapillary part of the duodenum, and endocrine pancreas were excluded. The median age of the patients with pancreatic cancer was 65 years (range 43 to 79 years). According to the TNM classification of the International Union Against Cancer,[24] there were 9 patients with stage I, 21 patients with stage II, 38 patients with stage III, and 6 patients with stage IV disease. Of the patients with stage I to III disease, a partial duodenopancreatectomy (Whipple resection) was carried out in 61 patients, a distal pancreatectomy in 6 patients, and a total duodenopancreatectomy in 1 patient. Of the six patients with stage IV disease, one patient underwent a Whipple resection in combination with a partial liver resection, and the others underwent a bypass procedure and tumor biopsy. All patients with stage IV disease were excluded from the survival analysis. Further, one patient with total duodenopancreatectomy died early after surgery and therefore was not included in the survival analysis. The final survival analysis included the data of 67 patients.

Freshly removed pancreatic tissue samples were immediately fixed in Bouin or paraformaldehyde solution for 12 to 24 hours and paraffin-embedded for routine histopathologic analysis and for immunohistochemical analysis and *in situ* hybridization. In addition, tissues destined for RNA extraction were frozen in the operating room in liquid nitrogen immediately on surgical removal and maintained at −80°C until use. All the studies were approved by the Ethics Committee of the University of Bern, Bern, Switzerland.

Northern Blot Analysis

Total RNA was extracted by the guanidinium isothiocyanate method. Twenty μg of total RNA was size-fractionated on 1.2% agarose/1.8 M formaldehyde gels and stained with ethidium bromide for verification of RNA integrity and loading equivalency.[5,25,26] The RNA was electrotransferred onto nylon membranes (Gene Screen, DuPont, Boston, MA) and cross-linked by ultraviolet irradiation. The filters were

then prehybridized, hybridized, and washed under conditions appropriate for the alpha-^{32}P-labeled antisense riboprobe (bcl-xL) or cDNA probe (7S), as previously described in detail.[5,25,26]

For the riboprobe, the blots were prehybridized for 6 hours in 50% formamide, 0.5% sodium dodecyl sulfate (SDS), 5 × SSC (sodium chloride/sodium citrate buffer), 5 × Denhardt's solution (1 × Denhardt's solution = 0.02% ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 250 mg/l salmon sperm DNA, and 50 mmol/l sodium phosphate buffer (pH 6.5). The blots were then hybridized for 18 hours at 65°C in the presence of 1×10^6 cpm/ml of the labeled antisense riboprobe, washed twice at 65°C in a solution containing 1 × SSPE (150 mmol/l NaCl, 10 mmol/l NaH_2PO_4, and 1 mmol/l EDTA) and 0.5% SDS, and washed twice at 65°C in a solution containing 0.1 × SSPE and 0.5% SDS.[5,25,26]

For the 7S cDNA probes, prehybridization was carried out for 4 to 8 hours at 42°C in a buffer that contained 50% formamide, 1% SDS, 0.75 mol/l NaCl, 5 mmol/l EDTA, 5 × Denhardt's solution, 100 mg/l salmon sperm DNA, 10% dextran sulfate, and 50 mmol/l sodium phosphate buffer, pH 7.4. Hybridization was carried out at 42°C for 18 hours after adding 1×10^5 cpm/ml of the ^{32}P-labeled 7S cDNA probe to the prehybridization buffer. After the hybridization solution was removed, the blots were washed under stringent conditions consisting of two rinses in 2 × SSC at room temperature followed by three washings of 20 minutes each at 55°C in 0.2 × SSC and 2% SDS.

Blots were then exposed to Fuji x-ray films with intensifying screens (DuPont) at −80°C, and the intensity of the radiographic bands was quantified by video image analysis (Image-Pro plus for Windows, Media Cybernetics, Silver Spring, MD), as previously reported.[5,27]

For statistical analysis the ratio between the bcl-xL mRNA signal and the corresponding 7S signal was calculated for each sample.

In Situ Hybridization

In situ hybridization was performed as previously reported.[5,28] Briefly, using consecutive tissue slides, one slide was incubated with the digoxigenin (DIG)-labeled antisense riboprobe, and simultaneously the other was incubated with the corresponding sense probe under the same incubation conditions. The tissue sections (3 μm) were deparaffinized, rehydrated with 1 × phosphate-buffered saline (PBS) and incubated in 0.2 mol/l HCl for 20 minutes at room temperature. After the slides were rinsed in 2 × SSC, the sections were treated with proteinase K (Boehringer Mannheim, Mannheim, Germany) at a concentration of 20 μg/ml for 15 minutes at 37°C. After postfixation with 4% paraformaldehyde in PBS (5 minutes) and washing in 2 × SSC, the samples were prehybridized at 59°C for at least 2 hours in 50% formamide (v/v), 4 × SSC, 2 × Denhardt's reagent, and 250 μg RNA/ml. Hybridization was performed overnight at 59°C in 50% (v/v) formamide, 4 × SSC, 2 × Denhardt's reagent, 500 μg RNA/ml, and 10% dextran sulfate (w/v). The final concentrations of the DIG-labeled probes were approximately 2.5 ng/μl. After hybridization, excess probe was removed by washing the slides in 2 × SSC and by RNase treatment: 100 U/ml RNase T1 (Boehringer Mannheim) and 0.2 μg/ml RNase (DNase-free) (Boehringer Mannheim) at 37°C for 30 minutes. After washing in 2 × SSC for 20 minutes at 63°C, and in 0.14 × SSC and 30% formamide at 63°C for 20 minutes, the samples were incubated with the antidigoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim). For the final color reaction, 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma, Buchs, Switzerland) were used.[5,28]

Evaluation of the *in situ* hybridization results was performed semiquantitatively by two independent observers blinded to the patients' status.[5,28] According to the intensity of specific hybridization signals, the expression levels of bcl-xL mRNA were classified as absent/faint (−), weak (+), moderate (++) and intense (+++).

Probe Synthesis for Northern Blot Analysis and *In Situ* Hybridization

Riboprobes of bcl-xL were used both in Northern blot analysis and in *in situ* hybridization. The bcl-xL cRNA probe consisted of a ClaI/Xba I fragment of human bcl-xL cDNA prepared by reverse transcriptase polymerase chain reaction.[23] The cDNA fragment was subcloned into the pGEM3Zf vector (Promega, Biotechnology, Madison, WI) carrying promoters for the DNA-dependent T3 and T7 RNA polymerases. The authenticity of the bcl-xL fragment was confirmed by sequencing using the dye terminator method (ABI 373A, Perkin Elmer, Rotkreuz, Switzerland).

The 7S cDNA probe consisted of a 190 base pair BamHI fragment of the mouse 7S cytoplasmic cDNA that cross-hybridizes with human 7S RNA.[5,25,26] This probe was used to verify equivalent RNA loading in the Northern blot experiments.

For Northern blot analysis, the bcl-xL cRNA antisense probe was radiolabeled with [α-^{32}P]CTP (DuPont International, Regensdorf, Switzerland). The 7S cDNA probe was labeled with [α-^{32}P]dCTP (DuPont International) using a random primer labeling system (Boehringer Mannheim).[5,25,26]

For *in situ* hybridization, the bcl-xL cRNA probe was labeled with digoxigenin.[5,28] After linearization, the cDNA was transcribed using the Ribomax System (Promega). The transcription resulted in digoxigenin-labeled antisense riboprobe specific for the bcl-xL mRNA. The corresponding sense probe was prepared in an analogous manner. For *in situ* hybridization, the sense and antisense probes were shortened to a length of about 150 bases and stored in diethylpyrocarbonate-treated water at −70°C until further use.[5,28]

Immunohistochemistry

Immunohistochemical analysis was performed with the streptavidin–phosphatase technique.[27,28] After deparaffinization and hydration, the slides (3 μm) were washed in Tris-buffered saline (TBS) buffer (10 mmol/l Tris-HCl, 0.85% sodium chloride, pH 7.4) containing 0.1% bovine serum albumin, immersed in 10 mM citrate buffer (10 mM sodium citrate, pH 6.0), and then exposed to microwave irradiation twice for 5 minutes with a cooling period of 4 minutes between the sessions.[27] After cooling to room temperature and repeated washing with fresh TBS buffer, the slides were incubated with 10% normal goat serum at 23°C for 30 minutes to block unspecific binding before adding specific polyclonal bcl-x antibodies (overnight, 4°C) (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The anti-bcl-x antibodies were produced by immunizing a rabbit against amino acids 2 to 19, mapping at the amino terminus of bcl-xL/S of human origin. There was no cross-reactivity between these antibodies and other members of the bcl-2 gene family. After washing with TBS buffer, biotinylated goat antirabbit immunoglobulin IgG secondary antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was added at 23°C for 35 minutes. After washing in TBS buffer and incubation with the streptavidin–phosphate complex at 23°C for 20 minutes, the slides were incubated with the phosphate ester of 6-bromo-2-hydroxy-3-naphthoic acid (Kirkegaard & Perry Laboratories) as the substrate (red color reaction).[27,29] The slides were then counterstained with Mayer's hematoxylin.

To ensure specificity of the immunostaining reactions, consecutive tissue sections were incubated either in the absence of the primary antibody or with a nonimmunized IgG antibody. In both cases, no immunostaining was detectable.

Evaluation of the immunohistochemical results was performed by two independent observers blinded to the patients' status. The intensity of the immunohistochemical signals was classified into four levels: 0 = no staining; 1 = weak staining; 2 = moderate staining; 3 = intense staining. In addition, the percentage of the immunoreactive cancer cells was recorded. The immunostaining score and the percentage of the immunoreactive cancer cells were multiplied, and the resulting numbers were used for further statistical analysis.[27,30] A final score of <0.8 was taken as negative, and a score of 0.8 or more was taken as positive. According to the value of scores assessed, positive samples were further divided into three different immunostaining groups: low immunoreactivity (>0.8 but <1.5), medium immunoreactivity (1.5 to 2.2), and high immunoreactivity (> 2.2) expression.

Statistical Analysis

The data are expressed as median and range. Statistical analysis was carried out using the SYSTAT Intelligent software (Evanston, IL). For statistical analysis, the chi square test, Mann-Whitney test, and log-rank test were used. Survival data were plotted according to the method of Kaplan and Meier and compared by the log-rank test.[31] Significance was defined as $p < 0.05$.

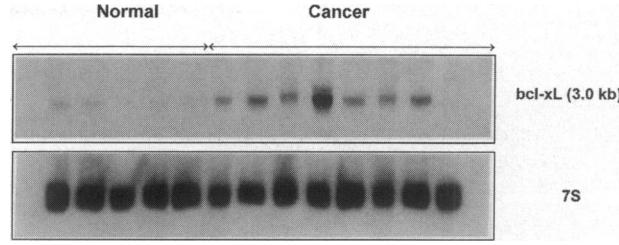

Figure 1. Northern blot analysis of bcl-xL mRNA expression in the normal pancreas (first 5 lanes) and in pancreatic cancer. Bcl-xL mRNA migrated as a 3 kb band. 7S RNA, migrating as a 0.4 kb band, was used to assess equivalent RNA loading.

RESULTS

Northern Blot Analysis of bcl-xL

Northern blot analysis using 20 μg total pancreatic RNA was carried out to determine bcl-xL mRNA expression in the normal and the cancerous pancreas. In all normal pancreatic tissue samples, bcl-xL mRNA expression was present at low levels (Fig. 1). In some normal pancreatic tissue samples, the 3.0 kb bcl-xL mRNA transcript was faint and was visible only on the original autoradiographs. No differences in bcl-xL mRNA levels were found between the organ donor pancreases and the pancreases of patients in whom pancreatic resection was done as an extension of another intraabdominal resection. In contrast, hybridization signals of bcl-xL mRNA were markedly higher in many pancreatic cancer samples than in normal controls (see Fig. 1). Bcl-xL mRNA levels were enhanced in 54% of the cancer samples in comparison with the normal controls. Densitometric analysis of the Northern blots indicated that the bcl-xL mRNA levels were 3.4-fold higher ($p < 0.01$) when all pancreatic cancer samples (samples with or without enhanced expression) were compared with the normal samples. When densitometric analysis was performed only in cancer samples with enhanced expression, there was a fivefold increase in pancreatic cancer ($p < 0.01$). None of the normal or the cancer samples revealed any aberrant bcl-xL mRNA transcript.

In Situ Hybridization of bcl-xL

In situ hybridization analysis was performed to determine the exact site of bcl-xL mRNA synthesis in the normal and cancerous pancreas. In normal pancreatic tissue samples, faint or no *in situ* hybridization signals of bcl-xL mRNA (Fig. 2A) were observed. The bcl-xL mRNA signal was present only in a few acinar cells and in some ductal cells.

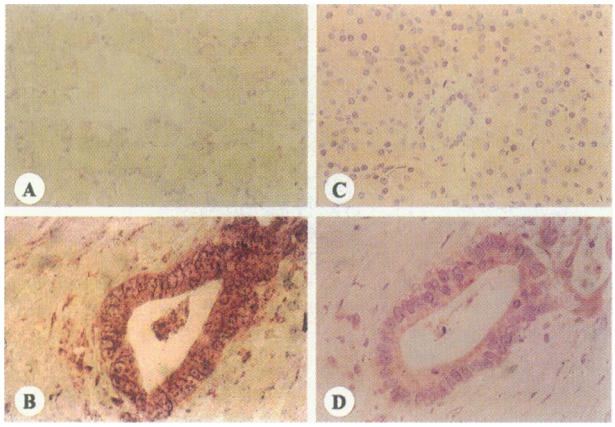

Figure 2. *In situ* hybridization (A,B) and immunohistochemical staining (C,D) of bcl-xL in the normal pancreas (A,C) and in pancreatic cancer (B,D). Panels A/C and B/D represent consecutive tissue sections. In the normal pancreas, only faint bcl-xL mRNA expression was present (A), whereas pancreatic cancer cells (B) exhibited high bcl-xL mRNA expression. The consecutive tissue sections used for immunohistochemistry exhibited no or faint bcl-x immunostaining in the normal pancreas (C), whereas moderate to strong immunoreactivity was found in the cytoplasm (red color) of the pancreatic cancer cells (D). Magnification × 400.

In contrast, 61% (45/74) of the pancreatic cancer samples exhibited greater bcl-xL mRNA staining than in the normal controls (Fig. 2B). Pancreatic cancer samples with enhanced bcl-xL mRNA levels using Northern blot analysis also showed strong mRNA expression in the pancreatic cancer cells using *in situ* hybridization. The percentages of cancer samples with weak, moderate, and intense bcl-xL mRNA expression were 35% (26/74), 19% (14/74), and 7% (5/74), respectively. In 39% (29/74) of pancreatic cancer samples, bcl-xL mRNA signals were absent or faintly detectable, comparable to the pattern in the normal controls. In pancreatic cancer tissues, bcl-xL mRNA signals were present only in the cancer cells; areas adjacent to the cancer lesion consisting of connective tissue and fibroblasts were devoid of any bcl-xL mRNA staining.

Immunohistochemistry of bcl-x

Immunohistochemical staining was performed in normal and cancerous pancreatic tissue sections simultaneously to guarantee comparability of the staining intensity between these groups. No or only faint bcl-x immunoreactivity was present in the acinar or ductal cells in normal pancreatic tissue samples (Fig. 2C). Further, in the interlobular and periductal stroma of the normal pancreas, no bcl-x immunostaining was found. There was no difference in bcl-x immunoreactivity between pancreatic tissue sections of organ donors and control pancreases obtained from patients who underwent pancreatic resection as a result of nonpancreatic disease. In contrast, pancreatic cancer cells exhibited stronger bcl-x immunoreactivity than was found in the normal controls (Fig. 2D). In 84% (62/74) of the pancreatic cancer samples, bcl-x immunoreactivity was stronger than in the control samples. In the cancer tissues, 39% (29/74), 31% (23/74), and 18% (13/74) had low, medium, and high immunoreactivity staining scores, respectively. Nine of 74 (12%) pancreatic cancer samples were devoid of any bcl-x immunoreactivity. Normal pancreatic tissue regions around the cancer mass exhibited an immunohistochemical staining pattern comparable to that of the normal controls. However, regions adjacent to the pancreatic cancer mass in which chronic pancreatitis-like morphologic changes were present showed weak to moderate bcl-x immunoreactivity in the remaining ductal and acinar cells. In contrast, there was no bcl-x immunostaining in the connective tissue and in fibroblasts in the pancreatic cancer tissues.

Association of Immunohistochemical and *In Situ* Hybridization Findings With Clinicopathologic Parameters

To determine whether the expression of bcl-xL mRNA or the presence of bcl-x in pancreatic cancer cells is of clinical significance for the course of the disease, the immunohistochemical and *in situ* hybridization data were correlated with clinical parameters such as gender, age, tumor differentiation, tumor stage, and survival after tumor resection. Six patients with stage IV disease and one patient who died early after surgery were excluded from the survival analysis. We excluded patients with stage IV disease because no tumor resection was performed (except in one patient), and therefore the survival prognosis was different from that of patients who underwent resection.

We found no relation between the presence of either bcl-x immunoreactivity or bcl-xL mRNA expression in pancreatic cancer cells and the patient's age, gender, tumor differentiation, tumor stage, or tumor size (Table 1).

The survival data were plotted according to the method of Kaplan and Meier. Statistical analysis of survival after surgery and bcl-xL mRNA expression levels was carried out by the log-rank test.

Patients whose tumors exhibited moderate bcl-xL mRNA expression levels lived significantly shorter (median 5 months) than did patients with weak, faint, or absent bcl-xL mRNA expression levels in their tumors (median 12 months) (Fig. 3). In five patients, strong bcl-xL mRNA expression was found in the tumors. However, when survival analysis was done in these patients, they tended to live longer (median 14 months, no statistical analysis done because of low sample number) than patients with moderate bcl-xL mRNA expression levels.

DISCUSSION

The bcl-x gene was identified in 1993 by low-stringency hybridization of chicken lymphoid cells with a murine bcl-2 cDNA.[23] It shows 44% homology with bcl-2 and mediates similar functions. In humans, two subforms of bcl-x (bcl-xL

Table 1. RELATIONSHIP BETWEEN BCL-XL MRNA EXPRESSION AND CLINICOPATHOLOGIC PARAMETERS

		Bcl-xL mRNA			
		Absent/faint n = 29	Weak n = 26	Moderate n = 14	Intense n = 5
Age	<60	11	8	2	1
	60–70	10	12	5	2
	>70	8	6	7	2
Gender	Male	16	15	8	3
	Female	13	11	6	2
Grading	Well	6	5	3	1
	Moderate	10	9	5	1
	Poor	12	11	5	2
	Undifferentiated	1	1	1	1
Tumor stage	I	2	3	3	1
	II	10	5	5	1
	III	15	16	5	2
	IV	2	2	1	1
Tumor size	<3 cm	13	12	8	2
	≥3 cm	16	14	6	3

Statistical analysis revealed no relationship between bcl-xL mRNA expression and these parameters.

and bcl-xS) exist; they can be differentiated by their molecular weight and their biologic functions. The bcl-xL cDNA encodes a 233-amino acid protein of 21 kDa. mRNA splicing in exon 1 of the bcl-x gene transcript leads to the loss of 63 amino acids, resulting in a protein of approximately 19 kDa that is named bcl-xS.[18,23]

In the present study, we analyzed for the first time the expression of the antiapoptotic gene bcl-xL in human pancreatic cancer. By Northern blot analysis and *in situ* hybridization, enhanced bcl-xL mRNA expression was present in approximately half of the pancreatic cancer tissue specimens. Analysis of the molecular data with clinical parameters such as age, gender, and tumor size, stage, and differentiation revealed no relation with bcl-xL MRNA levels, indicating that disturbances of apoptotic pathways are not related to the histologic phenotype of the tumor or to a specific stage in pancreatic cancer pathogenesis. However, we found an association between the presence or absence of bcl-xL and the survival time. Patients whose tumors exhibited moderate bcl-xL mRNA expression lived significantly shorter than patients whose tumors exhibited faint or weak bcl-xL mRNA expression or in whom no bcl-xL expression was present. Subanalysis of five patients whose tumors showed the highest bcl-xL mRNA levels revealed, surprisingly, that these patients tended to live longer than patients with faint, weak, or absent bcl-xL mRNA levels. However, only 7% of the pancreatic cancer samples exhibited strong bcl-xL mRNA expression, whereas in the remaining ones bcl-xL mRNA was either lower (54%) or absent (39%). Nevertheless, it is a surprising and interesting observation that high expression of this antiapoptotic gene might be associated with longer survival, because we would expect that high expression levels of the antiapoptotic gene bcl-xL would contribute to high viability of cancer cells and subsequently to worse survival.

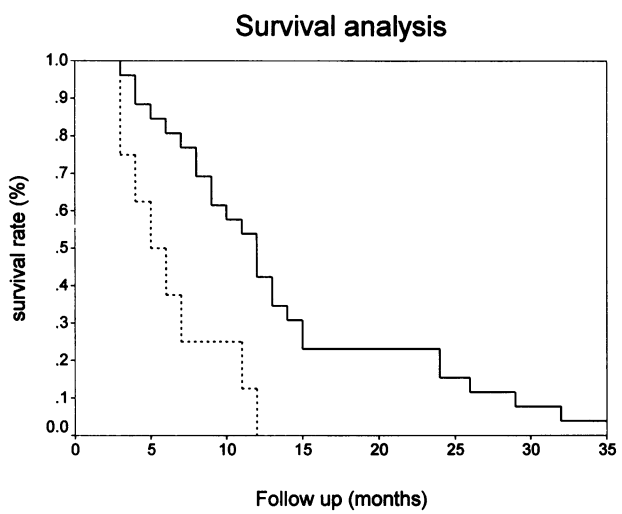

Figure 3. Survival curve. Kaplan–Meier plot of the survival period after surgery in patients whose tumors exhibited moderate bcl-xL mRNA expression (n = 13, broken line) *vs.* patients whose tumors had absent, faint, or weak bcl-xL mRNA expression (n = 49, solid line). Log-rank analysis of the survival periods after surgery indicated a significant difference ($p < 0.05$).

This phenomenon might be explained by the multiple competing dimerizations theory. At high levels of bcl-xL, the higher rate of bcl-xL heterodimers with other apoptotic genes may lead to structural changes that result in reduced biologic function of bcl-xL homodimers, as previously reported.[32] However, it also underlines the fact that multifac-

torial mechanisms are involved in the proliferation and maintenance of viability of pancreatic cancer cells, and that their distinct combination determines the final biologic growth behavior of the tumor. Further, recent data indicate that antiapoptotic members of the bcl-2 family not only negatively influence apoptosis but also restrain cell cycle entry. Although both functions seem to be mechanistically distinct, they might also contribute to enhance cell viability against damaging environmental factors.[33,34]

At present it is not possible to correlate the bcl-xL expression data obtained by Northern blot analysis or *in situ* hybridization with the immunohistochemical findings, because of the unavailability of antibodies that specifically differentiate between bcl-xL and bcl-xS. This differentiation might be important because the bcl-x subtypes exhibit opposite biologic effects on apoptosis: bcl-xL inhibits and bcl-xS promotes apoptosis.[18] This lack of differentiation between the two bcl-x subtypes might be one of the reasons why only a few studies have up to now focused on further defining the role of bcl-x in cancer pathogenesis. In our analysis, the expression data obtained by Northern blot analysis and *in situ* hybridization were similar. However, there was some divergence between the results of mRNA expression, which specifically measured bcl-xL, and the immunohistochemical findings, which detected both bcl-x subtypes together, suggesting that in human pancreatic cancer samples both bcl-xL and bcl-xS are present.

Bcl-xL strongly blocks apoptosis induced by a variety of stimuli under certain circumstances in different cell types.[35] Although in the bcl-2 gene family several apoptosis-inhibiting members are characterized, bcl-xL is much stronger at preventing apoptosis than bcl-2 itself and other bcl-2 family members.[18] Further, several observations indicate that bcl-xL and bcl-2 do not possess overlapping biologic functions.[36] For example, in normal, terminally differentiated plasma cells, apoptosis is regulated by bcl-xL rather than by bcl-2.[37] In addition, in normal human tissues except normal lymph nodes, bcl-xL expression levels are generally higher than those of bcl-2,[18,38] suggesting that bcl-xL plays a more dominant role in regulating apoptosis under physiologic conditions in a variety of cell populations.

Bcl-xL mRNA expression has been analyzed in several malignancies, including leukemia,[39,40] breast cancer,[41] gastric adenomas and carcinomas,[42] colorectal carcinomas,[43] neuroblastoma,[44] and Kaposi sarcoma tumor cells.[45] Fifty percent to 78% of these tumors exhibited increased bcl-xL levels, and the presence of bcl-xL in the cancer cells was associated with protection of p53-mediated apoptosis and with a higher viability of cancer cells against chemotherapy.[44] Further, suppression of bcl-xL promotes the susceptibility of normal and cancer cells to mutagenic substances, and several studies have provided evidence that bcl-xL participates in an acquired form of multimodal resistance to chemotherapeutic agents and radiation.[46,47] These *in vitro* and *in vivo* findings indicate that resistance of cancer cells against chemotherapy is at least partly mediated by activation of antiapoptotic genes and/or the deactivation of apoptosis-promoting pathways. In our study, by Northern blot analysis 54% of the pancreatic cancer samples overexpressed the bcl-xL mRNA gene compared with normal controls. Although overexpression of bcl-2, which represents the prototype of the antiapoptotic-acting members of the bcl-2 gene family, does not correlate with survival in pancreatic cancer patients, we found in our present study that bcl-xL mRNA expression did.[48] This observation suggests that the bcl-xL gene strongly influences the viability of pancreatic cancer cells *in vivo* and that its presence might contribute to the unresponsiveness of these tumors to anticancer treatment.

In summary, we can conclude that activation of antiapoptotic pathways occurs in pancreatic cancer. This activation might influence the viability of pancreatic cancer cells and thereby cell survival. Together with additional molecular alterations, such as overexpression of growth factors and growth factor receptors, mutations of p53 and k-ras, enhanced expression of tumor-associated proteinases, and loss of transmembranous glycoproteins, disturbances in apoptotic pathways may contribute to malignancy and to the aggressive proliferating phenotype of pancreatic cancer.[5,49–51]

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