ANTICANCER RESEARCH *25*: 1361-1368 (2005)

Expression of Retinoblastoma and p53 Pathway-related Proteins in Resectable Invasive Ductal Carcinoma of the Pancreas: Potential Cooperative Effects on Clinical Outcome

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Abstract. *Background: The retinoblastoma protein (RB) is an important cell cycle regulator. RB also plays an important role in the regulation of apoptosis, which is mediated by interaction of p53 signaling mediators. The present study was designed to assess the clinicopathological significance of RB and p53 pathway-related proteins (p53, $p21^{WAF1/CIP1}$ and Bax) expression in resectable invasive ductal carcinoma (IDC) of the pancreas. Materials and Methods: The present study included 79 pancreatic IDC patients, who received surgery between 1982 and 2002. The expression of RB and p53 pathway-related proteins (p53, $p21^{WAF1/CIP1}$ and Bax) were analyzed by immunohistochemistry. Results: RB was expressed in 45 (57%) of the 79 patients. RB expression correlated significantly with histological grade and grade of nodal involvement. The positive rate of p53, $p21^{WAF1/CIP1}$ and Bax expression was 49%, 48% and 67%, respectively. RB expression alone did not have a significant effect on patient survival. However, coexpression analysis of RB and p53 pathway-related proteins indicated that, in the patients with RB (+) IDC, the $p21^{WAF1/CIP1}$ (+) group had a significantly higher survival rate than the $p21^{WAF1/CIP1}$ (–) group. On the other hand, in the patients with RB (–) IDC, the Bax (+) group had a significantly higher survival rate than the Bax (–) group. Multivariate analysis indicated that, in the RB (–) group, pTNM stage, adjuvant chemotherapy and Bax expression were significant variables. Conclusion: The evaluation of RB expression combined with the mediators of the p53 pathway, $p21^{WAF1/CIP1}$ and Bax, may provide more accurate information regarding clinical outcome, beyond that which is provided by RB expression alone.*

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Key Words: Pancreatic cancer, retinoblastoma, p53, $p21^{WAF1/CIP1}$, Bax.

Invasive ductal carcinoma (IDC) of the pancreas is one of the most common causes of cancer death in developed countries (1). IDC of the pancreas is usually resistant to chemotherapy, radiotherapy and immunotherapy (2, 3). The high mortality associated with the disease is related to its propensity for extensive local invasion and early metastasis. One of the reasons for this extremely malignant potential is the fact that the biological characteristics and oncogenetic background of this tumor might be quite different from those of other carcinomas.

The retinoblastoma tumor-suppressor protein (RB) is an important cell-cycle regulator in controlling G1/S cell cycle transition by binding to the members of the E2F family of transcription factors (4). For cell cycle progression, RB must be phosphorylated and inactivated by cyclin D in association with cyclin-dependent kinases (CDKs) 4 and 6 (5). RB phosphorylation is also regulated by the $p16^{INK4a}$ protein by binding competitively to CDK4-CDK6, thereby preventing the cell cycle progression (6, 7). Certain human tumors have been reported to abrogate the RB pathway by multiple distinct mechanisms (8-11). In most pancreatic cancer, the RB pathway is almost exclusively abrogated by inactivation of the $p16^{INK4a}$ gene, rather than losing RB entirely (12). Deregulation of the RB pathway would, therefore, be one of the most important mechanisms for the development and progression of pancreatic cancer. However, the significance of RB expression in IDC of the pancreas is still unclear.

Apart from being a cell cycle regulator, RB also plays an important role in the regulation of apoptosis (13-15). Interestingly, recent studies have revealed that the regulation of apoptosis by RB is mediated by interaction of p53 signaling mediators and RB (13, 14). p53, which is one of the most important tumor suppressors, has a dual role as

a cell cycle regulator and as a inducer of apoptosis, in part, through the induction of $p21^{WAF1/CIP1}$ and the regulation of Bax expression, respectively (16-18). Most human tumors deregulate either the RB or p53 pathway (5, 7, 19). Furthermore, cross-talk between the RB and p53 pathways has been identified (20, 21), suggesting that simultaneous inactivation of multiple mediators in both pathways would increase the malignant potential, which clinically results in extensive progression, and subsequently poor prognosis associated with the tumor.

In this study, we focused on the significance of RB in human pancreatic IDC and investigated the expression of RB and p53 pathway-related proteins (p53, $p21^{WAF1/CIP1}$ and Bax) in 79 resectable cases of the disease, assessing clinicopathological significance. Furthermore, we evaluated the correlation between RB expression and expression of the other proteins.

Materials and Methods

Patients. Seventy-nine patients (41 women, 38 men; age range, 35-80 years; mean age, 65.7 years) with resectable pancreatic IDC, who had not received any cancer treatment before surgery, underwent pancreatectomy between 1982 and 2002 at the First Department of Surgery, Shimane University School of Medicine, Japan. The profile of the patients is summarized in Table I. Standard or pylorus-preserving pancreatoduodenectomy was carried out in 40 patients, distal pancreatectomy was performed in 26 patients, and total pancreatectomy was performed in 13 patients. The tumors were staged according to the UICC classification (TNM classification) (22) and patient outcome was surveyed in December 2003. None of the patients had received any type of treatment prior to the diagnostic or surgical procedures. Forty-nine of the patients received adjuvant chemotherapy after surgery and most patients were given oral UFT (a mixture of uracil and ftorafur at 4:1) alone or with oral cyclophosphamide, while some received intensive intravenous regimens, including 5-fluorouracil (5-FU) or gemcitabine.

Antibodies. The anti-RB mouse monoclonal antibody (mAb) (Ab-5, LM95.1), anti-p21 mouse mAb (Ab-1, EA10) and anti-p53 mouse mAb (Ab-6, DO-1) were purchased from Oncogene Science (Uniondale, NY, USA) and were used at a dilution of 1:10, 1:100 and a concentration of 2 mg/ml, respectively. The anti-Bax rabbit polyclonal antibody (Ab) (A3533) was purchased from Dako Corporation (Carpenteria, CA, USA), and was diluted 1:100 for the experiments.

Immunohistochemistry. Formalin-fixed paraffin-embedded specimens were cut into 4-μm sections. Slides were deparaffinized in xylene three times for 5 minutes each, hydrated in 100%, 95% and 45% ethanol, and finally in phosphate-buffered saline (PBS). After antigen retrieval by microwave oven, the endogenous peroxidase activity and non-specific binding were blocked by treatment with 0.3% H_2O_2 in methanol for 15 minutes and with 10% normal serum for 10 minutes. The sections were incubated with anti-RB Ab at 4˚C overnight, and with anti-p21, anti-p53 and anti-Bax Abs at room temperature for 2 hours. After rinsing twice in PBS, the specimens were incubated with biotinylated secondary Ab (Nichirei, Tokyo, Japan) for 15 minutes at 37˚C, washed twice in PBS and incubated with peroxidase-labelled avidin-biotin (Nichirei) for 10 minutes at room temperature, and again washed twice in PBS. The immunoreaction of the specimens was visualized with 0.05% 3,3'-diaminobenzidine (Nichirei) solution for several minutes at room temperature. After washing in distilled water, the specimens were counterstained with hematoxylin and mounted.

Table I. *Relationship between RB expression and clinicopathological characteristics.*

Parameter		No. of patients	No. expressing (%)
Overall		79	45 (57%)
Age	<65	29	13 (45%)
	≥65	50	32 (64%)
Gender	Male	38	19 (50%)
	Female	41	26 (63%)
Grade	1. Well-dif.	35	14 (40%)[a]
	2. Moderately-dif.	38	26 (68%)[a]
	3. Poorly-dif.	6	5 (83%)[a]
	4. Undif.	0	0
pTNM stage	I	12	8 (67%)
	II	3	2 (67%)
	III	39	20 (51%)
	IV	25	15 (60%)
pT	1	7	3 (43%)
	2	27	20 (74%)
	3	25	10 (40%)
	4	20	12 (60%)
Pn	0	8	7 (88%)[b]
	1	27	20 (74%)[b]
	2	44	18 (41%)[b]

Dif., differentiated; pT (pathologic classification of primary tumor): 1, limited to pancreas, <2.0 cm; 2, limited to pancreas, >2.0 cm; 3, extended to peripancreatic structures including the duodenum, bile duct, mesentery, mesocolon, ometum and peritoneum; 4, extended to adjacent structures including stomach, spleen, colon, portal vein, celiac artery and the superior mesenteric and common hepatic arteries and vein; pN, pathologic classification of nodal involvement.
[a]$p=0.019$ by χ^2 test.
[b]$p=0.004$ by χ^2 test.

Evaluation of immunostaining. For the evaluation of RB, p53 and $p21^{WAF1/CIP1}$ expression, immunostaining was scored as positive only when the nucleus of the tumor cell was stained more than 10%, 20% and 5%, respectively (23, 24). For the evaluation of Bax expression, immunostaining was taken to be positive only when unequivocally strong cytoplasmic staining was present in >10% of the tumor cells (25).

Statistical analysis. The Chi-square test was used to compare the correlation between the clinicopathological factors and the expression of RB. The cumulative survival rates were calculated according to the Kaplan-Meier method and compared by the Cox-Mantel test. A multivariate analysis of maximum likelihood estimates with the Cox proportional hazard model was applied to obtain a conditional risk of death due to IDC of the pancreas. Statistically significant differences were defined as $p<0.05$.

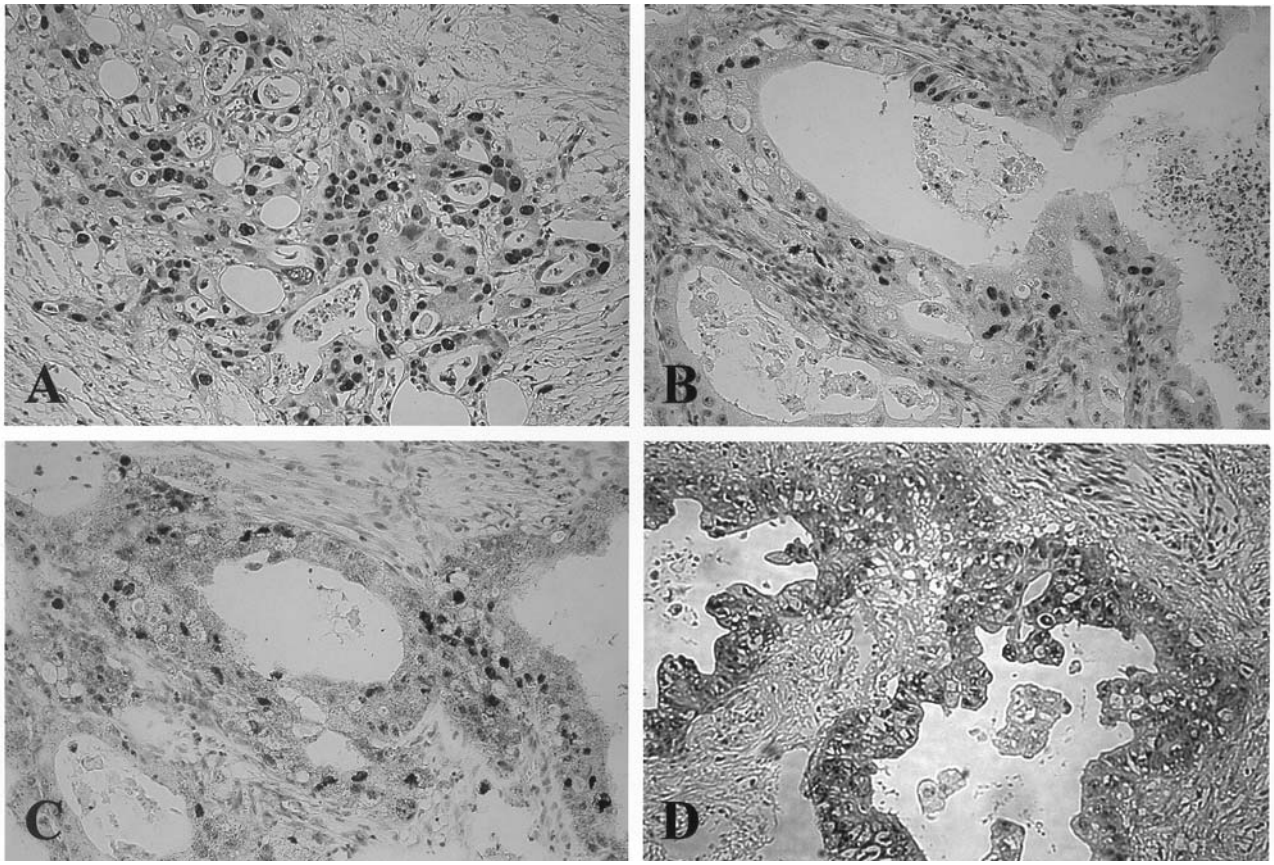


Figure 1. *Immunohistochemical staining of RB, p53, p21$^{WAF1/CIP1}$ and Bax in invasive ductal carcinoma of the pancreas (original magnification, X200). A: RB-positive immunostaining. The nucleus is positively-stained. B: p53-positive immunostaining. The nucleus is positively-stained. C: p21-positive immunostaining. The nucleus is positively-stained. D: Bax-positive immunostaining. The cytoplasm is positively-stained.*

Results

Expression of RB and p53-related proteins. Representative immunostaining for RB and p53 pathway-related proteins (p53, p21$^{WAF1/CIP1}$ and Bax) is shown in Figure 1. RB was expressed in 45 (57%) of the 79 patients. RB expression correlated significantly with histological grade and pN (Table I). The increase of histological grade was significantly associated with an increase in RB expression (p=0.019). On the other hand, the progression of lymph node metastasis was significantly associated with a decrease in RB expression (p=0.004). The positive rate of p53, p21$^{WAF1/CIP1}$ and Bax expression was 49%, 48% and 67%, respectively. There were significant correlations between RB expression and p21$^{WAF1/CIP1}$ expression (p=0.003) and RB expression and Bax expression (p=0.020) (Table II). p53 expression did not show any correlation with RB expression (Table II).

Survival curves and RB and p53 pathway-related proteins expression. RB expression alone did not have any significant effect on the patient survival (p=0.835) (Figure 2). Co-expression with p53 pathway-related proteins showed that, in the patients with RB (+) IDC, the p21$^{WAF1/CIP1}$ (+) group had a significantly higher survival rate than the p21$^{WAF1/CIP1}$ (–) group (p=0.008), although there was no significant difference in survival between the two groups in the RB (–) IDC (p=0.471) (Figure 3). On the other hand, in the patients with RB (–) IDC, the Bax (+) group had a

Table II. *Correlation between RB and p21$^{WAF1/CIP1}$, Bax and p53 expression.*

	RB (–)	RB (+)	Total	χ^2 test
p21$^{WAF1/CIP1}$(-)	24 (30.4%)	17 (21.5%)	41 (51.9%)	p=0.003
p21$^{WAF1/CIP1}$(+)	10 (12.6%)	28 (35.5%)	38 (48.1%)	
Bax (-)	16 (20.2%)	10 (12.7%)	26 (32.9%)	p=0.020
Bax (+)	18 (22.8%)	35 (44.3%)	53 (67.1%)	
p53 (-)	17 (21.5%)	23 (29.1%)	40 (50.6%)	p=0.922
p53 (+)	17 (21.5%)	22 (27.9%)	39 (49.4%)	
Total	34 (43.0%)	45 (57.0%)	79	

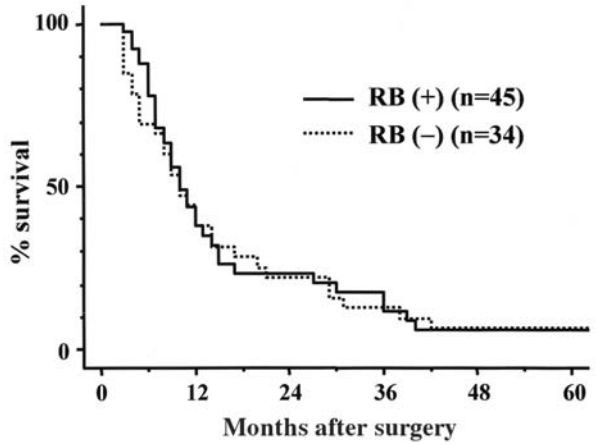
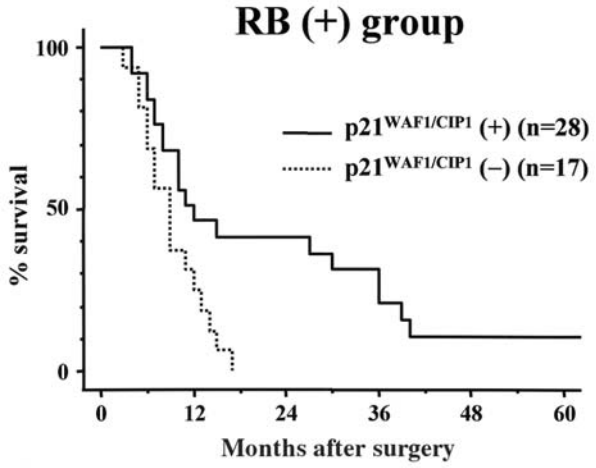


Figure 2. *Survival curves after pancreatectomy grouped RB expression. There was no significant difference between the RB (+) and RB (-) groups (p=0.835).*



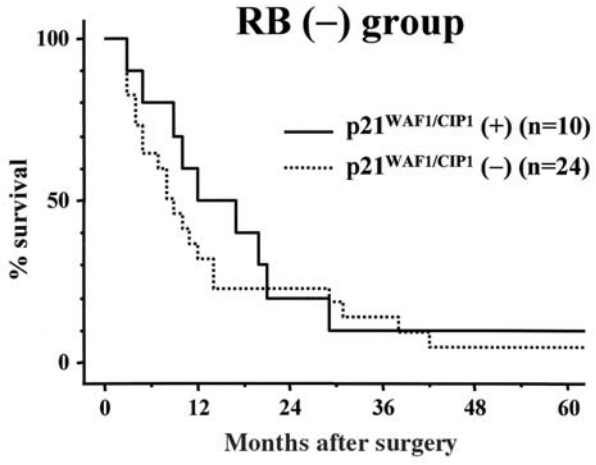
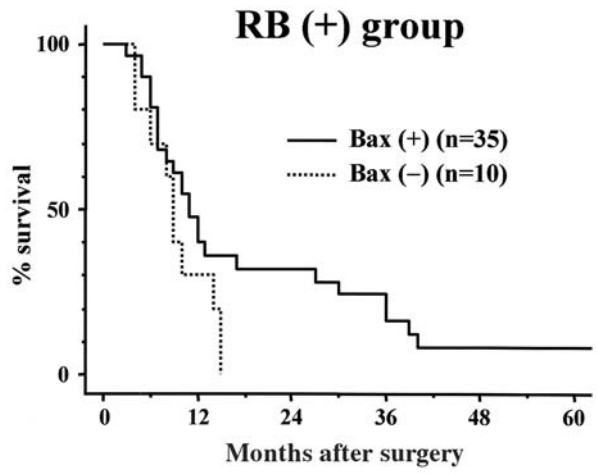


Figure 3. *Co-expression of RB and $p21^{WAF1/CIP1}$ and survival curves after pancreatectomy. There was a significant difference between $p21^{WAF1/CIP1}$ (+) and $p21^{WAF1/CIP1}$ (–) groups in the RB (+) group (p=0.008), but not between $p21^{WAF1/CIP1}$ (+) and $p21^{WAF1/CIP1}$ (–) groups in the RB (–) group (p=0.471).*



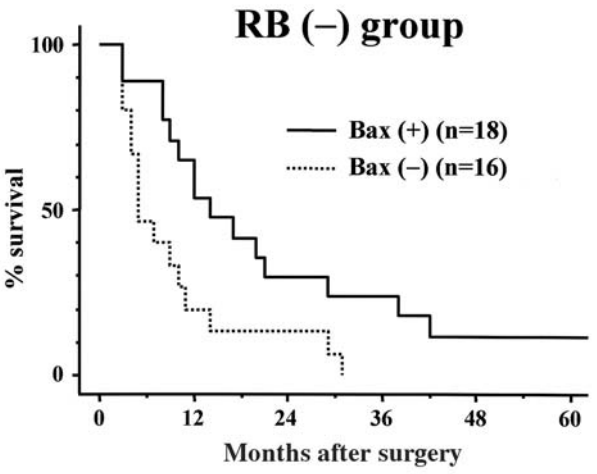


Figure 4. *Co-expression of RB and Bax and survival curves after pancreatectomy. There was a significant difference between the Bax (+) and Bax (–) groups in the RB (–) group (p=0.012), but not between the Bax (+) and Bax (–) groups in the RB (+) group (p=0.074).*

significantly higher survival rate than the Bax (–) group (p=0.012), although there was no significant difference in survival between the two groups in the RB (+) IDC (p=0.074) (Figure 4). However, RB expression status did not have any effect on the prognostic significance of p53 expression (Figure 5).

Multivariate analysis. To determine which factors exert significant influences on survival after pancreatectomy, multivariate analysis using the Cox proportional hazard risk model was applied. The results demonstrated that pTNM stage and adjuvant chemotherapy were independently significant variables for survival, but RB and p53 pathway-related proteins expression were not significant variables for survival (Table III). Furthermore, to investigate the effect of

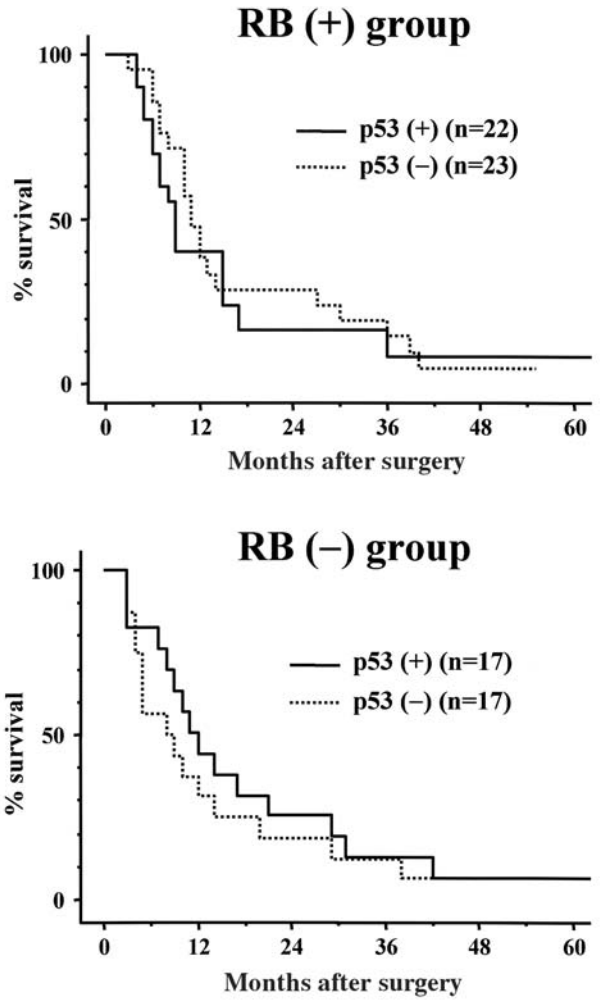


Figure 5. *Co-expression of RB and p53 and survival curves after pancreatectomy. There was no significant difference between the p53 (+) and p53 (–) groups in the RB (+) and RB (–) group.*

Table III. *Multivariate analysis on prognostic significance of clinicopathological and cytomolecular factors in the patients with resectable pancreatic IDC.*

variables	Conditional risk ratio (95% confidence limit)	*p* value (χ^2)
Age	1.006 (0.977-1.036)	0.702
Gender	1.107 (0.612-2.001)	0.737
Adjuvant chemotherapy	0.465 (0.254-0.853)	0.013
Histological grade	1.553 (0.983-2.453)	0.059
pTNM stage	1.707 (1.243-2.346)	0.001
RB expression	0.969 (0.534-1.756)	0.917
p53 expression	1.000 (0.596-1.679)	0.999
$p21^{WAF1/CIP1}$ expression	0.710 (0.380-1.326)	0.282
Bax expression	0.717 (0.395-1.302)	0.274

Multivariate analysis by Cox's proportional-hazard model: dependent variable, month; censoring variable, death due to pancreatic cancer.

RB expression status on survival, the patients were classified into two groups, the RB (+) group and the RB (–) group. In the RB (+) group, pTNM stage was a significant variable. $p21^{WAF1/CIP1}$ expression marginally failed to reach significance. However, pTNM stage, adjuvant chemotherapy and Bax expression were significant variables in the RB (–) group (Table IV).

Discussion

The RB pathway, which controls cell proliferation through regulation of the cell cycle at the G1/S cell cycle transition, is an important tumor suppressor pathway, and has been reported to be altered in various human malignancies (26, 27). However, the significance of RB expression in IDC of the pancreas has not been fully elucidated. In this study, we investigated the expression of RB, and showed that RB was expressed in 57% of the pancreatic IDCs. The incidence of RB expression in various human malignancies was reported to be 76-94% in esophageal carcinoma (28-30), 67% in gastric adenocarcinoma (31), 76-91% in non-small cell lung cancer (32-37), 63-74% in breast carcinoma (38-40) and 81% in bladder tumors (41). Previous reports on the immunohistochemical evaluation of RB expression in pancreatic cancer, albeit limited, showed a range of positivity varying between 50 and 100% (42-45). There are some possible reasons for these differences in the expression of RB, the variety of antibodies used and/or conditions for immunohistochemistry or variable evaluation criteria being the most likely. Moreover, the different ethnic and racial backgrounds of the patients may exert influences on the expression of RB, which was seen in the implication of p53 expression between Japanese, Chinese, Europeans and Americans (46).

Our study demonstrated the significant correlation of the loss of RB expression with advanced lymph node metastasis. This finding is in agreement with previous findings in adenocarcinoma of the lung (35), breast (38) and pancreatic cancer (45). In gastric adenocarcinoma, RB expression was lower in lymph node metastasis than in primary gastric tumors (31), and RB expression was significantly lower in liver metastasis than in primary colorectal tumors (47). Furthermore, RB expression was reported to inhibit bladder tumor cell invasion *in vitro* (48). Therefore, these data suggested that RB abnormalities may contribute to the progression of the metastatic process rather than simply having an effect on cell cycle dysregulation. On the other hand, RB expression significantly correlated inversely with

Table IV. *Multivariate analysis on prognostic significance of clinicopathological and cytomolecular factors in the patients with resectable pancreatic IDC.*

RB (+) group			RB (–) group		
variables	Conditional risk ratio (95% confidence limit)	*p* value (χ^2)	variables	Conditional risk ratio (95% confidence limit)	*p* value (χ^2)
Age	1.001 (0.959-1.044)	0.976	Age	1.023 (0.972-1.077)	0.382
Gender	1.266 (0.504-3.179)	0.616	Gender	0.405 (0.149-1.098)	0.076
Adjuvant chemotherapy	0.661 (0.274-1.594)	0.357	Adjuvant chemotherapy	0.272 (0.101-0.733)	0.010
Histological grade	1.823 (0.950-3.502)	0.071	Histological grade	1.536 (0.673-3.506)	0.308
pTNM stage	1.673 (1.107-2.530)	0.015	pTNM stage	3.310 (1.496-7.321)	0.003
$p21^{WAF1/CIP1}$ expression	0.460 (0.195-1.082)	0.075	$p21^{WAF1/CIP1}$ expression	1.704 (0.626-4.643)	0.297
Bax expression	0.862 (0.371-2.000)	0.729	Bax expression	0.315 (0.109-0.914)	0.034
p53 expression	1.274 (0.570-2.850)	0.555	p53 expression	0.435 (0.176-1.077)	0.072

Multivariate analysis by Cox's proportional-hazard model: dependent variable, month; censoring variable, death due to pancreatic cancer.

histological grade in this study. Although this is consistent with the previous report of stage I non-small cell lung cancer (49), it is difficult to understand given its tumor suppressor function. Moreover, the majority of studies have failed to find a correlation between RB expression and histological grade (29, 30, 31, 38, 50, 51). Taken together, it seems that RB may actually have little effect on tumor differentiation.

With regard to the prognostic value of RB expression, many conflicting results have been reported in various malignancies. Some studies demonstrated that patients with RB (–) tumors had a significantly poorer prognosis compared to those with RB (+) tumors, non-small cell lung cancer (32, 34, 36), bladder cancer (41) and gastric cancer (31). In contrast, patients with RB (+) tumors had a significantly poorer prognosis than those with RB (–) cancer of the ovary (52) and stomach (53). Other authors, however, reported that there is no correlation between RB expression and the prognosis of patients with esophageal (29, 30, 51), breast (40) and non-small cell lung cancer (35, 37, 39). In the case of pancreatic cancer, only one study reported that loss of RB expression could not be identified as a prognostic marker (44). In the present study, RB expression was also found to have no correlation with prognosis. Taken together, these data suggest that the alteration of RB expression alone may be of limited value as a prognostic marker.

The signaling of the p53 tumor suppressor pathway is frequently interconnected with the RB pathway (20, 21). We next performed combined analysis of RB and signaling mediators of the p53 pathway, $p21^{WAF1/CIP1}$ and Bax. When the patients were grouped by RB expression, the expression of $p21^{WAF1/CIP1}$ had a different influence on the survival of the patients. In the RB (+) group, the survival rate of the $p21^{WAF1/CIP1}$ (+) subgroup was significantly higher than that of the $p21^{WAF1/CIP1}$ (–) group, whereas in the RB (–) group, there was no difference between the $p21^{WAF1/CIP1}$ (+) and (–) subgroups. In addition to $p16^{INK4a}$, $p21^{WAF1/CIP1}$ prevents RB phosphorylation by inhibiting the activation of cyclinE/CDK2 complexes, which are required for RB phosphorylation (7). It has been shown that the *$p16^{INK4a}$* gene was inactivated in a majority of pancreatic cancers through intragenic mutation, homozygous deletion, or methylation-associated transcriptional silencing (12). It is, therefore, suggested that $p21^{WAF1/CIP1}$ may have an important role for cell cycle inhibition through the RB pathway in RB (+) pancreatic IDC; in contrast, cell cycle regulation of $p21^{WAF1/CIP1}$ may be impaired in tumors with RB (–).

On the other hand, in the RB (–) group, the survival rate of the Bax (+) subgroup was significantly higher than that of the Bax (–) group, whereas in the RB (+) group, there was no difference between the Bax (+) and (–) subgroups. These results were also confirmed by multivariate analysis. Bax is a central downstream effector of p53 in apoptosis (18, 54). Therefore, in RB (–) pancreatic IDC, regulation of apoptosis may play a more important part in the course and prognosis of pancreatic cancer compared to the effects of cell cycle regulation. Alternatively, since RB has been known to protect against apoptosis independently of its ability to inhibit cell cycle progression (13, 15), in RB (–) pancreatic IDC, Bax could easily induce apoptosis, which is indicative of favorable prognosis. A recent study reported that Bax induces apoptosis independently of Bcl-2 by directly mediating the activation of mitochondria for cytochrome c release (55), although it has been reported that the relative ratio of Bax and anti-apoptotic molecules, such as Bcl-2, determines the susceptibility of cells to apoptosis (56).

In the present study, RB expression significantly positively correlated with $p21^{WAF1/CIP1}$ and Bax expression respectively. It was also reported that RB expression of the p53-/p21+ group was significantly higher than that of the p53+/p21– group in esophageal cancer (51). In bladder

transitional cell carcinoma, when RB status was considered wild-type (1% to 50%), there was a significant association between the p21 expression and RB expression; the majority of tumors displaying RB-wild-type expression also had p21-wild-type protein expression (57). In contrast, there was no correlation between RB and p53 expression, which is consistent with the study of non-small cell lung cancer (37). Taken together, these results suggested that mediators of RB and p53 tumor suppressor pathways may influence the expression of the others, although further studies, including the examination of other mediators of these two tumor suppressor pathways, are required to elucidate the precise mechanism of interaction of these pathways.

In conclusion, the evaluation of RB expression, combined with the mediators of the p53 pathway, $p21^{WAF1/CIP1}$ and Bax, may provide accurate information regarding clinical outcome, beyond what is provided by RB expression alone.

Acknowledgements

We gratefully acknowledge Ms. Tomoko Toga, Ms. Miyuki Ishihara, Ms. Yasuko Sonoyama and Ms. Yuka Maniwa for their excellent assistance.

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Received September 7, 2004
Accepted December 29, 2004