Correlation of Expression of Apoptosis-related bcl-2-family (Bcl-2, Bax, Bcl-xL, Bcl-xS) and p53 in Human Adenocarcinoma of Pancreas

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Abstract **Objective** To clarify the association between p53 and bcl-2 family (Bcl-2, Bax, Bcl-xL, Bcl-xS) expression in PC. **Methods** A total of 35 PC patients were studied. The expression of p53 protein in PC was assessed with immunohistochemical method, which categorized the PC patients into two groups: group 1, immunonegative p53 (18 cases); and group 2, immunopositive p53 (17 cases). The expression of p53, Bcl-2, Bax, Bcl-xL, and Bcl-xS in the 35 PC cases detected by western bioting was quantified with a densitometer. The apoptosis of the 35 PC cases was determined by terminal deoxynucleotidyltransferase-mediated UTP end-labeling (TUNEL). **Results** The expression of Bcl-2 was remarkably up-regulated in group 2 (15 of 17), but was down-regulated in group 1 (5 of 18). The expression of Bax was up-regulated in both group (15 of 18 in group 1 and 15 of 17 in group 2). The expression of Bcl-xL was up-regulated in both group(15 of 18 in group 1) and 14 of 17 in group 2). Bcl-xS was remarkably down-regulated in group 2 compared to group1 (P=0.01. The apoptosis indexes (AI) of groups 1 and 2 were 12.1±2.47 and 9.1±1.48, respectively (P =0.023), there was no relationship between AI and the expression of Bcl-2, Bax, Bcl-xL and Bcl-xS (P>0.05, respectively). AI were remarkably increased in high bcl-2/bax ratio group, and in low bcl-2/bax ratio group (P =0.012). **Conclusion** Bcl-2 and Bcl-xS represented the significant anti-and proapoptotic proteins, respectively, modulated through a p53-dependent pathway in PC, and p53 modulated apoptosis mainly through bcl-2/bax ratio.

Key words Pancreatic carcinoma; apoptosis; p53; Bcl-2; Bax; Bcl-xL; Bcl-xS.

Pancreatic cancer is a malignacy with extremely poor prognosis. Less than 5% of patients with pancreatic adenocarcinomas survive more than 5 years [1]. The blocked apoptosis is one of the important roles in carcinogenesis of PC. The family of Bcl-2 related proteins constitutes one of the biologically most relevant classes of apoptosis regulatory gene products acting at the effector stage of apoptosis [2~4]. 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). The ratio of death antagonists to agonists determines whether a cell will respond to an apoptosis signal. The death-life rheostat is mediated, at least in part, by competitive dimerization between selective pairs of antagonista and agonists [5]. Furthermore, diverse effects of p53 on the transcription of the Bcl-2 family proteins that regulate apoptosis substantially affect the biological aggressiveness of PC [6, 7]. The transcription of bax, a proapoptotic member of the Bcl-2 family was activated by wild-type p53 while Bcl-2, which functions to prevent apoptosis, was transcriptionally repressed by wild-type p53 [6, 8]. Nevertheless, the relationship between Bcl-x and p53 remains unclear. In this article, we attempted to systematically quantify the expression of the Bcl-2 family regulated by p53 in human PC.

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MATERIALS AND METHODS

Tissue samples and patients characteristics

Pancreatic carcinoma tissues were obtained from 35 patients who underwent surgical operations for pancreatic cancer at the Department of General Surgery, University of Su Zhou. 35 pairs of tumor tissue of PC were obtained at the time of surgical resection. Each tissue sample was bisected; one half was processed for routine histopathologic examination and immunohistochemical study, and the other was frozen immediately in liquid nitrogen, and stored at 80℃ until use.

Immunohistochemical Staining for p53

Tumour tissue sections were cut (thickness 4μm), placed on slides coated with 0.05% poly−L−lysine hydrobromide, toasted 58℃ for 24h. The sections were dewaxed with xylene, dehydrated through a series of alcohol solutions (95%, 85%, 75%)for 2 min, and then incubated in 3% (v/v) hydrogen peroxide in absolute methanol for 20 min to quench endogenous peroxidase activity. After brief washing with distilledwater, tissue sections were processed in 10mM Mcitrate buffer (pH 6.0) and heated to 120℃ in an autoclave for 10 min for antigen retrieval. Slides were allowed to cool at room temperature for 20 min and then rinsed with PBS. To inhibit nonspecific binding activity, slides were incubated with blocking serum at room temperature for 30 min. Sections then were incubated with primary monoclonal antibody against p53 at 1:100 (ready to use), at 4℃. The sections were then incubated with biotinylated anti −mouseimmunoglobulins for 30 min at room temperature, with washing in PBS. 3,3´−Diaminobenzidine tetrahydrochloride was used as the color reagent, and hemoxylen was used as a counterstain. Colon cancer with known p53 gene mutation and p53 protein overexpression were used as positive controls. Negative controls were obtained by omitting the primary antibody. Only nuclear staining was considered to be immunohistochemically positive for p53. The staining intensity of the positive cells was classified into following grades: negative expression (−): positive cells less than 20%, positive expression (+): positive cells more than 20%.

Western blotting for p53, Bcl−2, Bax, Bcl−xL, and Bcl−xS

Small sections of frozen tissue were cut and homogenized in 200 ul of ice−cold lysis buffer. After incubation for 30 min on ice, the samples were centrifuged at 14,000 rpm, at 4℃ for 20 min and the supernantant transferred to a new tube. Total protein was measured with a Bio−Rad Bradford kit, then 30ug 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 TBS overnight at 4℃. The membrane was then probed with a primary antibody, washed several times with 0.3% Tween−200, and incubated with a horseradish peroxidase (HRPO) conjugated secondary antibody. Finally, the membrane was washed with an enhanced chemiluminescence system. The primary antibodies used were p53, Bcl −2, Bax, Bcl−xL, Bcl−xS, and p21. As a control for sample loading, the blot was stripped and reprobed with anti−glyceridehyde −3 −phosphate dehydrogenase (G3PDH) polyclonal Ab. Densiometric 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 G3PDH was defined as the protein expression.

Detection of apoptosis

DNA fragmentation characteristic of the apoptosis of the specimen was monitored by TUNEL staining using an apoptag peroxidase in situ apoptosis detection kit by following manufacture´s instructions. Briefly, after deparaffinization and incubation with 20μg/ml of proteinase K for 15 min at room temperature, the sections were rinsed with PBS for one time, then rinsed with deionized distilled water for 3 times(one time per 5min) The sections incubate with 3% H_2O_2 (50μl /section)in methanol for 10 min at room temperature and rinse slides 3 times (one time per 5min) with deionized distilled water. The slides were incubated in permeabilization solution (50μl /section)for 3 min at room temperature. After washing 3 times (one time per 5min) in deionized distilled water, the sections were incubated in TdT buffer containing TdT (0.6μl) and fluorescein−deoxy−UTP (dUTP 1.4μl) for 1 h at 37℃ in a humidified chamber. The coverslips were washed in PBS for 3 times (one time per 3min) and incubated with antibody

Table 1 Correlation of expression of Bcl−2, Bax, Bcl−xL, Bcl−xS with p53 in pancreatic carcinoma

Groups	Ratio≥1	Ratio<1	*P*
Bcl−2			
Group1	5	12	0.047
Group2	15	3	
bax			
Group1	15	3	0.274
Group2	15	2	
Bcl−2/bax			
Group1	4	14	0.012
Group2	14	3	
Bcl−xL			
Group1	15	3	0.334
Group2	14	3	
Bcl−xs			
Group1	13	5	0.021
Group2	3	14	
Bcl−Xl/bcl−xs			
Group1	14	4	0.215
Group2	12	5	

Table 2 Correlation of expression of Bcl−2, Bax, Bcl−xL, Bcl−xS with AI in pancreatic carcinoma

Groups	Cases	AI	*P*
Bcl−2/G3PDH			
Ratio≥1	20	10.36±2.26	0.056
Ratio<1	15	11.54±2.54	
bax/G3PDH			
Ratio≥1	30	11.39±3.82	0.364
Ratio<1	5	10.45±2.06	
Bcl−xL/G3PDH			
Ratio≥1	29	11.18±3.21	0.173
Ratio<1	6	10.45±1.36	
Bcl−xs/G3PDH			
Ratio≥1	16	12.83±2.66	0.069
Ratio<1	19	10.00±2.43	
Bcl−2/bax			
Ratio≥1	18	9.19±1.87	0.012
Ratio<1	17	13.02±3.45	
Bcl−Xl/bcl−xs			
Ratio≥1	26	11.72±2.58	0.082
Ratio<1	9	12.51±3.19	

conjugated to peroxidase for 30 min at 37℃ in a humidified chamber. The coverslips were incubated with Avidin−HRP(50μl) for 60 min at 37℃ in a humidified chamber. The coverslips were washed in PBS for 3 times(one time per 3min) , then stained with DAB, and counterstained as above. Then, using a microscope, we counted the tumor cells, up to 500 cells,the number of stained tumor cells was defined as the apoptosis index (AI).

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 as statistically significant.

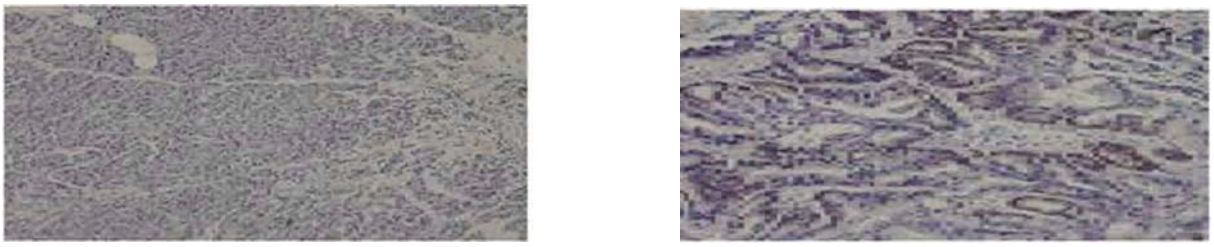
RESULTS

Western blot

Of the 35 PC cases, 18 were in the immunonegative p53 group (group1), and the remaining17 were in the immunopositive p53 group (group2) (Figure1). Western blottings of p53, and the Bcl−2 family, including Bcl−2, Bax, Bcl−xL, and Bcl−xS, in the35 PC cases are shown in Figure2. The expressions of Bcl−2, Bax, Bcl−xL, and Bcl−xS were tabulated for groups 1 and 2 (Table 1). In brief, Bcl−2 was remarkably up−regulated in group 2 (15 of 17), but was down−regulated in group 1 (5 of 18). Bax was up−regulated in both group: 15 of 18 in group 1, and 15 of 17 in group 2. The Bcl−2/Bax ratio was remarkably up−regulated in group 2 (14 of 17), but was down−regulated in group 1 (4 of 18). Bcl−xLwas up−regulated in both group: 15 of 17 in group 2, and 14 of 18 in group 1. Bcl−xS was down−regulated in group 2 (3 of 17) and up−regulated in group 1 (13 of 18). The Bcl−xL/Bcl−xS ratio was up−regulated in both group 1 (14 of 18) and group 2 (12 of 17).

Apoptosis

The apoptosis indexes of groups 1 and 2 were 12.1± 2.47 and 9.1±1.48, respectively ($P=0.023$), there was no relationship between AI and expression of Bcl−2, Bax, Bcl−xL and Bcl−xS ($P>0.05$, respectively). AI was re-



A B

Fig. 1 Immunohistochemical staining of p53 in human pancreatic hepatocellular carcinoma (PC).
A: Negative staining of nuclear p53 protein in the tumorous part of PC was shown(×200).
B: Strong staining of nuclear p53 protein in the tumorous part of PC was shown(×200).

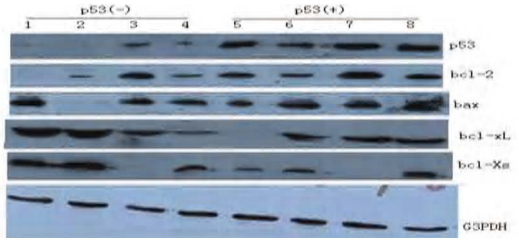


Fig. 2 Western blotting of p53, Bcl−2, Bax, Bcl−xL, and Bcl−xS in PC cases

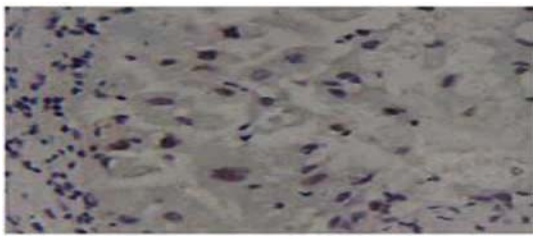


Fig. 3 Features of apoptotic PC cells detected by TUNEL (× 400).

markably increased in high bcl−2/bax ratio group, and in low bcl−2/bax ratio group(P=0.012). No relationship was seen between AI and Bcl−xL/Bcl−Xs ratio (P> 0.05), The representative features of apoptosis of PC are shown in Figure 3 and Table 2)

DISCUSSION

Wild−type p53 protein functions as a transregulator of the genes involved in DNA synthesis, DNA repair, and apoptosis. Because of its very short half−life and the minute amounts found in normal cells, wild−type p53 protein is almost undetecable by immunohistochemical stain. However, p53 that is inactivated through mutations,deletions, or binding to other proteins results in a p53 protein accumulation that can be detected by immunohistochemistry[7].

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 [9, 10]. However, p53 is required if programmed cell death is to be efficiently executed [11, 12]. In this study, 48.6% (17 of 35) of the PC cases were of immunopositive for p53. The apoptosis indexes of groups 1 and 2 were 12.1±2.47 and 9.1±1.48, respectively (P= 0.023), thus, the AI was dependent on p53 in PC.

p53 promotes mitochodrial 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 (6). Based on our results, Bcl−2 was remarkably over−expressed in group 2, possibly due to the absence of restraint of the functional p53; Bcl−2 was underexpressed in the majority of group 1 through functional p53 inhibition. The ratio of anti-apoptotic 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 [6, 7, 13].

In this study, Bax was unexpectedly overexpressed in a majority of the PC cases, in both group 1 and group 2. Accordingly, the Bcl−2/Bax ratio was remarkably elevated in PC cases with immunopositive p53 (14 of 18) compared to that of PC cases with immunonegative p53 (4 of 18). Again, Bcl−xL was up−regulated in a majority of PC cases with either immunopositive p53 or im-

munonegative p53. Bcl−xS was remarkably down−regulated in PCs with immunopositive p53 (4 of 17) compared to these with immunonegative p53 (13 of 18). Accordingly, the Bcl −xL/Bcl −xS ratio was similarly up−regulated in PCs with immunopositive p53 (12 of 17) compared to that of PC cases with immunonegative p53 (14 of 18). In our study, there was no relationship between AI and 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 high bcl −2/bax ratio group, and in low bcl −2/bax ratio group (P=0.012).This reveals the fact 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[14, 15].

Taken together, Bcl−2 and Bcl−xS represented the most significant individual antiapoptotic and proapoptotic proteins, respectively, expressed in human PC 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. and p53 modulated apoptosis mainly through bcl−2/bax ratio.

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basal cell carcinoma,while the expressions of Fra−1 and Fra−2 have no remarkably change. All these tell us in the development of skin tumor, Fos family subunits played much less role than Jun family subunits.

AP−1 distributes in different epidermis according to its different role in epidermal cell proliferation and differentiation. In neonatus epidermis, expression of c−jun was only limited in granular cell layer, and the expressions of JunB and JunD were in every layer in epidermis, but they were prominent in basal cell layer and prickle cell layer. c−fos, Fra−1 and Fra−2 expressed in prickle cell layer and granular cell layer, FosB expressed in prickle cell layer [10]. In epidermis close to cutaneous tumor, all subunits concentrate in basal cell layer, except JunB and JunD expressed in prickle cell layer and granular cell layer, c−fos in prickle cell layer, which means in this area, the appearance of epidermis are"normal", but the genes are abnormal. Co−expression of AP−1 subunit protein in basal cell layer in this area tells us that in the specific environment, the important effect of AP−1 is not differentiation but to regulate cell proliferation.

CONCLUSION

The expression of C−jun and p−c−jun, both the subunit of AP−1, increased obviously in basal cell carcinoma, squamous cell carcinoma, Bowens disease, and keratoacanthoma, and the expression of JunB decreased in basal cell carcinoma and keratoacanthoma. These showed that c−jun is the promoting agent for cutaneous tumor and cellular proliferation, but JunB is the inhibitor. These changes can not help us to distinguish whether a tumor is a malignant or not, but it provides the theory base for the gene therapy target to cutaneous tumor. Otherwise, to understand the relationship between expression of subunit of AP−1 and tumor differentiation, prognosis of the patients, much more data were needed to confirm.

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