Apoptosis and Expression of the Bcl-2 Family of Proteins and P53 in Human Pancreatic Ductal Adenocarcinoma

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Key Words

Pancreatic ductal adenocarcinoma • Apoptosis • P53 • Bcl-2 protein family

Abstract

Objective: The purpose of this study was to clarify the association between P53 and the Bcl-2 family (Bcl-2, Bax, Bcl-xL, Bcl-xS) expression and apoptosis in pancreatic ductal adenocarcinoma (PDAC). **Subjects and Methods:** A total of 70 patients with PDAC were studied. The expression of P53 protein in PDAC was assessed using the immunohistochemical method, which categorized the PDAC patients into two groups: group 1: 36 cases with immunonegative P53(–), and group 2: 34 cases with immunopositive P53(+). The expression of Bcl-2, Bax, Bcl-xL, and Bcl-xS in the 70 PDAC cases was detected by immunohistochemical and Western blotting methods. The apoptotic index (AI) was also measured in these samples by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method. The relation between P53 and the Bcl-2 protein family and apoptosis was then evaluated. **Results:** Bcl-2 and Bcl-xS expression was significantly associated with P53 ($p < 0.05$). No clear associations were found among P53, Bax and Bcl-xL expression ($p > 0.05$). The AI of groups 1 and 2 was 12.1 ± 2.47

and 8.1 ± 1.48 , respectively ($p = 0.023$). There was no relationship between AI and Bcl-2, Bax, Bcl-xL and Bcl-xS expression ($p > 0.05$, respectively). Bcl-2/Bax ratio was significantly associated with AI ($p < 0.01$). **Conclusion:** Bcl-2 and Bcl-xS represent significant anti- and proapoptotic proteins, respectively, modulated through a P53-dependent pathway in PDAC, and P53 modulated apoptosis mainly through Bcl-2/Bax ratio.

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Introduction

Apoptosis has been accepted as a fundamental component in the pathogenesis of cancer. The origin of cancer involves deregulated cellular proliferation and the suppression of apoptotic processes, ultimately leading to tumor establishment and growth [1]. A major challenge in oncology is the development of chemoresistance. This often occurs as cancer progresses and malignant cells acquire mechanisms to resist insults that would normally induce apoptosis [2]. Factors affecting the apoptotic function by any mechanism may also interfere with the prognosis of cancer patients.

The Bcl-2 family is composed of death antagonists (Bcl-2, Bcl-xL, Bcl-w, Bfl-1, Brag-1, Mcl-1, and A1) and death agonists (Bax, Bak, Bcl-xS, Bad, Bid, Bik and Hrk) [11–13]. The ratio of death antagonists to agonists determines whether or not a cell will respond to an apoptosis signal [14]. The death-life rheostat is mediated, at least in part, by competitive dimerization between selective pairs of antagonists and agonists [15, 16]. Furthermore, diverse effects of P53 on the transcription of the Bcl-2 protein family that regulate apoptosis substantially affect the biological aggressiveness of PADC [17, 18]. The transcription of Bax, a proapoptotic member of the Bcl-2 family, is activated by wild-type P53, while Bcl-2, which functions to prevent apoptosis, is transcriptionally repressed by wild-type P53 [19, 20]. Nevertheless, the relationship between the Bcl-2 protein family and P53 remains unclear. In this study, we analyzed the relationship between P53 and Bcl-2, Bax, Bcl-xL, Bcl-xS protein expression and apoptosis.

Tissue Samples

Immunohistochemical Staining

and then incubated in 3% (v/v) hydrogen peroxide in absolute methanol for 20 min to quench endogenous peroxidase activity. After brief washing with distilled water, the tissue sections were processed in 4 mM citrate buffer (pH 6.0) and heated to 120°C in an autoclave for 10 min for antigen retrieval. Slides were allowed to cool at room temperature for 20 min and then rinsed with phosphate-buffered saline (PBS). To inhibit nonspecific binding activity, slides were incubated with blocking serum at room temperature for 30 min. Sections were then incubated with primary monoclonal antibody against P53, Bcl-2, Bax, Bcl-xL and Bcl-xS, respectively at 1:400–1:1,000 (Santa Cruz Biotechnology), at 4°C. The sections were then incubated with biotinylated anti-mouse immunoglobulins for 30 min at room temperature, washed in PBS. 3,3'-Diaminobenzidine tetrahydrochloride, which was used as the color reagent, and hematoxylin as a counterstain. Immunostaining was evaluated by 2 independent pathologists to validate the diagnosis.

Immunohistochemical staining of P53 was defined as a detectable immunoreaction in the nucleus. Only nuclear staining was considered to be immunohistochemically positive for P53. The staining intensity of the positive cells was classified into the following grades [20]: negative immunoreactive staining intensity (-): positive cells less than 10%; positive immunoreactive staining intensity (+): positive cells more than 10%. Immunohistochemical staining of Bcl-2, Bax, Bcl-xL and Bcl-xS was defined as detectable immunoreaction in cytoplasm. The amounts of immunopositive cells were estimated semiquantitatively: grade '+' corresponds to 5–10%, grade '++' to 10–50%, and grade '+++' to more than 50% positive cells [21]. All series included positive and negative controls. The results of control staining were satisfactory.

Small sections of frozen tissue were cut and homogenized in 200 μ l of ice-cold lysis buffer. After incubation for 30 min on ice, the samples were centrifuged at 14,000 rpm, then at 4°C for 20 min and the supernatant was transferred to a new tube. Total protein was measured with a Bio-Rad Bradford kit, then 30 μ g of total protein was run on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane that was blocked with nonfat dry milk in Tris-buffered saline overnight at 4°C. The membrane was then probed with a primary antibody, washed several times with Tween-20 (0.3%), and incubated with a horseradish peroxidase-conjugated secondary antibody. Finally, the membrane was washed with an enhanced chemiluminescence system. The primary antibodies used were P53, Bcl-2, Bax, Bcl-xL and Bcl-xS (1:100–1:200, Santa Cruz Biotechnology). As a control for sample loading, the blot was stripped and reprobed with antiglyceraldehyde-3-phosphate dehydrogenase (GAPDH) polyclonal antibody. Densitometric quantification of the autoradiographs was performed with a Bio-Rad/GS 700 imaging densitometer. Protein levels in tumor tissues were quantified and the ratio of protein to GAPDH was defined as the protein expression.

DNA fragmentation characteristics of the apoptosis of the specimen were monitored by TUNEL staining using an Apoptag peroxidase in situ apoptosis detection kit by following the manufacturer's instructions. Briefly, after deparaffinization and incu-

bation with 20 µg/ml of proteinase K for 15 min at room temperature, the sections were rinsed with PBS once, then rinsed with deionized distilled water 3 times at 5-min intervals. The sections were incubated with 3% H₂O₂ (50 µl/section) in methanol for 10 min at room temperature and the slides were rinsed 3 times at 5-min intervals with deionized distilled water. The slides were incubated in a permeabilization solution (50 µl/section) for 3 min at room temperature. After washing 3 times at 5-min intervals in deionized distilled water, the sections were incubated in a TdT buffer containing TdT (0.6 µl) and fluorescein-deoxy-UTP (dUTP 1.4 µl) for 1 h at 37°C in a humidified chamber. The coverslips were washed in PBS 3 times at 3-min intervals and incubated with antibody conjugated to peroxidase for 30 min at 37°C in a humidified chamber. The coverslips were incubated with avidin-HRP (50 µl) for 60 min at 37°C in a humidified chamber and washed in PBS 3 times at 3-min intervals, then stained with diaminobenzidine and counterstained as above. A total of 500 cells were counted in each specimen. The apoptotic index was defined as follows: apoptotic index (%) = 100 × apoptotic cells/total cells. The tumor specimens were stratified according to TUNEL staining in <10 or >10% stained cells.

Statistical Analysis

All continuous variables are expressed as mean ± SD. Statistical analysis was done by using the χ^2 test or Fisher's test for qualitative data and Student's t test for quantitative data; $p < 0.05$ was considered statistically significant.

Results

Immunohistochemical Analysis

The immunostaining results are presented in table 1. Of the 70 samples, P53(+) was detected in 34 (48.6%) and P53(-) in 36 (51.4%). In the P53(-) samples, Bcl-xS and Bax showed strong immunoreactivity (++/+++), and Bcl-2 and Bcl-xL showed weak immunoreactivity (+). In P53(+) samples, Bcl-xS and Bax showed weak immunoreactivity (+), however, Bcl-2 and Bcl-xL showed strong immunoreactivity (++/+++). A significant association was found between P53 accumulation and down-regulation of Bcl-xS ($p = 0.003$). A highly significant association was found between P53 accumulation and up-regulation of Bcl-2 ($p = 0.026$). However, no significant association between P53 accumulation and Bcl-xL, Bax was shown ($p > 0.05$). The representative expression of P53, Bcl-2, Bcl-xL, Bcl-xS and Bax is shown in figure 1.

Western Blot Analysis

Western blot for P53 and the Bcl-2 protein family (Bcl-2, Bax, Bcl-xL, and Bcl-xS) was detected in the 70 pancreatic cancer cases. The relative expression levels of Bcl-2, Bax, Bcl-xL and Bcl-xS protein are shown in table 2. A highly significant association between P53 with Bcl-xS

Table 1. Protein accumulation of P53 relative to other proteins in pancreatic cancer

Protein	Staining intensity	n	P53(-) (n = 36)	P53(+) (n = 34)	p
Bcl-2	0/+	41	28	13	0.026
	++/+++	29	8	21	
Bcl-xS	0/+	48	23	25	0.003
	++/+++	22	13	9	
Bax	0/+	49	22	27	0.145
	++/+++	21	14	7	
Bcl-xL	0/+	33	21	12	0.093
	++/+++	37	15	22	

Table 2. Correlation of expression of Bcl-2, Bax, Bcl-xL and Bcl-xS with P53

		Ratio ≥1	Ratio <1	p
Bcl-2	group 1	11	25	0.017
	group 2	25	9	
Bax	group 1	23	13	0.173
	group 2	18	16	
Bcl-2/Bax	group 1	10	26	0.028
	group 2	22	12	
Bcl-xL	group 1	14	22	0.125
	group 2	19	15	
Bcl-xS	group 1	26	10	0.013
	group 2	11	23	
Bcl-xL/Bcl-xS	group 1	13	23	0.146
	group 2	14	20	

and Bcl-2 expression was observed ($p < 0.05$). No significant association between P53 and Bcl-xL, Bax expression was found ($p > 0.05$). The Bcl-2/Bax ratio was remarkably up-regulated in group 2, but was down-regulated in group 1 ($p < 0.05$). The representative samples of the 70 pancreatic cancer cases are shown in figure 2.

Apoptosis Analysis

The AI was 12.1 ± 2.47 and 8.1 ± 1.48 in groups 1 and 2, respectively. A remarkable correlation was found between P53 expression and AI (fig. 3, $p = 0.023$). The relationship between AI and the expression of Bcl-2 family proteins is presented in table 3. No relationship was found between the expression of Bcl-2, Bax, Bcl-xL, Bcl-xS and

Fig. 1. Tumor tissue from pancreatic cancer showing immunoreactivity for P53, Bcl-2, Bax, Bcl-xL and Bcl-xS proteins. P53 protein immunoreactivity was seen in cell nuclei. Bcl-2, Bax, Bcl-xL and Bcl-xS protein expression was detected in the cytoplasm. Magnification $\times 100$.

Fig. 2. Western blotting for P53, Bcl-2, Bax, Bcl-xL and Bcl-xS expression. Bcl-2 protein level was strikingly higher in P53(+) tissues ($p < 0.05$), and Bcl-xS protein level was strikingly higher in P53(-) tissues ($p < 0.05$). No significant association between P53 and Bcl-xL and Bax expression was found ($p > 0.05$).

Table 3. Correlation of Bcl-2, Bax, Bcl-xL and Bcl-xS with AI

		Cases	AI	p
Bcl-2/GAPDH	ratio ≥ 1	36	9.36 ± 2.26	0.063
	ratio < 1	34	11.54 ± 2.54	
Bax/GAPDH	ratio ≥ 1	41	10.89 ± 3.82	0.082
	ratio < 1	29	9.27 ± 2.06	
Bcl-xL/GAPDH	ratio ≥ 1	33	10.67 ± 3.21	0.105
	ratio < 1	37	10.25 ± 1.36	
Bcl-xS/GAPDH	ratio ≥ 1	37	10.23 ± 2.66	0.320
	ratio < 1	33	10.82 ± 2.49	
Bcl-2/Bax	ratio ≥ 1	32	7.19 ± 1.87	0.004
	ratio < 1	38	13.02 ± 3.45	
Bcl-xL/Bcl-xS	ratio ≥ 1	27	9.72 ± 2.58	0.163
	ratio < 1	43	11.51 ± 3.19

Fig. 3. Apoptosis detected by TUNEL. $\times 200$. TUNEL analysis was used to detect apoptosis in P53(+) and P53(-) groups. AI was remarkably increased in P53(-) groups ($p < 0.05$).

AI, respectively ($p > 0.05$). However, AI was remarkably increased in the low Bcl-2/Bax ratio groups, and decreased in the high Bcl-2/Bax ratio group ($p = 0.012$). No relationship was seen between AI and Bcl-xL/Bcl-xS ratio ($p > 0.05$). Representative pictures of apoptosis of PADC are shown in figure 3.

Discussion

Wild-type P53 protein functions as a transregulator of the genes involved in DNA synthesis, repair, and apoptosis. Because of its very short half-life and the minute amounts found in normal cells, wild-type P53 protein is almost undetectable by immunohistochemical staining [21–23]. However, P53 inactivated through mutations, deletions, or binding to other proteins results in a P53 protein accumulation that can be detected by immunohistochemistry [24, 25].

Apoptosis is known to be a gene-directed process and can be mediated through at least two pathways, which can be dependent upon or independent of the induction of P53 [26, 27]. However, P53 is required if programmed cell death is to be efficiently executed [26]. In this study, the findings of 48.6% of the PADC cases were immunopositive for P53, the apoptosis indexes of groups 1 and 2 were 12.1 ± 2.47 and 8.1 ± 1.48 , respectively ($p = 0.023$); thus, the finding that AI was dependent on P53 in PADC confirmed previous reports [28, 29]. P53 promotes mitochondrial leakage by up-regulating Bax and down-regulating Bcl-2, two antagonistic proteins that insert into mitochondrial membranes to inhibit (Bcl-2) or facilitate (Bax) the opening of mitochondrial permeability transition pores [30]. Based on our results, Bcl-2 was remarkably overexpressed in group 2, possibly due to the absence of restraint of functional P53; Bcl-2 was under-

expressed in the majority of group 1 cases through functional P53 inhibition. The ratio of antiapoptotic to proapoptotic protein expression, like Bcl-2 to Bax and Bcl-xL to Bax, represents a rheostat that determines a cell's life or death response to an apoptotic stimulus [31, 32].

In this study, Bax was unexpectedly overexpressed in a majority of the PADC cases, in both group 1 and group 2. Accordingly, the Bcl-2/Bax ratio was remarkably elevated in PADC cases with immunopositive P53 compared to that of PADC cases with immunonegative P53. Again, Bcl-xL was up-regulated in a majority of PADC cases with either immunopositive or immunonegative P53. Bcl-xS was remarkably down-regulated in PADCs with immunopositive P53 compared to those with immunonegative P53. Accordingly, the Bcl-xL/Bcl-xS ratio was similarly up-regulated in PADCs with immunopositive P53 compared to that of PADC cases with immunonegative P53. In our study, there was no relationship between AI and the expression of Bcl-2, Bax, Bcl-xL, Bcl-xS and Bcl-xL/Bcl-xS ratio alone ($p > 0.05$, respectively), but AI was remarkably increased in the high Bcl-2/Bax ratio group, and decreased in the Bcl-2/Bax ratio group ($p = 0.012$). This demonstrates that it is not individual apoptosis-related proteins, but the net effect of the antagonistic/agonistic proteins that determines the fate of the affected cells.

Conclusion

Taken together, Bcl-2 and Bcl-xS represented the most significant individual antiapoptotic and proapoptotic proteins, respectively, expressed in human pancreatic cancer in the current study, and the Bcl-2/Bax ratio was more deeply modulated by the P53-dependent pathway than was the Bcl-xL/Bcl-xS ratio. P53 modulated apoptosis mainly through Bcl-2/Bax ratio.

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