Low p27 Expression Is an Independent Predictor of Survival for Patients with Either Hilar or Peripheral Intrahepatic Cholangiocarcinoma

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ABSTRACT

Purpose and Experimental Design: The prognosis for intrahepatic cholangiocarcinoma (ICC) depends mainly on the feasibility of complete surgical resection. In the absence of demonstrated biological predictors of survival, we evaluated the prognostic value of the cyclin-dependent kinase inhibitor p27 by immunohistochemistry in a series of routine specimens from 47 ICC patients, 22 with the hilar and 25 with the peripheral subtype. Proliferation rate was also evaluated in the same cases by the MIB1 index. Tumors were scored as high, low, and negative p27 expressers (≥50%, <50%, and no positive nuclei, respectively).

Results: High, low, and negative p27 expression was recorded in 18 (38%), 17 (36%), and 12 (26%) cases, respectively. No significant correlation was found between p27 expression and gender, age, tumor grade, tumor location, vascular or perineural invasion, or proliferative index. Tumors with low or absent p27 expression were associated with poor survival compared with the high-expresser group. Kaplan-Meier curves comparing different p27 expression levels with survival showed highly significant separation (P = 0.0001, log-rank test). With univariate Cox proportional hazard regression, only p27 score among all of the parameters was found to influence survival (P = 0.0003).

Conclusion: We conclude that in ICC, low or absent p27 expression can predict poor survival, regardless of tumor location, pathological features, and tumor proliferation. Immunohistochemical detection of p27 on routine sections may provide the first biological prognostic marker for ICC, thus influencing the therapeutic strategies for these patients.

INTRODUCTION

The p27 cyclin-dependent kinase inhibitor Cdki is a negative cell-cycle regulator that belongs to the Cip/Kip group of cyclin-dependent kinase inhibitors and shares sequence homology with p21 and p57 (1). The p27 protein penetrates the nucleus, where it preferentially binds to cyclin A-E/Cdk2 complexes and inactivates them, thereby preventing the cell from entering the S-phase (2, 3). Expression of the p27 gene is posttranscriptionally regulated by proteolytic degradation via the ubiquitin-proteasome pathway (4). Although some effective point mutations have been described, the main control system for the function of p27 seems to be retention of the protein in the cytoplasm, where ubiquination takes place (4, 5).

Low or absent nuclear immunohistochemical expression of p27 is an important negative prognostic parameter for many human epithelial tumors in the breast, colon, stomach, prostate, lung, liver, and elsewhere (6–20). Regarding tumors of biliary origin, a recent study revealed the existence of an inverse correlation between p27 and cyclin D1 expression that can significantly predict survival in patients with resectable carcinoma of the extrahepatic bile ducts (21). Moreover, loss of p27 expression in gallbladder carcinoma patients has been associated with poor prognosis and tumor progression (22).

ICCs are commonly classified as PCs (arising form small bile ducts) and HCs (originating from the large bile ducts of the hepatic hilus), also known as Klatskin tumors (23, 24). The prognosis for ICC is generally poor, with long-term as well as short-term survival almost exclusively depending on the feasibility of surgical resection (25). None of the many proposed biological prognostic parameters, such as p53 mutations, c-erbB-2, c-met, and Muc1 expression, has proven reliable enough to be systematically adopted in clinical practice (26–30). It should also be noted that many studies have lumped ICCs together with distal extra-hepatic biliary tumors and carcinoma of the gallbladder, thus causing confusion in a field where tumor location is itself a prognostic parameter.

In the present study we analyzed the expression of p27 by IHC in 47 human resected ICC specimens (25 PC and 22 HC) and in the adjacent nonneoplastic tissues. Expression of p27 was analyzed with respect to the main pathological prognostic variables of ICC, proliferation index and overall survival.

MATERIALS AND METHODS

Patient Population. We performed a retrospective study on 47 patients (22 men and 25 women; mean age, 60.7 years)
who underwent liver resection for ICC between 1984 and 1998 in our hospital. Pathological, demographic, and survival data of the patients were retrieved from the files of the Pathology Unit at the “Addaari” Institute of Oncology and the Department of Surgery at the University of Bologna. Twenty-two tumors were HC, whereas 25 were PC. All tumors were histologically diagnosed, graded, and grouped as moderately differentiated (grade 2; \( n = 29 \)) or poorly differentiated (grade 3; \( n = 18 \)). Tumors were also grouped according to the presence (\( n = 15 \)) or absence (\( n = 32 \)) of neoplastic vascular thrombosis and the presence (\( n = 16 \)) or absence (\( n = 31 \)) of neoplastic perineural invasion (Table 1). The follow-up interval ranged from 1 to 60 months from the date of surgery (mean, 12.7 months). None of the patients received postoperative adjuvant therapy. After surgical resection, specimens from neoplastic and surrounding non-neoplastic tissue were fixed in 10% buffered formalin. Informed consent was obtained for all patients.

**IHC.** Four-\( \mu \)m tissue sections were cut, placed on silane-pretreated slides, deparaffinized, and rehydrated through graded alcohols. Antigen retrieval was performed by microwave heating at high power (750 W) in 10 mM sodium citrate buffer (pH 6) for four cycles of 5 min each. Slides were then allowed to cool for 30 min prior to incubation with either the p27 monoclonal antibody (Transduction Laboratories, Lexington, KY) diluted 1:200 or the anti-Ki67 monoclonal antibody (clone MIB1; Biogenex, San Ramon, CA) diluted 1:50 for 1 h at room temperature. Staining was performed with the Envision Monoclonal System (Dako, Carpinteria, CA). The reaction was developed with DAB. Slides were counterstained with Mayer’s hematoxylin and mounted. Nonimmune mouse serum was used as negative control. Lymphocytes were used as the internal positive control for p27. Identical reaction times allowed accurate comparison of all samples. Immunohistochemical staining was evaluated independently by two pathologists (M. F. and A. D.).

**IHC for p27** was scored as the ratio of nuclei displaying clear-cut positivity with respect to the total number of cells, as described previously (20). Tumors were categorized into three groups: high p27 expressers (\( \geq 50\% \) positive nuclei; score 2), low p27 expressers (<50% positive nuclei; score 1), and p27-negative tumors; score 0). The proliferation index was calculated as the number of MIB1-positive nuclei over the total number of neoplastic cells, and tumors were scored as low proliferating (<10% positive neoplastic cells) or high proliferating (\( \geq 10\% \) positive neoplastic cells; Table 1). At least 20 high-power fields were randomly chosen, and 2000 cells were always counted.

**Statistical Analysis.** Associations between the p27 score and other clinicopathological variables were assessed by Pearson’s \( \chi^2 \) test (31). Survival times were calculated from the date of initial diagnosis. Associations between the relative risk of case fatality and the various categories of single prognostic variables were evaluated according to Cox’s proportional hazard regression model for censored data after dummy code transformation of the predictor (32). Survival curves were computed using the Kaplan-Meier product-limit method and compared using the log-rank test (33, 34). Analyses were performed using StatView 5.0 statistical software (SAS Institute, Cary, NC).

### RESULTS

**p27 Protein Expression in ICC and Adjacent Liver Tissues.** In normal liver tissue adjacent to the ICC, the p27 antibody stained inflammatory cells and the mature bile duct cells of both large and small intrahepatic ducts, whereas hepatocytes were only occasionally positive. The location of the p27 protein was nuclear in all of the mature bile ducts.

The 47 ICC samples showed a heterogeneous pattern of positivity for p27 in terms of the percentage of cells displaying clear-cut nuclear staining. In 12 of 47 (26%) ICC samples, p27 expression was absent (score 0). Seventeen (36%) were low p27 expressers (score 1; Fig. 1, B and E), and 18 (38%) were high expressers (score 2; Fig. 1, C and F). Among the high p27 expressers, 10 were grade 2 and 8 were grade 3 ICCs. Among the low expressers, 12 were grade 2 and 5 were grade 3 (Table 1). According to tumor location, 9

### Table 1  p27 expression and clinicopathological variables in 47 cholangiocarcinoma specimens

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total</th>
<th>High</th>
<th>Low</th>
<th>Absent</th>
<th>( P^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( G_2 )</td>
<td>29</td>
<td>10</td>
<td>12</td>
<td>7</td>
<td>( \chi^2 = 0.91; P = 0.633 )</td>
</tr>
<tr>
<td>( G_3 )</td>
<td>18</td>
<td>8</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Vascular invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>32</td>
<td>12</td>
<td>12</td>
<td>8</td>
<td>( \chi^2 = 0.08; P = 0.962 )</td>
</tr>
<tr>
<td>Present</td>
<td>15</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Perineural invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>31</td>
<td>12</td>
<td>11</td>
<td>8</td>
<td>( \chi^2 = 0.02; P = 0.99 )</td>
</tr>
<tr>
<td>Present</td>
<td>16</td>
<td>6</td>
<td>6</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Proliferation (MIB1) ( ^b )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>39</td>
<td>13</td>
<td>15</td>
<td>11</td>
<td>( \chi^2 = 2.48; P = 0.289 )</td>
</tr>
<tr>
<td>High</td>
<td>6</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Central</td>
<td>22</td>
<td>6</td>
<td>7</td>
<td>9</td>
<td>( \chi^2 = 5.36; P = 0.068 )</td>
</tr>
<tr>
<td>Peripheral</td>
<td>25</td>
<td>12</td>
<td>10</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \) Pearson \( \chi^2 \).

\( ^b \) Only 45 cases were stained for MIB1 IHC.
of 22 HCs (41%) had score 0, 7 had score 1 (32%), and 6 (27%) had score 2, whereas among the 25 PCs, 3 (12%) had score 0, 10 (40%) had score 1, and 12 (48%) had score 2 (Table 1).

Expression levels of p27 did not significantly correlate with gender, age, tumor grade, tumor location, and vascular or perineural invasion (Table 1).

**p27 Expression Does Not Correlate with the MIB1 Proliferation Index.** The MIB1 proliferation index was assessed in 45 patients. Thirty-nine (87%) ICCs (20 PCs and 19 HCs) displayed a low MIB1 index, whereas the remaining 6 (13%; 4 PCs and 2 HCs) showed a high MIB1 index (≥10% of the neoplastic cells; Table 1). \( \chi^2 \) analysis showed no association between p27 expression and MIB1 index. Likewise, proportional hazard regression analysis showed no association between the MIB1 index and overall survival (Table 2).

**Only p27 Is Predictive of Survival.** Survival data were available for 47 patients. The observation period ranged from 1 to 60 months, with a median survival of 20 months (confidence limits, 12 and 42 months) and a median time to censoring of 14 months (confidence limits, 10 and 20 months). At the most recent follow-up, 29 patients were alive and 18 had died. Kaplan-Meier survival curves did not show any significant relationship between tumor grade, tumor location, vascular or perineural invasion and survival \( (P > 0.35 \text{ for all by log-rank test}) \).

Only 2 of 18 (11%) patients in the high-expressor group died of disease, compared with 11 of 12 (92%) in the low-expressor group (Table 2). The Kaplan-Meier survival curves comparing high, low, and no expressers of p27 showed a highly significant separation \( (P < 0.0001; \text{Fig. 2}) \). As can be seen from Table 2, when the variables were separately compared using univariate Cox proportional hazard regression, only p27 was found to influence survival \( (P = 0.0003) \), with absent p27 expression carrying a 17-fold higher risk of mortality than high p27 expression. Because only p27 (of all of the examined variables) correlated with outcome, regression analysis was not explored further.

**DISCUSSION**

ICC is a highly aggressive tumor with a generally poor prognosis. The possibility of long-term survival depends on the feasibility of performing surgical resection. Although alterations in various oncogenes and tumor suppressor genes, such as K-ras, c-erb-b2, and p53, have been reported, none of them have
been introduced as prognostic markers in routine clinical practice (25). In other biliary tumor types, decreased expression of the p27 cell-cycle inhibitor has been found to have prognostic significance (21, 22). Furthermore, the high levels of p27 protein that were found to be present in the nonneoplastic ductal cells of the extrahepatic biliary tree progressively fell off in preneoplastic dysplastic lesions and cancer (22).

Our data demonstrate that, as in other epithelial tumor types, ICC patients bearing tumors that express high levels of p27 fare better than those with tumors that lack p27 expression, independent of tumor grade or subtype. Although high p27 expression was less frequent in the HC than in the PC subtype, this difference was not statistically significant. No association was found between p27 expression and tumor grade. These findings underscore the concept that p27 expression is a specific biological trait that characterizes individual tumors independently of their morphological appearance. As in the study on extrahepatic cholangiocarcinomas by Hui et al. (22), we found that all of the nonneoplastic mature biliary structures always expressed p27 and that loss of expression was invariably associated with severe dysplasia or cancer. This finding could be helpful for differentiation between normal and dysplastic/neoplastic biliary structures in histological or cytological samples, particularly in the HC subtype where extremely well-differentiated neoplastic ducts are often intermingled with mature nonneoplastic ones. However, the advanced stage of our cases at the time of diagnosis prevented us from providing more information on p27 expression during the development of ICC.

Regarding proliferation, a high MIB1 index has been associated with worse outcome in both ICC and extrahepatic cholangiocarcinoma (26, 35). Moreover, in cases of extrahepatic cholangiocarcinoma, the cyclin D1 index inversely correlated with p27 expression, and the cyclin D1/p27 ratio was predictive of poor survival (21). Nevertheless, in our series of ICCs, the MIB1 index did not correlate with either p27 expression or survival. In contrast to other studies on bile duct cancers, only a minority of our ICC samples (13%) showed a high MIB1 index (35). This finding need not be attributed to technical artifacts because lymphocyte aggregates within the tumors provided reliable internal controls. The generally low growth fraction of the ICCs might be related to the fact that they were already bulky and in an advanced stage at the time of surgery.

**Table 2** Proportional hazard regression analysis of single predictors of survival

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>β coefficient (SE)</th>
<th>Test</th>
<th>OR&lt;sup&gt;b,c&lt;/sup&gt; survival (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>47</td>
<td>0.024 (0.024)</td>
<td>χ² = 1.03; P = 0.309</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>22</td>
<td>-0.002 (0.475)</td>
<td>χ² = 0.00; P = 0.99</td>
<td>1.0 (0.39–2.53)</td>
</tr>
<tr>
<td>F</td>
<td>25</td>
<td>0.488 (0.758)</td>
<td>χ² = 0.57; P = 0.449</td>
<td>1.45 (0.56–3.77)</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G&lt;sub&gt;2&lt;/sub&gt;</td>
<td>29</td>
<td>0.384 (0.475)</td>
<td>χ² = 0.65; P = 0.419</td>
<td>1.47 (0.58–3.72)</td>
</tr>
<tr>
<td>G&lt;sub&gt;0&lt;/sub&gt;</td>
<td>18</td>
<td>0.422 (0.476)</td>
<td>χ² = 0.79; P = 0.375</td>
<td>1.52 (0.6–3.87)</td>
</tr>
<tr>
<td>Vascular invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>32</td>
<td>0.414 (0.477)</td>
<td>χ² = 0.75; P = 0.386</td>
<td>1.51 (0.59–3.85)</td>
</tr>
<tr>
<td>Present</td>
<td>15</td>
<td>0.051 (0.643)</td>
<td>χ² = 0.06; P = 0.937</td>
<td>1.05 (0.3–3.71)</td>
</tr>
<tr>
<td>Perineural invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>31</td>
<td>0.418 (0.477)</td>
<td>χ² = 0.75; P = 0.386</td>
<td>1.51 (0.59–3.85)</td>
</tr>
<tr>
<td>Present</td>
<td>16</td>
<td>2.84 (0.79)</td>
<td>χ² = 2.91; P = 0.087</td>
<td>4.22 (0.81–22.1)</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral</td>
<td>25</td>
<td>0.449 (1.45)</td>
<td>χ² = 12.89; P = 0.0003</td>
<td>17.07 (3.63–80.35)</td>
</tr>
<tr>
<td>Central</td>
<td>22</td>
<td>0.123 (0.12)</td>
<td>χ² = 13.92; P = 0.0001</td>
<td>5.03 (1.59–15.56)</td>
</tr>
<tr>
<td>Proliferation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>41</td>
<td>0.372 (0.375)</td>
<td>χ² = 0.79; P = 0.375</td>
<td>1.52 (0.6–3.87)</td>
</tr>
<tr>
<td>High</td>
<td>6</td>
<td>0.917 (0.937)</td>
<td>χ² = 2.91; P = 0.087</td>
<td>4.22 (0.81–22.1)</td>
</tr>
<tr>
<td>p27</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>18</td>
<td>0.419 (0.429)</td>
<td>χ² = 0.75; P = 0.386</td>
<td>1.51 (0.59–3.85)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>17</td>
<td>0.418 (0.477)</td>
<td>χ² = 0.75; P = 0.386</td>
<td>1.51 (0.59–3.85)</td>
</tr>
<tr>
<td>Negative</td>
<td>12</td>
<td>0.449 (1.45)</td>
<td>χ² = 12.89; P = 0.0003</td>
<td>17.07 (3.63–80.35)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Wald test for the regression coefficient.

<sup>b</sup> OR, odds ratio; CI, confidence interval.

<sup>c</sup> OR = e<sup>b</sup>; odds ratios are given only for categorical variables.

Fig. 2  Kaplan-Meier estimated survival rates according to p27 expression (log-rank test, χ² = 23.86; df, 2; P < 0.0001). The median survival was 6 months in the absent group, 42 months in the low group, and had not been reached at the time of publication in the high group. Censored observations are marked by small vertical bars. Cum., cumulative.
In our series of 47 ICCs, proportional hazard regression analysis (Table 2) showed that p27 expression was the only significant predictor of survival (P = 0.0003). In keeping with the concept that pathological parameters do not have prognostic value in ICCs, vascular or perineural invasion and tumor grade all failed to correlate with outcome. These observations are in line with studies on extrahepatic biliary tumors, where p27 was predictive of survival and recurrence (21, 22).

In conclusion, as with extrahepatic bile duct tumors, immunohistochemical detection of p27 should provide the first biological predictor of survival for ICC patients. Low p27 expression is also a better prognostic indicator than MIB1 index because the former predicts poor survival regardless of tumor grade and stage. The highest 5-year survival rates for ICC patients are achieved when complete tumor resection with negative margins can be obtained (25, 36–39). Thus, assessment of p27 expression could help identify patients that might benefit from particularly aggressive surgical strategies, and it could also be useful for planning adjuvant therapies during follow-up.

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REFERENCES


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