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Clinical Significance of Cell Cycle– and Apoptosis-Related Markers in Biliary Tract Cancer

A Tissue Microarray–Based Approach Revealing a Distinctive Immunophenotype for Intrahepatic and Extrahepatic Cholangiocarcinomas

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Abstract

Cholangiocarcinoma is the second most common malignant tumor of the liver. We analyzed, immunohistochemically, the significance of cell cycle– and apoptosis-related markers in 128 cholangiocarcinomas (42 intrahepatic, 70 extrahepatic, and 16 gallbladder carcinomas) combined in a tissue microarray. Follow-up was available for 57 patients (44.5%).

In comparison with normal tissue (29 specimens), cholangiocarcinomas expressed significantly more frequently p53, bcl-2, bax, and COX-2 ($P < .05$*). Intrahepatic tumors were significantly more frequently bcl-2+ and p16+, whereas extrahepatic tumors were more often p53+ (*$P < .05$*). Loss of p16 expression was associated with reduced survival of patients.*

Our data show that p53, bcl-2, bax, and COX-2 have an important role in the pathogenesis of cholangiocarcinomas. The differential expression of p16, bcl-2, and p53 between intrahepatic and extrahepatic tumors demonstrates that there are location-related differences in the phenotype and the genetic profiles of these tumors. Moreover, p16 was identified as an important prognostic marker in cholangiocarcinomas.

Cholangiocarcinoma is the second most common primary hepatic tumor, and its incidence is increasing.[1-3] Prevention of this cancer is very difficult, and only a few risk factors have been so far identified. Cholangiocarcinoma arises from the biliary epithelial cells and can occur anywhere along the biliary tract. Tumors are divided anatomically into intrahepatic and extrahepatic cholangiocarcinomas, as well as adenocarcinomas of the gallbladder.[4] Despite their similar morphologic features, differences have been found among these subtypes in relation to their clinical course, genetic profiles, and immunophenotypes.[5,6]

Studies have reported that the expression of cell cycle modulating proteins, such as p16, p21, and p27, is associated with aggressive tumor behavior in several human malignancies and in cholangiocarcinomas.[7-10] The p53 protein has been reported to be immunohistochemically detectable in 20% to 80% of cholangiocarcinomas.[11-14] Mutations in the *p53* gene have not been found to correlate with apoptosis rates.[15] Therefore, alteration of the p53 pathway leading to the loss of cell cycle control may be critical to the cellular pathogenesis of cholangiocarcinoma.

COX-2 has been reported to be up-regulated in a variety of gastrointestinal tumors[16,17] and is induced by a variety of cytokines and mitogens under certain pathologic conditions.[18] COX-2 overexpression has been observed in chronic cholangitis and in cholangiocarcinoma cells but not in normal biliary epithelial cells, suggesting that this enzyme may have an important role in bile duct carcinogenesis and tumor progression.[19-22]

The best characterized modulators of mitochondrial apoptotic function are the members of the bcl-2 family. The relative abundance of proapoptotic (eg, bax) vs

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antiapoptotic (eg, bcl-2) bcl-2 family proteins seems to be involved in the activation of the mitochondrial death pathway.[23] Immunohistochemical studies of cholangiocarcinomas support a relative abundance of antiapoptotic mediators, including bcl-2 family members.[24,25]

To identify and/or further analyze possible diagnostic and prognostic markers for cholangiocarcinomas, in relation to anatomic site, we studied, by immunohistochemical analysis, the frequency and clinical significance of the aforementioned markers in a series of 128 cholangiocarcinomas, including 42 intrahepatic and 70 extrahepatic cholangiocarcinomas and 16 gallbladder carcinomas, as well as 29 normal control samples combined in a tissue microarray (TMA) format. Clinical follow-up information was available for 57 patients (44.5%).

Materials and Methods

Patients and Specimens

Formalin-fixed and paraffin-embedded tumor and control specimens were retrieved from the archives of the Institute of Pathology, University of Bern, Bern, Switzerland. All tumor and control specimens were reviewed by one pathologist (E.K.). Representative tumor areas were selected for the construction of the TMA. The TMA consisted of 128 cholangiocarcinoma cases including 42 intrahepatic and 70 extrahepatic cholangiocarcinomas and 16 gallbladder carcinomas, as well as 29 normal control samples. The 128 patients comprised 70 men and 58 women with a mean ± SD age of 64 ± 2 years (range, 30-90 years).

This study was approved by the ethical committee of the University of Bern.

Assessment of Behavior

Medical charts were available for 57 (44.5%) of 128 patients. Of these 57 patients, 45 (79%) died of the disease, and 6 (11%) were alive with recurrent or metastatic disease. An additional 6 patients (11%) were alive without disease. The median follow-up was 11.5 months (mean ± SD, 21.05 ± 4.9 months; range, 0-108 months).

Construction of the TMA

The TMA was built as previously described.[26] Briefly, 1 core tissue biopsy specimen with a diameter of 0.6 mm was taken from a representative region of individual paraffin-embedded cholangiocarcinomas (donor blocks) and placed into a new recipient paraffin block using a semiautomated tissue arraying device. The presence of tumor tissue on the TMA was verified on an H&E-stained slide. For biomarker analysis, 2 or 3 tissue cores of each tumor were available. Immunohistochemical examination used 5-µm sections cut by using an adhesive-coated slide system (Instrumedics, Hackensack, NJ). The number of samples varied slightly between individual markers because of variability in the number of interpretable specimens on TMA sections.

Immunohistochemical Staining

The TMA sections were used for immunohistochemical staining with 7 different antibodies. Standard indirect immunoperoxidase procedures (ABC-Elite, Vector Lab, Burlingame, CA) were used for the detection of the secondary antibodies. The primary antibodies and their dilutions are listed in **Table 1**. Diaminobenzidine was used as a chromogen. The primary antibody was omitted for negative control experiments. For a positive control experiment, a TMA with various normal tissue samples was stained in parallel. Nuclear immunoreactivity for p53, p16, p21, and p27 was visually scored. A case was considered positive if immunoreactivity was detected in at least 10% of the tumor cell nuclei, on the basis of previous reports.[10] Only for p27, the cutoff was 50% of positive tumor cell nuclei.[8] For bcl-2 and bax, staining in more than 10% of tumor cells within a tissue spot was required to define positivity.[10,24] For COX-2, tumor samples were considered positive when more than 20% of the cells were immunoreactive.[22]

Statistics

The χ^2 test was used to study the relationship between the expression of different markers and histologic subgroups. Kaplan-Meier survival curves were determined for each protein, and the log-rank test was used to assess differences in survival time. A Cox proportional hazards regression analysis was performed in a multivariate setting to assess the independent prognostic effect of p16. The hazard ratio and 95% confidence interval were obtained, in addition to *P* values. The levels of statistical significance were set at least at a *P* value of less than .05 (2-sided).

Table 1
Antibodies Used for Immunohistochemical Analysis

Protein (Antibody)	Source	Dilution
COX-2	DAKO, Glostrup, Denmark	1:25
p53	Novocastra, Newcastle-on-Tyne, England	1:200
bcl-2	DAKO	1:200
bax	NeoMarkers, Fremont, CA	1:100
p16	MTM, Heidelberg, Germany	1:400
p21	DAKO	1:25
p27	DAKO	1:200

Results

The immunohistochemical findings are summarized in **Table 2** and **Table 3**. Representative IHC stainings are shown in **Image 1** and **Image 2**.

Cholangiocarcinomas vs Normal Control Samples

In comparison with normal tissue (29 specimens), cholangiocarcinoma cases expressed COX-2, p53, bcl-2, and bax significantly more frequently. In more detail, the majority of the tumors (84.0%) were found to be COX-2+, whereas only 12% of the normal tissues (3/25) exhibited a positive reaction ($P < .005$). Positive p53 nuclear staining was observed in 23.5% of the tumors, whereas no nuclear staining was noted in the normal bile ducts ($P = .0225$). Regarding bcl-2 and bax immunoreactivity, 23.6% and 41.3% of the cholangiocarcinomas, respectively, showed a positive cytoplasmic reaction. Normal bile ducts remained consistently immunonegative for bcl-2 ($P = .0409$), whereas only 5% of the control cases showed a positive reaction for bax ($P = .0037$).

Differences in the Immunophenotype Between Intrahepatic and Extrahepatic Tumors

Distinctive immunophenotypes were observed regarding localization of the neoplasms. Intrahepatic tumors more frequently expressed bcl-2 and p16 (ie, 15/35 [43%] and 15/33 [45%], respectively) than did extrahepatic cholangiocarcinomas, in which bcl-2 and p16 immunostaining was noted in 10 (14%) of 73 and 12 (18%) of 66 cases, respectively ($P = .0036$ and $P = .0133$). In contrast, p53 immunoreactivity was restricted to extrahepatic tumors, being found in 20 (32%) of 62 extrahepatic cholangiocarcinomas and 4 (31%) of 13 gallbladder carcinomas, whereas no p53 nuclear staining (0%) was observed in their intrahepatic counterparts ($P = .0035$).

Prognostic Significance

Regarding survival, patients with p16+ tumors survived significantly longer than patients with p16– tumors ($P < .001$) **Figure 1**, and loss of p16 expression was associated with reduced survival. The expression of the other markers (COX-2, p53, bcl-2, bax, p21, and p27) did not show any correlation with the survival. In multivariate analysis that included patient age, tumor grade, tumor diameter, and localization, p16 expression was found to have independent prognostic value ($P < .001$; hazard ratio, 0.16; 95% confidence interval, 0.06-0.5). Information on T and N stage was available only for 12 and 10 patients, respectively, and, therefore, the value of p16 adjusting for these factors could not be determined.

Table 2
Immunohistochemical Findings in Cholangiocarcinomas in Comparison With Normal Tissue*

Positive Markers	Tumor	Normal	*P*
COX-2	89/106 (84.0)	3/25 (12)	.005
p53	24/102 (23.5)	0/24 (0)	.0225
bcl-2	29/123 (23.6)	0/27 (0)	.0409
bax	43/104 (41.3)	1/20 (5)	.0037
p16	29/115 (25.2)	1/20 (5)	.0754
p21	21/98 (21)	1/20 (5)	.1877
p27	9/91 (10)	0/24 (0)	.3494

* Data are given as number/total (percentage).

Table 3
Immunohistochemical Findings in Cholangiocarcinomas in Association With Localization*

Positive Markers	Intrahepatic	Extrahepatic	Gallbladder	*P*
COX-2	26/34 (76)	50/58 (86)	13/14 (93)	.6144
p53	0/27 (0)	20/62 (32)	4/13 (31)	.0035
bcl-2	15/35 (43)	10/73 (14)	4/15 (27)	.0036
bax	18/32 (56)	21/60 (35)	4/12 (33)	.2873
p16	15/33 (45)	12/66 (18)	2/16 (13)	.0133
p21	7/29 (24)	10/58 (17)	4/11 (36)	.3349
p27	3/27 (11)	4/52 (8)	2/12 (17)	.6233

* Data are given as number/total (percentage).

Discussion

Cholangiocarcinoma is a devastating cancer with presently no effective treatment. The primary aim of this study was to examine cholangiocarcinomas from different anatomic sites to identify similarities and differences in the expression of diagnostic and prognostic factors that would eventually provide a guide for developing novel therapeutic strategies for this lethal disease.

A major finding in this regard was the differential expression of the p16 protein, which was significantly more frequently present in intrahepatic cholangiocarcinomas than in their extrahepatic counterparts ($P = .0133$; Table 3). It is interesting that patients with p16+ tumors survived significantly longer than did patients with p16– tumors ($P = .0031$), and loss of p16 expression was associated with a poor outcome in our series of cholangiocarcinomas. This finding shows that the anatomic site of the tumor may have a role in survival differences in patients with cholangiocarcinoma. Previous studies have shown that inactivation of the tumor suppressor gene *p16,* most commonly by promoter methylation, is a frequent event in cholangiocarcinomas but failed to establish p16 as a prognostic factor in their cases.[9,27,28]

Further site-related differences found in the present study include the overexpression of bcl-2 by intrahepatic tumors ($P = .0036$) and p53 expression exclusively by the extrahepatic and gallbladder carcinomas ($P = .0035$). Our findings are partly in agreement with those of Jarnagin et al,[6] who also reported site-related differences in cholangiocarcinomas, with p27 and bcl-2 more frequently expressed by intrahepatic

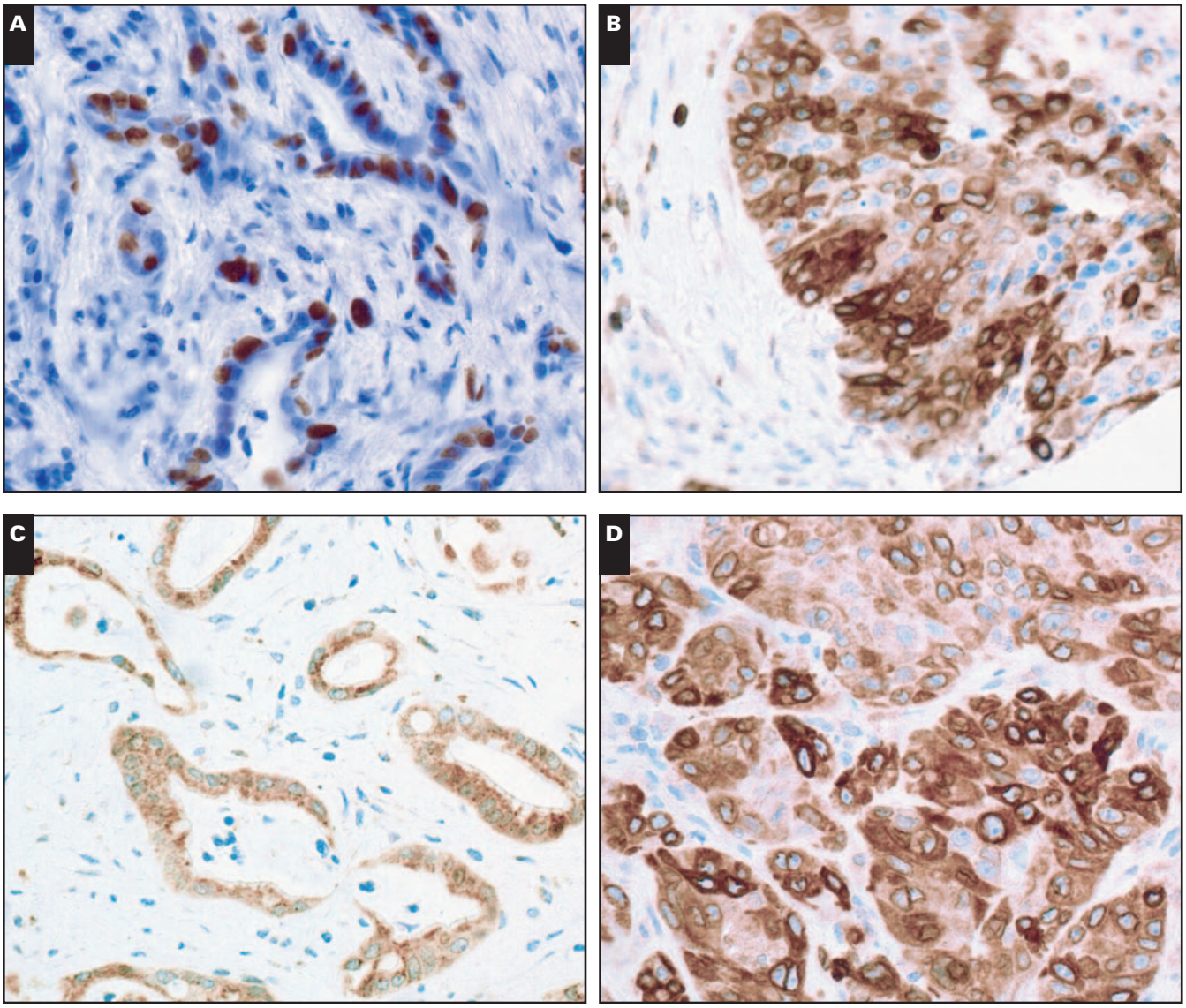

Image 1 Immunohistochemical detection of Ki-67 (**A**), p53 (**B**), bcl-2 (**C**), and bax (**D**) in cholangiocarcinoma. Typical examples are shown (**A**-**D**, ×400).

tumors and p53 more frequently found in extrahepatic tumors. In their analysis, cases of cholangiocarcinoma with low or absent p27 expression were associated with poor survival compared with the high expression group. In our study, p27 positivity was found in roughly 10% of the tumors, showing no association with localization or with survival of patients.

Other studies[6,11,29] have also stressed the fact that the frequency of p53 overexpression is significantly higher in extrahepatic compared with intrahepatic bile duct cancers. Although *p53* mutations have been reported in cholangiocarcinomas, they have been detected in only 5% to 35% of intrahepatic cholangiocarcinomas.[13,15,30] Our findings suggest that inactivation and/or accumulation of inactive p53 in intrahepatic cholangiocarcinomas may not occur to a level sufficient for the immunohistochemical detection of the protein.

In the present study, expression of COX-2 exhibited a strong association with malignant phenotype—almost 84% of cholangiocarcinomas but only 12% of normal bile ducts were found to be positive ($P < .05$), thus suggesting a pathogenetic role of this protein in the carcinogenesis of cholangiocarcinoma. However, no association between COX-2 expression and survival of patients was documented in our cases. Kim and coworkers[22] reported no association between COX-2 expression and clinicopathologic features, including survival, in their series of cholangiocarcinomas, whereas Schmitz and coworkers[31] found that patients with cholangiocarcinoma with strong COX-2 expression had significantly decreased overall survival. The results regarding prognostic significance of COX-2 in other tumors seem also to be contradictory. Although in many tumor types COX-2 has been reported to

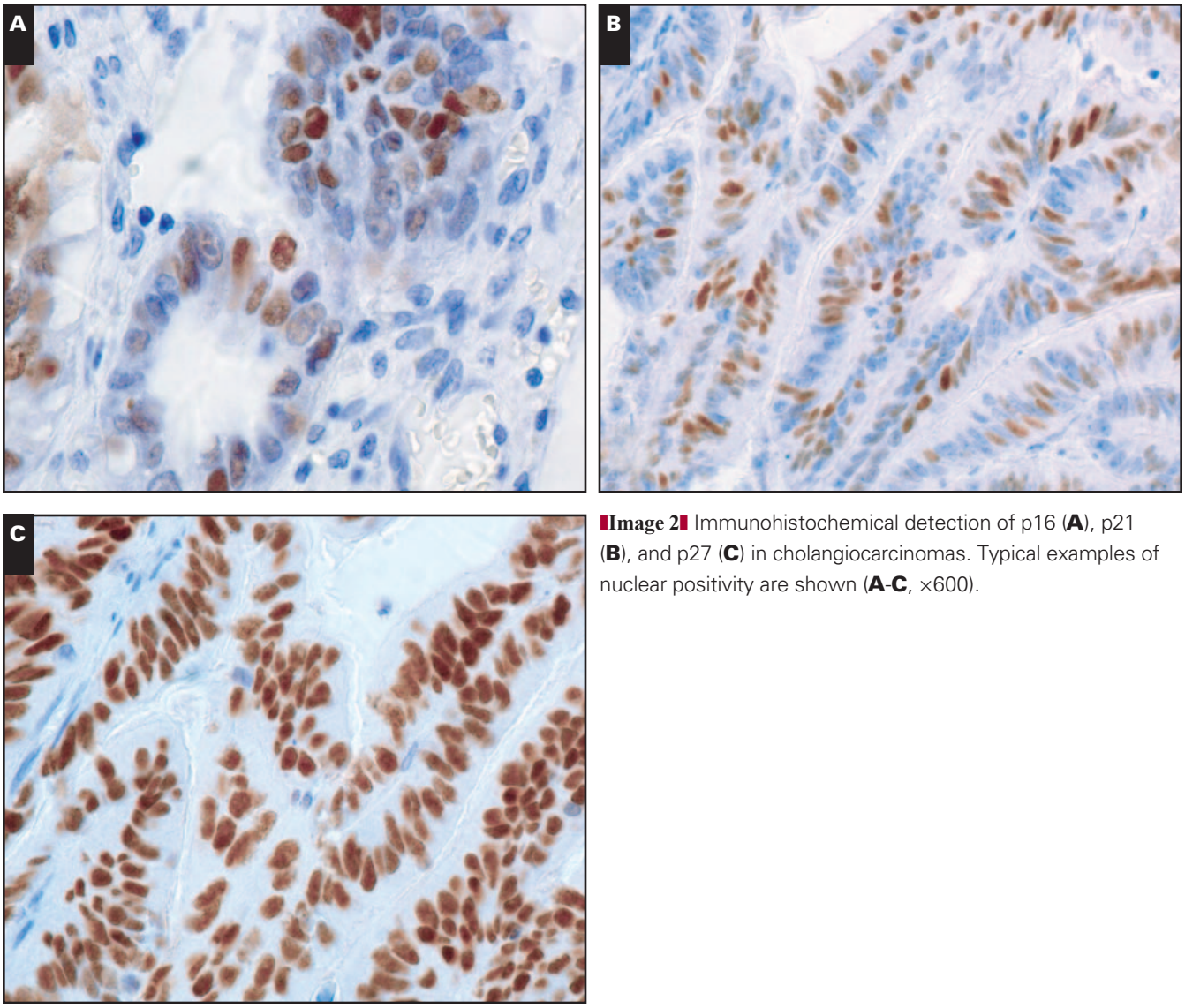

Image 2 Immunohistochemical detection of p16 (**A**), p21 (**B**), and p27 (**C**) in cholangiocarcinomas. Typical examples of nuclear positivity are shown (**A**-**C**, ×600).

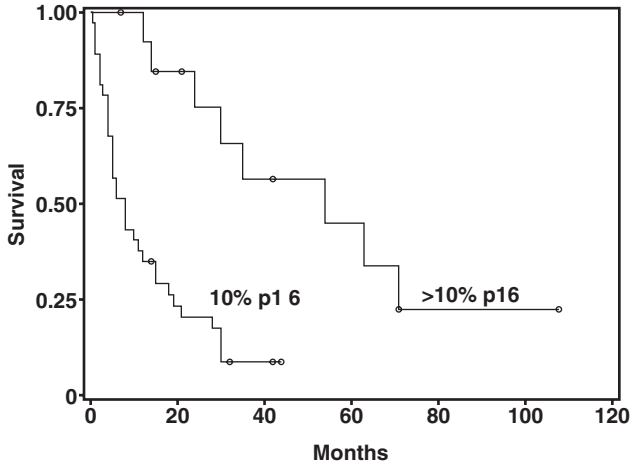

Figure 1 Kaplan-Meier survival curve for patients with positive (>10%) and negative (10%) p16 expression. $P < .001$.

be associated with a worse prognosis,[32,33] in others no such association was proven,[34] and still in others, an association with a favorable prognostic phenotype was shown.[35]

In a previous study, the expression of bcl-2 was inversely related to lymph node metastasis, vascular invasion, perineural invasion, the Ki-67 labeling index, aberrant p53 expression, and the incidence of apoptotic cells in cholangiocarcinomas.[24] In the present study, bcl-2 expression was found in 23.6% of cholangiocarcinomas exhibiting, as stated earlier, a strong association with the localization of the tumors. This finding, along with the absence of bcl-2 immunoreactivity in the normal bile ducts, suggests that bcl-2 is probably fulfilling an important role in the carcinogenesis of cholangiocarcinoma, especially the intrahepatic type. Immunoreactivity for bax was found in 38% of all cases of cholangiocarcinoma without any association with localization. Expression of neither bcl-2 nor bax correlated with survival of patients, suggesting that these proteins do not have prognostic significance in cholangiocarcinoma.

In the present study, the expression profiles of the gallbladder carcinomas were found to be similar to those of the extrahepatic cholangiocarcinomas (ie, increased p53 and very low p16 and bcl-2 expression). Our findings are in agreement with those of Parwani et al,[36] who reported that loss of p16 and abnormal accumulation of p53 were the most common abnormalities noted in invasive non–small cell carcinomas of the gallbladder. Moreover, this finding could suggest similarities in the pathogenesis between gallbladder carcinomas and extrahepatic cholangiocarcinomas. Indeed, in a recent study,[37] an increased incidence of dysplasia and adenocarcinoma of the gallbladder was found in patients with primary sclerosing cholangitis, a well-known risk factor for the development of extrahepatic and intrahepatic cholangiocarcinomas, whereas cholelithiasis has been implicated as a risk factor not only for gallbladder carcinomas, but also for the development of extrahepatic cholangiocarcinomas.[38]

In comparison with normal tissue, cholangiocarcinoma cases were found in the present study to be significantly more frequently p53–, COX-2–, bcl-2+, and bax+ ($P < .05$). This finding suggests that the aforementioned proteins have an important role in the pathogenesis of cholangiocarcinoma. Moreover, they may have diagnostic significance, being useful in the distinction between malignant and benign lesions in difficult cases.

A major finding of this study was the demonstration of a clearly distinctive immunoreactive pattern between intrahepatic and extrahepatic cholangiocarcinomas. The seemingly inverse immunophenotype between intrahepatic (absence of p53 and increased bcl-2 and p16 expression) and extrahepatic (increased p53 and very low bcl-2 and p16 expression) cholangiocarcinomas suggests different pathogenetic mechanisms and genetic profiles for these tumors. Moreover, it can be postulated that the differential expression of the aforementioned proteins can have a diagnostic importance, being helpful in the distinction between extrahepatic and intrahepatic cholangiocarcinomas in difficult cases.

In addition, our data show that the expression of COX-2, p53, bcl-2, and bax seems to have an important role in the pathogenesis of cholangiocarcinoma. Moreover, we demonstrated that the loss of p16 protein expression is linked to a worse prognosis in cholangiocarcinomas.

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