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Apoptotic Index or a Combination of Bax/Bcl-2 Expression Correlate With Survival After Resection of Pancreatic Adenocarcinoma

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Abstract In the present study, the prognostic impact of factors involved in the apoptosis pathway were tested on 67 consecutive patients treated with surgical resection. Included in the study were all patients resected for pancreatic adenocarcinoma from 1988 to 2003. Expression analysis for p53, Bax, and Bcl-2 were performed by immunohistochemical staining. Apoptotic cells were identified by the TUNEL method. These data were correlated with survival. Sixty-seven tumor specimens were included in the study. A strong positive correlation was recorded between p53 overexpression and Bax expression levels ($P < 0.001$). By univariate analysis, overall survival seemed to be improved with Bcl-2 and Bax expression (respectively, $P = 0.0379$ and 0.0311). The median survival time in patients with low apoptotic index was better versus those with a high index ($P = 0.0127$). Lymph node involvement was the only clinico-pathologic parameter that significantly correlated with overall survival ($P = 0.0202$). By a multivariate Cox regression analysis, the only immunohistochemical parameter that influenced overall survival was the apoptotic index ($P = 0.040$). Tumor's overexpression of both Bax and Bcl-2 resulted the strongest independent prognostic factor ($P = 0.013$). This is the first study to report a statistically significant association of apoptosis to overall survival for pancreatic cancer patients treated with surgical resection. The contemporary overexpression of Bax and Bcl-2 represents the strongest prognostic factor. J. Cell. Biochem. 97: 98–108, 2006.

Key words: pancreas cancer; apoptosis; bax; bcl-2; prognosis; survival; adjuvant therapy; p53; nodal status

Ductal adenocarcinoma of the pancreas is the fifth leading cause of cancer-related death in Western societies [Stat Bite, 2002]. The annual mortality rate from this disease almost parallels its incidence [Kimura et al., 1998].

The most favorable prognostic markers are the absence of lymph node involvement and the status of resection margins. Inconclusive are the effects on survival by extended resections of the gland or its lymph nodes as well as biological factors such as DNA ploidy or degree of differentiation [Geer and Brennan, 1993; Nitecki et al., 1995; Kawesha et al., 2000; Sohn et al., 2000].

Interest has focused on molecular markers to select patients with better prognosis and perhaps those that might benefit by a more specific treatment.

There is compelling evidence implicating the loss of cell-cycle control in the development and progression of most human cancers; therefore,

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disregulation of the normal cell-cycle regulatory machinery has recently been examined, especially the mechanism of apoptosis [Sherr, 1996]. Apoptosis is the usual form of cell death that occurs as the result of a variety of insults including radiation. Apoptosis is characterized by fragmentation of DNA caused by enzymatic cleavage of DNA into 180–200 bp segments also termed nucleosomes [Ueda and Shah, 1994]. For some reason apoptosis does not occur in neoplastic tissue fostering new cell growth. The mechanism of apoptosis involves the Bcl-2 family of proteins such as Bax, Bcl-2, and Bcl-XL. The Bcl-2 family is represented by an antiapoptotic subgroup including Bcl-XL, and the proapoptotic BAX, BAK, and BH3-domain-only subgroups [Oltvai et al., 1993].

It has been reported that Bax inactivates Bcl-2 proteins through heterodimerization and that the ratio of Bax to Bcl-2 proteins increases during the apoptosis induction [Miyashita et al., 1994]. The ratio of Bax to Bcl-2 proteins is involved in the susceptibility to apoptotic stimuli in normal hematopoietic cell lines [Canman et al., 1995] and in a variety of cancer cell lines such as leukemia, breast cancer, bladder cancer, and lung cancer [Clarke et al., 1993; Ryan et al., 1993]. The Bcl-XL protein is also a member of the Bcl-2 family and is thought to be an inhibitor of apoptosis [Zhan et al., 1996]. Bcl-2 family regulatory function seems to be dependent on p53 protein. The induction of Bcl-XL depends on normal p53 functioning [Olopade et al., 1997] and overexpression of Bcl-XL is associated with a high tumor grade and an acquired resistance to chemotherapy and radiation [Kuhl et al., 1997].

Moreover, it is widely accepted that the Bax promoter contains a typical p53 tumor suppressor gene-binding site and that p53 exerts its role as an inducer of apoptosis partly by transactivating the expression [Miyashita and Reed, 1995]. Wild-type p53 gene products can inhibit Bcl-2 expression through their interaction with a p53-dependent negative response in the *Bcl-2* gene [Perego et al., 1996]. The biology of pancreatic adenocarcinoma could help to identify prognosis and potential clinically relevant molecular targets for therapy. To this purpose, we examined the prognostic significance of the expression of Bax, Bcl-2, and p53 proteins and separately measured the apoptotic index in a large cohort of resected pancreatic cancer patients. Moreover, we explored whether a combination of the prognostic information given by the molecular prognostic factors for each patient could help to distinguish patients who have a better prognosis.

PATIENTS AND METHODS

Clinical Data and Tumor Specimen Acquisition

All patients were treated at Catholic University School of Medicine of Rome and at the University Campus Bio-Medico from January 1986 through April 2003. In order to obtain the most possible consistent and homogeneous group of patients, only R0 patients were considered evaluable for this study. Staging consisted of computed tomography of the thorax, abdomen, and pelvis and intraoperative liver palpation. The location of these tumors were in the pancreatic head in 54 cases (84.7%), in the body and/or in the tail in six cases (8.9%). A diffuse neoplasm was present in 7 (10.4%). When indicated, intraoperative ultrasound of the liver was performed. Finally, the surgeon assessed for the absence of distant metastases and infiltration of mesenteric vessels and/or portal vein. All patients underwent standard lymphadenectomy. Surgery consisted of 54 pancreaticoduodenectomies (Whipple: 14 cases, Pylorus-preserving: 40 cases), six distal pancreatectomies and seven total pancreatectomies. Exclusion criteria from our analyses were perioperative mortality and presence of macroscopic residual disease after resection.

Data on clinical parameters, including sex, age, preoperative assessment of disease state, and type of operative procedure, were gathered retrospectively from patient records. Pathologic findings (tumor size, tumor location, involvement of surrounding structures, and lymph node status) were obtained from the pathologists' original reports. In addition to the original pathology reports, microscopic findings (tumor type, degree of differentiation, and TNM classification) were reassessed. Tumors were categorized using the 5th edition of the International Union Against Cancer [Sobin and Wittekind, 2002].

Survival was determined from the date of initial surgery. Follow-up was available for all patients. Subjects that died of causes other than pancreatic cancer during the follow-up period were considered in the survival analysis.

Histology

The formalin-fixed, paraffin-embedded samples were sectioned at 5 μm and stained with

hematoxylin and eosin. The histological diagnosis was re-examined. In addition, the most representative blocks were selected to be cut into new 5 μm-thick sections for immunohistochemical studies.

Immunohistochemistry and Quantification of the Immunoreactivity

Immunohistochemical staining (ICH) was performed by the streptoavidin-biotin method. In brief, sections were de-paraffinized and antigen retrieval was achieved by steaming slides for 35 min in a target retrieval solution (Dako Corporation, Carpinteria, CA). Endogenous peroxidase was blocked using 3% hydrogen peroxide solution in PBS for 5 min. The antibodies used were a monoclonal mouse antibody against p53 protein (DO7: Dakopatts A\S, Denmark) in a 1:50 dilution at room temperature for 1 h, a monoclonal mouse antibody against Bcl-2 protein (clone 124 Dakopatts A\S) in a 1:40 dilution at room temperature for 1 h, a monoclonal mouse antibody against Bax protein (clone 2D2, Zymed Laboratories, San Francisco, CA) in a 1:80 dilution at room temperature for 1 h. After washing three times with TBS (trishydroxymethyl–aminomethane buffered saline), sections were incubated with biotinylated goat anti-mouse\anti-rabbit immunoglobulin (Dako A\S, Basturp, Denmark) for 10 min. They were then washed three times with TBS, treated with streptoavidin-peroxidase reagent (Dakopatts A\S) for 10 min and then washed again with TBS three times. Finally, specimens were incubated in diaminobenzidine (DAB) for 5 min, followed by counterstained with Mayer hematoxylin (Poly Scientific, Bay Shore, NY) for 1 min, rinsed twice in distilled water, and dehydrated with ethanol followed by xylene. Semiquantitative evaluation of the immunohistochemical results was performed by two independent observers blinded to patient status.

The criteria for achieving a positive score for each of the antigens studied were based on published criteria: p53 was considered positive when there was a homogeneous staining pattern with more than 5% of cells demonstrating nuclear p53 protein accumulation [Campani et al., 2001]; Bcl-2 was considered to be overexpressed when a homogeneous staining pattern was observed with more than 5% of nuclei staining [Evans et al., 2001]; Bax was considered positive when a homogeneous staining pattern was seen with more than 10% of nuclei staining [Sturm et al., 1999]. For each reaction, we have replaced the primary antibody with mouse or goat or rabbit serum for negative controls.

Detection of Apoptosis

Apoptotic cells were identified by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate biotin nick-end labeling (TUNEL) method. Dewaxed and rehydrated specimens were incubated in proteinase K 40 μg/ml for 1 h at 37°C and were treated with 3% H_2O_2 in methanol for 30 min at room temperature. After adding equilibration buffer for 5 min at room temperature, terminal TdT enzyme was pipetted onto the sections and incubated at 37°C for 2 h. The reaction was stopped by incubating the sections in stop buffer for 30 min at 37°C. Anti-digoxigenin peroxidase was added to the slides, followed by incubation for 30 min at 37°C. Slides were stained with diaminobenzine for 10 min and counterstained with hematoxylin. A total of 500 cells were counted in each specimen. The apoptotic index was defined as follows: apoptotic index (%) = 100 × apoptotic cells/total cells. We stratified tumor specimens according to TUNEL staining in $\leq$10% or >10% stained cells.

Statistical Analysis

Spearman correlation test was used to assess relationship between original ordinal data before binary re-categorizations (correlation matrix between immunostaining parameters).

Univariate survival analysis for each prognostic variable on overall survival was estimated according to the Kaplan–Meier method [Kaplan and Meier, 1958]. The terminal event was death attributable to cancer or non-cancer causes. The statistical significance of the differences in survival distribution among the prognostic groups was evaluated by the log-rank test [Peto et al., 1997]. The Cox proportional hazards model was applied to the multivariate survival analysis [Cox, 1972]. The prognostic variables tested for overall survival included: age, gender, TUNEL staining, Bcl-2, Bax, and p53 expression.

P values <0.05 was regarded as statistical significant in two tailed tests. SPSS software (version 10.00, SPSS, Chicago) was used for statistical analysis.

TABLE I. Patients' Characteristics

Total number	67
Median age (range)	63 (45–83) years
Gender	
Male vs. female	45 vs. 22 (67.2% vs. 32.8%)
Pancreatic cancer site	
Head	54 (80.6%)
Tail/body	6 (9.0%)
Diffuse	7 (10.4%)
T factor	
T1	8 (11.9%)
T2	17 (25.4%)
T3	40 (59.7%)
N factor	
Negative	33 (49.3%)
Positive	34 (50.7%)
Grading	
Well differentiated	14 (20.9%)
Moderate differentiated	28 (41.8%)
Poor differentiated	15 (22.3%)
Post-operative radiotherapy	
Yes	19 (28.4%)
No	48 (71.6%)
Post-operative chemotherapy	
Yes	28 (41.8%)
No	39 (58.2%)
Post-operative chemoradiation	
Yes	11 (16.4%)
No	56 (83.6%)
Median follow-up time (median; range)	22 (3–100) months
Median overall survival (median; range)	18.5 (3–92) months

RESULTS

Patients' Characteristics

The cohort (Table I) consisted of 67 resected patients with a diagnosis of pancreatic adenocarcinoma (45 men and 22 women). The median age at diagnosis was 63 years (range 45–83).

The main histopathological tumors features are summarized in Table I.

Median follow-up after surgery was 22 months (range: 3–100 months). The minimum follow-up for the patients who did not have recurrence was 5 months. Fourteen patients were still alive at the moment of census period (July, 2003).

Of the 67 patients, 42 (93.3%) died of pancreatic cancer and 3 (6.7%) died of other causes. None were lost to follow-up.

The overall median survival time was 18.5 months (range: 3–92 months). The overall disease-specific 1-year survival rate was 76.2%, with a 5-year survival rate of 22.8%.

Adjuvant therapy has not been routinely offered in the hospitals involved in the study. Overall 58 (86.6%) patients underwent adjuvant therapy. We identified 28 (41.8%) patients who received adjuvant chemotherapy within 3 months of their operation, 19 patients (28.4%) adjuvant radiotherapy and 11 (17.5%) chemoradiation. No patients were treated with preoperative concomitant chemoradiation. Protocols for chemotherapy were not standardized, but chemotherapy was 5-fluorouracil or gemcitabine-based, both when only chemotherapy was administered as adjuvant treatment and when administered in combination with radiotherapy.

P53, Bax, Bcl-2, and TUNEL Staining

Immunohistochemical analysis of the 67 pancreatic adenocarcinoma specimens showed that Bcl-2 was expressed in 45 (67.2%) out of 67 cases; immunostaining was faint to moderate. Cellular distribution of the positive staining was restricted to the cytoplasm, which is typical for Bcl-2. Stronger staining was observed in peritumoral lymphoid follicles, which were used as an internal positive control. Immunostaining specific for Bax was cytoplasmic: 36 (53.7%) cases were defined as positive and 31 as negative (46.3%). We have used as positive control for immunostaining of both Bax and Bcl-2 lymph nodes with follicular hyperplasia. P53 nuclear expression was present in 32 (47.2%) out of 67 pancreatic cancer tissues. In 28 (41.8%) pancreatic cancer specimens out 67 TUNEL staining was present in more than 10% of the observed cancer cells. For an example of positive immunostaining for each of the antibodies used and for positive TUNEL stain see Figure 1. Interestingly, normal tissues of the pancreas did not express altered p53, but were always positive for Bcl-2 accordingly with data reported by Hu et al. (data not shown).

Correlation Between Immunohistochemical and Pathological Parameters

By rank correlation matrix, a positive statistically significant correlation was observed between Bcl-2 and Bax expression level ($P = 0.004$). Moreover, a positive correlation was recorded between Bcl-2 and p53 expression ($P = 0.023$). In regard to the apoptotic index (evaluated by TUNEL staining), a negative correlation was identified with Bax expression ($P = 0.006$) and a positive correlation with nodal involvement ($P < 0.001$). Finally, a strong positive correlation was recorded between p53 overexpression and Bax expression levels ($P < 0.001$). These results are summarized in Table II.

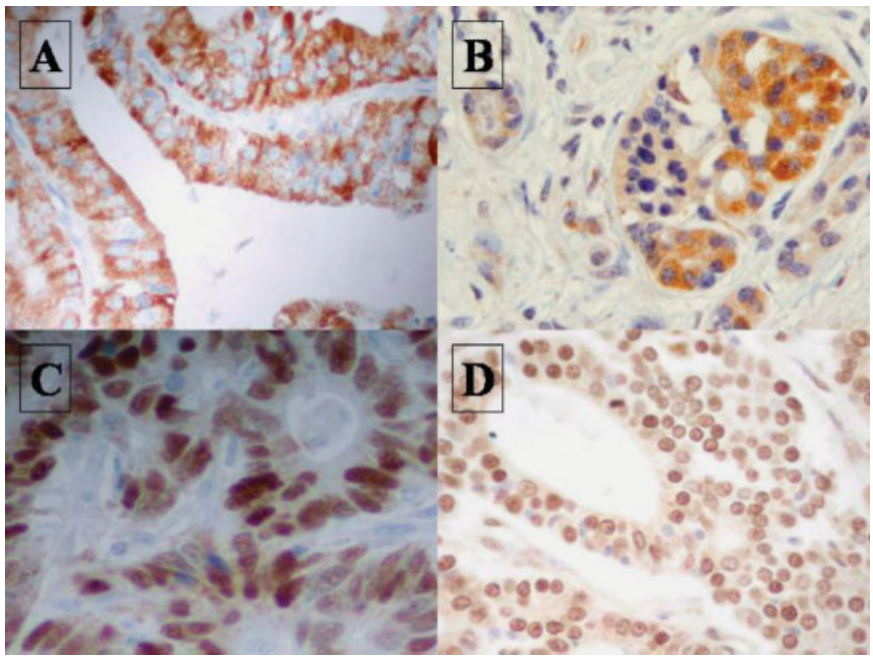

Fig. 1. Pancreatic ductal adenocarcinoma: (**A**) diffuse and marked cytoplasmic positivity for bcl2 (×400); (**B**) intense cytoplasmic positivity for bax (×400); (**C**) p53 diffuse positivity (×400); (**D**) Tunel nuclear positive staining (×400).

Immunohistochemical and Clinico-Pathological Parameters and Patient Survival

By univariate analysis overall survival seems to be influenced by Bcl-2 and Bax expression. In particular, those patients with overexpression of Bcl-2 or Bax showed a longer overall survival (respectively $P = 0.0379$ and 0.0311). Moreover, the median survival time in patients with low apoptotic index, evaluated by TUNEL method, was 20 months versus 8 months in those with high index ($P = 0.0127$). On the other hand, p53 and the histologic type did not influence the overall survival in our patients' population (Table III).

Figure 2 depicts Kaplan–Meier survival plots for all patients showing a statistically significant association between either low apoptotic index (A) or high levels of Bcl-2 (B) and Bax (C) and better outcome (respectively $P = 0.0127$, 0.0379 and 0.0311), while p53 levels (D) did not significantly associate with outcome. Moreover, combining Bcl-2 and Bax expression we identified two subset of patients (group A and B):

TABLE II. Correlation Between Immunohistochemical and Pathological Parameters in Radically Resected Pancreatic Adenocarcinoma Patients

	Bcl-2	TUNEL	Bax	p53	N factor
Bcl-2	—				
ρ		−0.201	0.351	0.285	0.097
P		n.s.	0.004	0.023	n.s.
TUNEL		−0.341			
ρ	−0.021	—	0.006	−0.341	0.430
P	n.s.		0.006	−0.341	0.001
Bax					
ρ	0.351	0.341	—	0.464	−0.151
P	0.004	−0.006		<0.001	n.s.
P53					
ρ	0.285	−0.341	0.464	—	−0.063
P	0.023	n.s.	<0.001		n.s.
N factor					
ρ	−0.097	0.430	−0.151	0.063	—
P	n.s.	<0.001	n.s.	n.s.	

ρ, correlation coefficient; n.s., non significant.

TABLE III. Univariate Analysis of Survival in Resected Pancreatic Adenocarcinoma Patients

	Median survival (months)	95% confidential interval	*P* value
Gender			
Female	13.00	7.99–18.01	0.4020
Male	19.00	13.40–24.60	
Age			
<65 years	12.00	10.23–13.77	0.2890
≥65 years	22.00	16.36–27.64	
T factor			
T1–2	21.00	4.71–37.29	0.4360
T3	16.00	11.67–20.33	
N factor			
N0	22.00	9.12–34.88	0.0202
N+	13.00	9.70–16.30	
Any adjuvant therapy			
No adjuvant therapy	15.00	12.33–19.34	0.8670
Any adjuvant therapy	20.00	13.67–25.11	
p53 expression			
p53 negative	13.00	10.28–15.72	0.1611
p53 positive	20.00	9.28- 30.72	
Bcl-2 expression			
Bcl-2 negative	12.00	3.74–20.26	0.0379
Bcl-2 positive	19.00	10.12–27.88	
Bax expression			
Bax negative	11.00	9.41–14.59	0.0311
Bax positive	19.00	5.34–32.66	
TUNEL staining			
TUNEL ≤10	20.00	14.42–25.58	0.0127
TUNEL >10	8.00	2.55–13.45	

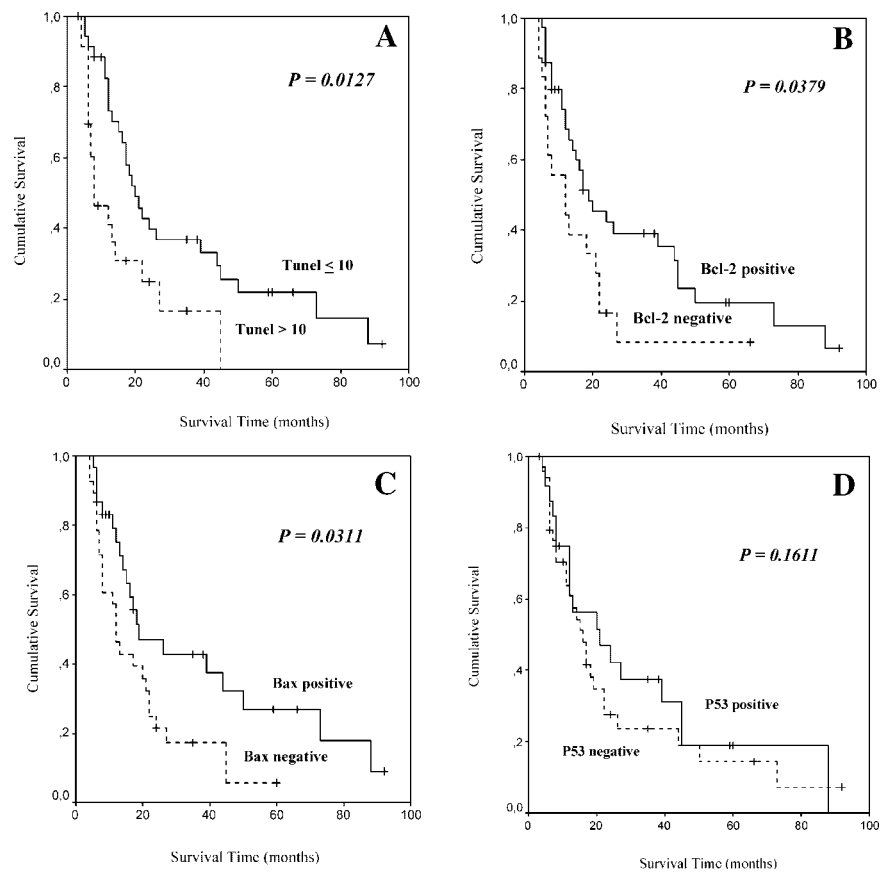

Fig. 2. Kaplan–Meier survival curves for radically resected pancreatic cancer patients: (**A**) Tunel staining (>10% vs. ≤10%); (**B**) Bcl-2 expression (Bcl-2 positive vs. Bcl-2 negative) (**C**) Bax expression (Bax positive vs. Bax negative); (**D**) p53 expression (p53 positive vs. p53 negative).

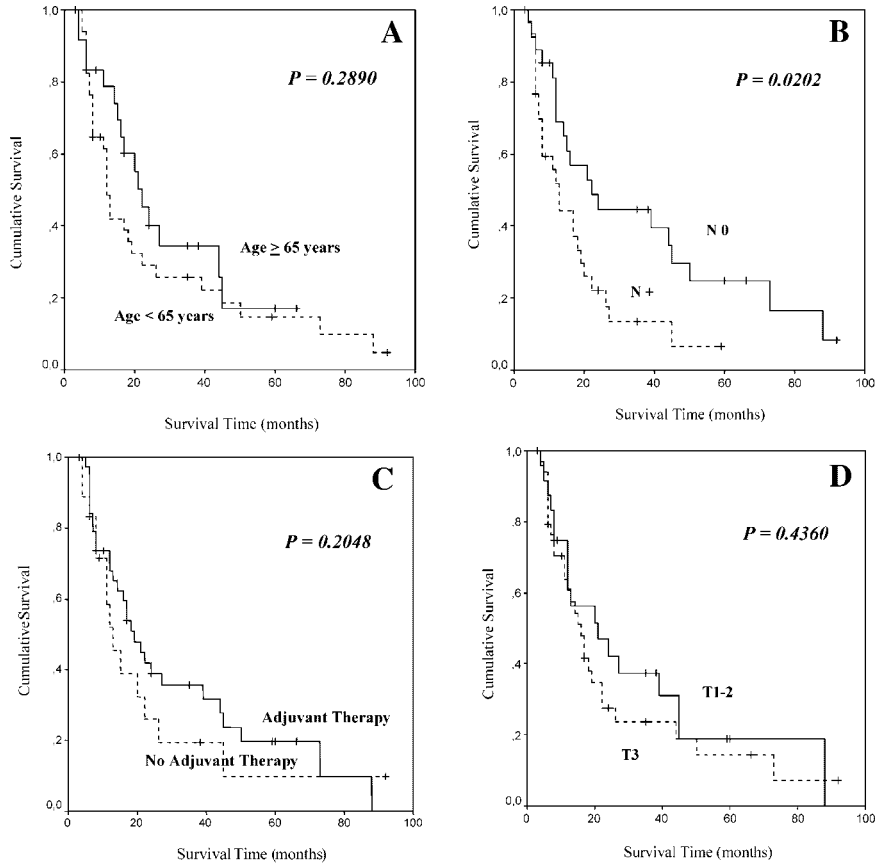

Fig. 3. Kaplan–Meier survival curves for radically resected pancreatic cancer patients: (**A**) Age (< 65 years vs. ≥65 years); (**B**) N stage (nodal involvement vs. no nodal involvement); (**C**) adjuvant therapy (any adjuvant therapy vs. no adjuvant therapy); (**D**) T stage (T1-2 vs. T3).

group A is represented of 32 patients with both overexpression of Bax and Bcl-2, while group B was represented of 35 patients with both negative expression of Bax and Bcl-2 or only one overexpressed.

Patients' group with both expression of Bcl-2 and Bax (group A) showed a statistically longer survival 39 months (95% CI: 8.01–69.99) when compared with survival of all the other patients (median survival: 12 months; 95% CI: 6.68–17.32) ($P = 0.0006$).

Figure 3 includes Kaplan–Meier survival plots in relation to the main clinical–pathologic patients' features. The only clinical–pathologic parameter that significantly correlates with overall survival was the presence of lymph nodes involvement ($P = 0.0202$) (Fig. 3B). Remarkably, adjuvant therapy did not show any influence on overall survival (any adjuvant therapy: $P = 0.2048$; postoperative chemotherapy: $P = 0.4790$; radiotherapy: $P = 0.1102$) (Fig. 3C). However, those patients who received chemoradiation as adjuvant therapy after resection showed a longer median overall survival (19.00 months) than those patients who did not (13.00 months), even if this difference does not reach a statistical significance ($P = 0.0960$) (data not shown). Probably, this non-significant difference is due to the small number of patients who received chemoradiation. Moreover, conclusive remarks can not be achieved due to the heterogeneity of the adopted adjuvant therapy.

Finally, by a multivariate Cox regression analysis, the only immunohistochemical parameter that resulted to influence overall survival was the apoptotic index evaluated by the TUNEL staining. The calculated relative risk of death in pancreatic cancer patients with low apoptotic index resulted significantly lower (0.436; $P = 0.040$). The other two immunohistochemical parameters significantly associated with prognosis in univariate analysis (Bcl-2 and Bax) did not influence the overall survival when

TABLE IV. Multivariate Analysis of Survival in Radically Operated Pancreatic Adenocarcinoma Patients

	Relative risk	95% confidential interval	*P*
N factor			
N+	1	—	0.453
N0	0.761	0.372–1.555	
Bcl-2 expression			
Bcl-2 negative	1	—	0.807
Bcl-2 positive	0.909	0.422–1.958	
Bax expression			
Bax negative	1	—	0.113
Bax positive	0.371	0.109–1.263	
TUNEL staining			
TUNEL positive	1	—	0.040
TUNEL negative	0.436	0.233–0.916	
Bcl-2/Bax combination			
Bcl2+/Bax−; Bcl2−/Bax+	1	—	0.013
Bcl2−/Bax−; Bcl2+/Bax+	5.413	1.420–20.637	

evaluated by multivariate analysis as single prognostic factors (respectively $P = 0.807$ and $P = 0.113$). However, if evaluated in combination Bax/Bcl-2 overexpression was the strongest independent prognostic factor in multivariate analysis ($P = 0.013$). These results are summarized in Table IV and Figure 4.

DISCUSSION

In the present paper, we evaluated the influence of clinical, pathological, and molecular features in a cohort of resected pancreatic cancer patients. Among the pathological and clinical features only N factor seems to represent a prognostic factor for overall survival in univariate analysis. Molecular studies, however, showed some interesting findings related to apoptosis.

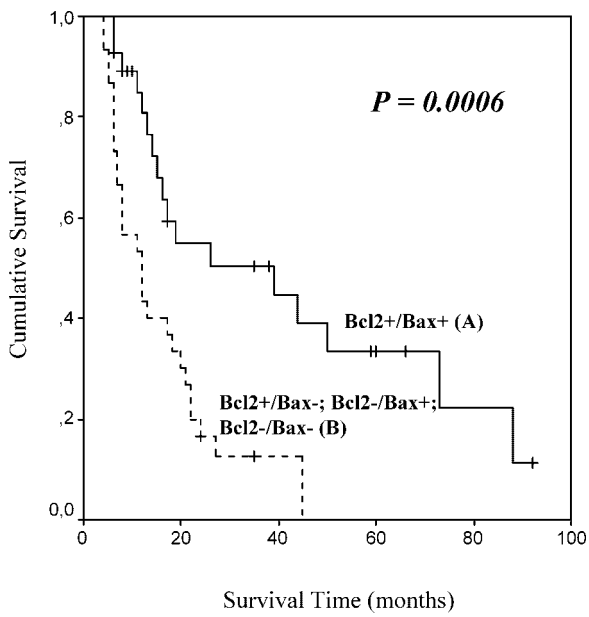

Fig. 4. Kaplan–Meier survival curve for Bcl-2+/Bax+ pancreatic cancers versus Bcl2+/Bax−; Bcl2−/Bax+; Bcl2−/Bax− pancreatic cancers.

A positive correlation for survival between Bcl-2 and p53 expression was found in our series of pancreatic carcinomas. These data are in contrast with previous reports. Hu et al. [1999], using similar techniques, reported an inverse correlation between Bcl-2 and p53 expression in a series of pancreatic carcinomas.

Apoptotic programmed cell death pathways are activated by a different array of extrinsic and intrinsic signals, most of which are ultimately coupled to the activation of effector caspases. Central to the regulation of the mitochondrial checkpoint is a complex network between members of the BCL-2 family, which are comprised of an anti-apoptotic subgroup including BCL-2 itself, and the proapoptotic BAX, BAK, and BH3-domain-only subgroups [Breckenridge et al., 2003].

In the present experience, apoptosis was not related to p53 status suggesting that apoptosis in our pancreatic cancer patients was mediated by a p53-independent pathway. Moreover, in our cohort p53 failed to be a prognostic survival factor, while overexpression of Bax and Bcl-2 were associated with longer survival in univariate analysis suggesting the p53-independent apoptotic process was not involved. Most of the studies concerning p53 expression in pancreatic cancer did not find any correlation with survival [Yokoyama et al., 1994; Coppola et al., 1998; Makinen et al., 1998]. Only a few showed a

correlation with poorer disease-free and overall survival [Sessa et al., 1998].

In addition, the results of the present paper provide evidence that p53-independent apoptosis may represent a significant prognostic factor in resected pancreatic cancer patients. In our report, the apoptotic index was a statistically significant prognostic factor: those patients with a higher apoptotic index showed a shorter median survival if compared with those with lower apoptotic index. Remarkably, performing multivariate analysis only the apoptotic index continued to be a prognostic factor for overall survival. The correlation between lower apoptotic index and longer survival could be explained by the fact that apoptosis could be an epiphenomenon that parallels active proliferation. Therefore, active proliferating cells could display also a higher apoptotic index.

A few studies are available about the prognostic role of the apoptotic index in patients with pancreatic cancer. Sarela et al. [2002] failed to identify any prognostic relevance of apoptotic index in pancreatic adenocarcinoma patients (advanced or resected).

Nio et al. [2001] confirmed that the apoptotic index did not have a prognostic role in a cohort of 66 resected pancreatic cancer patients.

After multivariate analysis only apoptotic index was a statistically significant prognostic factor and if both Bax and Bcl-2 were overexpressed had a statistically significant prognosis for survival. In particular, comparing patients with concomitant overexpression of Bax and Bcl-2 with all the others, the second group of patients showed a shorter median survival and an odds ratio of 5.413 in multivariate analysis. A possible explanation of the latter effect is the relative importance of Bax in determining the inhibition of anti-apoptotic function of Bcl-2. Therefore, the tissues that have high levels of both proteins are more prone to apoptotic events. On the other hand, tissue that express high levels of Bax, but not of Bcl-2 likely present other mechanisms of survival (mcl-1, bcl-xl, or others) that are not promptly inhibited by Bax.

The ratio of pro-apoptotic Bax-like proteins to anti-apoptotic Bcl-2-like proteins is a crucial determinant of both cellular susceptibility to apoptosis. Bcl-2/Bax-regulated apoptosis usually occurs in a p53-dependent manner. Wild-type p53 can induce apoptosis through transcriptional modulation of Bcl-2 and Bax [Miyashita and Reed, 1995] and through the inhibition of a potent Bcl-2 gene promoter [Budhram-Mahadeo et al., 1999].

Nevertheless, Bcl-2/Bax-regulated apoptosis can occur in a p53-independent manner [Butt et al., 2000]. Currently, we do not know what the downstream mediators are for the p53-independent apoptosis [Tsuji et al., 2002].

Only a few studies have evaluated the prognostic significance of Bcl-2 in pancreatic cancer, with contradictory results. Fujii et al. [1997] and Friess et al. [1998] did not find any correlation in their series of 81 and 60 cases, respectively. In addition, in the study performed by Makinen et al. [1998] Bcl-2 turned out to be an indicator of good outcome, while there was only a trend towards improved survival according to Sinicrope et al. [1996]. The role of Bax expression levels and prognosis in pancreatic cancer patients is controversial, as well. Some authors stated that Bax overexpression may represent a strong prognostic factor in pancreatic cancer patients. Bax maintains a statistically significant prognostic impact even when evaluated in multivariate analysis and when compared with standard prognostic factors, such as the clinical or pathological stage [Friess et al., 1998]. On the other hand, Nio et al. reported that Bax overexpression is not related to a better prognosis in resected pancreatic cancer patients, even if it may predictive of response to adjuvant chemotherapy. In the same paper, univariate analysis demonstrated that the Bax+/Bcl-2+ group had a significantly higher survival than the other groups, as reported by multivariate analysis in our study [Tsuji et al., 2002].

Some reasons may be involved in the explanation of these controversial findings in the previous reports: different antibodies and/or conditions for fixation of specimens and for immunostaining or different evaluation criteria may be the most likely reasons. Moreover, insufficient follow-up time in some study may partially explain the dissimilar results about the prognostic role of molecular markers in pancreatic cancer patients.

To the best of our knowledge, the present study is the first one to assess the clinical relevance of apoptotic index and Bax/Bcl-2 combination in resected pancreatic cancer. Knowledge of the factors that have an independent influence on prognosis is crucial in the development and interpretation of prospective

randomized trials in which patients are stratified according to these prognostic determinants.

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REFERENCES

Breckenridge DG, Germain M, Mathai JP, Nguyen M, Shore GC. 2003. Regulation of apoptosis by endoplasmic reticulum pathways. Oncogene 22(53):8608–8618.

Budhram-Mahadeo V, Morris PJ, Smith MD, Midgley CA, Boxer LM, Latchman DS. 1999. p53 suppresses the activation of the Bcl-2 promoter by the Brn-3a POU family transcription factor. J Biol Chem 274:15237–15244.

Butt AJ, Firth SM, King MA, Baxter RC. 2000. Insulin-like growth factor-binding protein-3 modulates expression of Bax and Bcl-2 and potentiates p53-independent radiation-induced apoptosis in human breast cancer cells. J Biol Chem 275:39174–39181.

Campani D, Esposito I, Boggi U, Cecchetti D, Menicagli M, De Negri F, Colizzi L, Del Chiaro M, Mosca F, Fornaciari G, Bevilacqua G. 2001. Bcl-2 expression in pancreas development and pancreatic cancer progression. J Pathol 194(4):444–450.

Canman CE, Gilmer TM, Coutts SB, Kastan MB. 1995. Growth factor modulation of p53-mediated growth arrest versus apoptosis. Genes Dev 9:606–611.

Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML, Wyllie AH. 1993. Thymocyte apoptosis induced by p53 dependent and independent pathway. Nature 362:849–852.

Coppola D, Lu L, Fruehauf JP, Kyshtoobayeva A, Karl RC, Nicosia SV, Yeatman TJ. 1998. Analysis of p53, P21WAF1, and TGF-beta1 in human ductal adenocarcinoma of the pancreas: TGF-beta1 protein expression predicts longer survival. Am J Clin Pathol 110:16–23.

Cox DR. 1972. Regression models and life tables. J R Stat Soc 34:187–220.

Evans JD, Cornford PA, Dodson A, Greenhalf W, Foster CS, Neoptolemos JP. 2001. Detailed tissue expression of bcl-2, bax, bak, and bcl-x in the normal human pancreas and in chronic pancreatitis, ampullary, and pancreatic ductaladenocarcinomas. Pancreatology 1(3):254–262.

Friess H, Lu Z, Graber HU, Zimmermann A, Adler G, Korc M, Schmid RM, Buchler MW. 1998. bax, but not bcl-2, influences the prognosis of human pancreatic cancer. Gut 43:414–421.

Fujii H, Inagaki M, Kasai S, Miyokawa N, Tokusashi Y, Gabrielson E, Hruban RH. 1997. Genetic progression and heterogeneity in intraductal papillary-mucinous neoplasms of the pancreas. Am J Pathol 151:1447–1454.

Geer RJ, Brennan MF. 1993. Prognostic indicators for survival after resection of pancreatic adenocarcinoma. Am J Surg 165:68–73.

Hu YX, Watanabe H, Ohtsubo K, Yamaguchi Y, Ha A, Motoo Y, Okai T, Sawabu N. 1999. Bcl-2 expression related to altered p53 protein and its impact on the progression of human pancreatic carcinoma. Br J Cancer 80:1075–1079.

Kaplan EL, Meier P. 1958. Nonparametric estimation from incomplete observations. J Am Stat Assoc 53:457–481.

Kawesha A, Ghaneh P, Andren-Sandberg A, Ograed D, Skar R, Dawiskiba S, Evans JD, Campbell F, Lemoine N, Neoptolemos JP. 2000. K-ras oncogene subtype mutations are associated with survival but not expression of p53, p16(INK4A), p21(WAF-1), cyclin D1, erbB-2, and erbB-3 in resected pancreatic ductal adenocarcinoma. Int J Cancer 89:469–474.

Kimura W, Morikane K, Esaki Y, Chan WC, Pour PM. 1998. Histologic and biologic patterns of microscopic pancreatic ductal adenocarcinomas detected incidentally at autopsy. Cancer 82:1839–1849.

Kuhl JS, Krajewski S, Duran GE, Reed JC, Sikic BI. 1997. Spontaneous overexpression of the long form of Bcl-X protein in a highly resistant P388 leukaemia. Br J Cancer 75:268–274.

Makinen K, Hakala T, Lipponen P, Alhava E, Eskelinen M. 1998. Clinical contribution of bcl-2, p53, and Ki-67 proteins in pancreatic ductal adenocarcinoma. Anticancer Res 18:615–618.

Miyashita T, Reed JC. 1995. Tumor suppressor p53 is a direct transcriptional activator of human *bax* gene. Cell 80:293–299.

Miyashita T, Harigai M, Hanada M, Reed JC. 1994. Identification of a p53-dependent negative response element in the *bcl-2* gene. Cancer Res 54:3130–3135.

Nio Y, Iguchi C, Yamasawa K, Sasaki S, Takamura M, Toga T, Dong M, Itakura M, Tamura K. 2001. Apoptosis and expression of Bcl-2 and Bax proteins in invasive ductal carcinoma of the pancreas. Pancreas 22(3):230–239.

Nitecki SS, Sarr MG, Colby TV, et al. 1995. Long-term survival after resection for ductal adenocarcinoma of the pancreas: Is in really improving? Ann Surg 221: 59–66.

Olopade OI, Adeyanju MO, Safa AR, Hagos F, Mick R, Thompson CB, Recant WM. 1997. Overexpression of Bcl-X protein in primary breast cancer is associated with high tumor grade and nodal metastases. Cancer J Sci Am 3:230–237.

Oltvai ZN, Milliman CL, Korsmeyer SJ. 1993. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerated programmed cell death. Cell 74:609–611.

Perego P, Giarola M, Righetti SC, Supino R, Caserini C, Delia D, Pierotti MA, Miyashita T, Reed JC, Zunino F. 1996. Association between cisplatin resistance and mutation of *p53* gene and reduced Bax expression in ovarian carcinoma cell system. Cancer Res 56:556–562.

Peto R, Pike MC, Armitage P, Breslow NE, Cox DR, Howard SV, Mantel N, McPherson K, Peto J, Smith PG. 1997. Design and analysis of randomised clinical trials requiring prolonged observation of each patient. Br J Cancer 35:1–39.

Ryan JJ, Danish R, Gottlieb CA, Clarke MF. 1993. Cell cycle analysis of p53-induced cell death in murine etythroleukemia cells. Mol Cell Biol 13:711–719.

Sarela AI, Verbeke CS, Ramsdale J, Davies CL, Markham AF, Guillou PJ. 2002. Expression of survivin, a novel inhibitor of apoptosis and cell cycle regulatory protein, in pancreatic adenocarcinoma. Br J Cancer 86(6):886–892.

Sessa F, Bonato M, Bisoni D, Ranzani GN, Capella C. 1998. Ki-ras and p53 gene mutations in pancreatic ductal carcinoma: A relationship with tumour phenotype and survival. Eur J Histochem 42:67–76.

Sherr CJ. 1996. Cancer cell cycles. Science 274:1672–1677.

Sinicrope FA, Evans DB, Leach SD, Cleary KR, Fenoglio CJ, Lee JJ, Abbruzzese JL. 1996. Bcl-2 and p53 expression in resectable pancreatic carcinoma: Association with clinical outcome. Clin Cancer Res 2:2015–2022.

Sobin LH, Wittekind C. 2002. TNM Classification of Malignant Tumours: UICC International Union Against Cancer. Edition 5. New York, NY: Wiley-Liss.

Sohn TA, Yeo CJ, Cameron JL, Koniaris L, Kaushal S, Abrams RA, Sauter PK, Coleman J, Hruban RH, Lillemoe KD. 2000. Resected adenocarcinoma of the pancreas—616 patients: Results, outcomes, and prognostic indicators. J Gastrointest Surg 4:567–579.

Stat Bite: 2002. Pancreatic cancer incidence in U.S. blacks and whites. 1973-1999. J Natl Cancer Inst 94(22):1671.

Sturm I, Kohne CH, Wolff G, Petrowsky H, Hillebrand T, Hauptmann S, Lorenz M, Dorken B, Daniel PT. 1999. Analysis of the p53/BAX pathway in colorectal cancer: Low BAX is a negative prognostic factor in patients with resected liver metastases. J Clin Oncol 17(5):1364–1374.

Tsuji K, Mizumoto K, Sudo H, Kouyama K, Ogata E, Matsuoka M. 2002. p53-independent apoptosis is induced by the p19ARF tumor suppressor. Biochem Biophys Res Commun 295(3):621–629.

Ueda N, Shah SV. 1994. Apoptosis. J Lab Clin Med 124:169–177.

Yokoyama M, Yamanaka Y, Friess H, Buchler M, Korc M. 1994. p53 expression in human pancreatic cancer correlates with enhanced biological aggressiveness. Anticancer Res 14:2477–2484.

Zhan Q, Alamo I, Yu K, Boise LH, Cherney B, Tosato G, O'Connor PM, Fornace AJ, Jr. 1996. The apoptosis associated gammaray response of Bcl-X(L) depends on normal p53 function. Oncogene 13:2287–2293.