Altered Expression of Cell Cycle and Apoptotic Proteins in Human Liver Pathologies

ANTIGONY MITSELOU1, DAMIANOS KARAPIPERIDES2, IOANNIS NESSERIS3, THEODOROS VOUGIOUKLAKIS1 and NIKI J. AGNANTIS3

Departments of 1Forensic Pathology and 2Pathology, University School of Medicine, Ioannina, Greece; 3Department of Surgery, Agios Dimitrios Hospital, Thessaloniki, Greece

Abstract. The expression of cell cycle (P53, Ki-67, P21, and P27) and apoptotic proteins (BCL-2 and BAX) was investigated by immunohistochemistry in paraffin-embedded formalin-fixed tissues of normal and pathologic liver. An increased frequency of expression of P21 in cirrhosis and hepatocellular carcinoma (HCC) (p=0.003 and p=0.001 respectively) was found; P27 protein expression was more frequent in hepatitis (p=0.001) and HCC (p=0.003) when compared with normal tissue. BCL-2 protein was markedly more frequent in steatohepatitis (p<0.05) as compared to normal liver, in hepatitis cases (p=0.002) and in metastases (p<0.033). The expression of BAX was more frequent in hepatitis (p=0.001) and cirrhosis (p<0.001). We demonstrated in our study the expression of these proteins at different levels in liver pathologies. These findings have implications for understanding the evolution from liver inflammation to cirrhosis and associated carcinogenesis.

Hepatocyte proliferation is a well-described adaptive response to acute liver injuries, such as those resulting from viruses, toxins, and partial hepatectomy. Hepatocyte proliferation is also thought to play an important role in maintaining hepatic function in chronic liver diseases (1). In many progressive chronic liver diseases, ongoing liver injury leads to apoptosis and necrosis of hepatocytes, resulting in impaired hepatic function and associated morbidity (2). Hepatocyte proliferation is thought to offset the loss of hepatocytes in chronic liver diseases, such as viral hepatitis, but may also contribute to the development of hepatocellular cancer (3). Diminished hepatocyte proliferation has been associated with a poor prognosis in liver diseases (4).

Progression through the cell cycle is controlled by the activity of cyclin-dependent kinases (CDKs), which in turn is regulated by cyclins, CDK-inhibitory proteins (CDKIs), and changes in the phosphorylation status of CDKs (5). CDK2 activation plays an essential role in the transition of cells into the S-phase and in DNA synthesis (6). The activity of these kinases is influenced by numerous mitogenic and antiproliferative signals. The best-characterized cyclin partner of CDK4 is cyclin D1. This protein is induced during progression through the G1-phase, which is thought to be a key growth factor-dependent step during proliferation (7). CDK2 is associated with the E- and A-type cyclins during the G1-S transition and S-phase, respectively. In addition, binding to cyclins, full activation of the CDKs requires phosphorylation by the CDK-activating kinase (CAK), which is constitutively expressed in many cells (8).

The activity of cyclin/CDK complexes is negatively regulated by the CDKIs, which are grouped into two structurally related families (9). The INK4 family (P15, P16, P18, and P19) inhibits CDK4 and CDK6, while the Cip/Kip family (P21, P27, and P57) inhibits numerous CDKs. Of these, P21 and P27 have been the most extensively studied. P21 acts downstream of numerous signaling pathways and can be induced by p53, transforming growth factor β (TGFβ), and other antmitogenic stimuli (10). In tissue culture systems, P21 is up-regulated in proliferating cells and appears to play a role in governing progression through the cell cycle (11). In addition, P21 is induced in some cell types during senescence and terminal differentiation, and it is thought to play a key role in down-regulating CDK activity in these settings. Finally, at low stoichiometric concentrations, P21 may serve as an assembly factor for active cyclin/CDK complexes (12).

P27 is constitutively expressed in many cells, and the level of this protein is elevated in the G0 phase and declines as cells in culture enter the cell cycle. P27 may play an important role in governing the growth factor restriction point by ensuring that CDK activity is suppressed during G0- and early G1-phases (13). P27 appears to play an important role in regulating cell proliferation during development.
The *p53* gene was the first tumor-suppressor gene to be identified and has been found to be inactivated in most types of human cancer (14). *P53* protein prevents division of stressed cells or causes them to undergo programmed cell death (apoptosis) (15). The stresses that activate *P53* are diverse, ranging from DNA damage to oxidative stress, hypoxia, and heat shock. Thus, the *P53* protein is thought to be a guardian against cellular stresses, and has been extensively studied and is well established as a tumor suppressor. However, much less is known about other roles of *P53* beyond tumor suppression. Notably recent reports document that *P53* is involved in the molecular mechanisms of some types of hepatocellular injury (16, 17).

Ki-67 antigen can be detected reliably by the monoclonal antibody MIB-1 in paraffin wax-embedded sections that reacts with a nuclear protein expressed in the G1-, G2-, S- and M-phases of the cell cycle, which is absent from resting cells, and is useful in detecting differences in proliferative activity among different pathologies (3).

Apoptosis is a genetically programmed form of cell death that plays a major role in development and tissue homeostasis in addition to pathological processes. In the intrinsic pathway of apoptosis, BCL-2 family proteins play a pivotal role in modulating membrane integrity and the release of apoptogenic factors from mitochondria. Anti-apoptotic BCL-2 and BCL-XL localize predominantly at the mitochondria and inhibit apoptosis. BAX, a pro-apoptotic protein, resides in the cytoplasm and stimulates cell death after translocation to mitochondria. The balance between anti-apoptotic and pro-apoptotic protein expression determines the susceptibility of the cell to apoptogenic stimuli (18). As in other cell types, apoptosis normally occurs in liver cells during development and in renewal of hepatocytes in the adult liver. However, programmed cell death can also be triggered in various viral, immunological, malignant or drug-induced liver diseases (19).

In the present study, we aimed to evaluate the expression of cell cycle and apoptotic proteins in normal liver tissue and various pathologies, including hepatitis, steatohepatitis, cirrhosis, hepatocellular carcinoma (HCC), and metastasis in liver.

### Patients and Methods

A total of 116 cases were collected from the Departments of Forensic Pathology and Pathology of Ioannina Medical School. Autopsy samples of 71 individuals were taken within 5-6 hours postmortem and 45 percutaneous liver biopsies were retrieved from the files of the Pathology Department. The cases were selected to represent a spectrum of liver pathologies. There were 59 (50.86%) men and 57 (49.14%) women with a mean age of 55.7 years, ranging from 33 to 82 years. This study included 50 cases of normal liver tissue, 6 cases of steatosis; 12 cases of steatohepatitis; 6 cases of hepatitis B; 18 cases of cirrhosis; 9 of well-differentiated HCC and 15 cases of metastasis in liver. No patients received adjuvant chemotherapy or radiotherapy at the time of diagnosis. Tissues were routinely fixed immediately in 4%-buffer formalin and embedded in paraffin. The tissue blocks were subjected to repeat sectioning and routinely stained with hematoxylin-eosin, Masson’s trichrome for collagen fibers, reticulin for reticulin fibers, Orcein for elastic fibers, Perl’s reaction for iron, Alcian blue and periodic acid-Schiff before and after diastase digestion for mucin. The diagnosis of the liver pathologies was based on established histological criteria.

### Immunohistochemistry

The standard streptavidin-biotin immunoperoxidase method (Dakopatts, Glostrup, Denmark) was used for immunostaining with *P53* (Clone DO-7, from Dako), *P21W AF/Cip1* (Clone 4D10, Dako), *P27Kip1* (Clone 1B4, Dako), *Ki-67* (MIB-1, Dako), *BCL-2* (Clone 124, Dako), and BAX (Ig fraction, Dako). The slides were cut into 4 μm-thick sections, plassed on poly-L-lysine-coated glass slides. In brief, sections were deparaffinized and dehydrated used a xylene alcohol sequential wash. Following this, all sections were subjected to antigen retrieval by heating 0.01 M citrate buffer (pH 6.0) in a pressure cooker. Subsequently, all sections were treated for 30 min with 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. After blocking with 10% goat serum for two hours, the sections were incubated with primary antibodies at 4°C. Antibodies directed against *P21*, *P27*, *P53*, *Ki-67*, *BCL-2*, and BAX were applied. Linking antibody and streptavidin peroxidase complex were added consecutively for 10 minutes at room temperature and washed in Trisbuffer. Peroxidase activity was visualized with 0.03% 3,3-diaminobenzidine tetrahydrochloride (Dakopatts, Glostrup, Denmark) applied for 5 minutes. The sections were then washed and finally, the slides were counterstained with Harri’s hematoxylin, dehydrated and mounted for examination. To assess the specificity of the reaction, in negative controls were included, where tissues sections were not incubated with the primary antibodies. The sources of primary antibodies and dilutions as well as the retrieval method used in this study are indicated in Table I.

### Quantitative analysis

The immunostained sections were examined using an Olympus microscope. Evaluations of *P21*, *P27*, *P53*, and *Ki-67* were based on nuclear staining. Cytoplasmic or membranous staining assessed evaluation of *BCL-2* and *BAX* proteins. Quantification of positively stained hepatocytes was undertaken by two independent observers (A.M. and J.N.), by counting 1,000 hepatocytes at a magnification of ×400 in five to ten randomly selected fields. The positively stained hepatocytes were expressed as a percentage of the total cells counted in each case and values for the subsequent analysis were obtained from the median of the two different observations.

### Table I. Antibodies used in the present study.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P21W AF/Cip1</em></td>
<td>4D10</td>
<td>Dako</td>
<td>1:40</td>
</tr>
<tr>
<td><em>P27Kip1</em></td>
<td>Clone 1B4</td>
<td>Dako</td>
<td>1:40</td>
</tr>
<tr>
<td><em>P53</em></td>
<td>DO-7, IgG2b</td>
<td>Dako</td>
<td>1:200</td>
</tr>
<tr>
<td><em>Ki-67</em></td>
<td>MIB1</td>
<td>Dako</td>
<td>1:10</td>
</tr>
<tr>
<td><em>BCL-2</em></td>
<td>Clone 124</td>
<td>Dako</td>
<td>1:10</td>
</tr>
<tr>
<td><em>BAX</em></td>
<td>Ig fraction, Dako</td>
<td>Dako</td>
<td>1:1000</td>
</tr>
</tbody>
</table>
P27 expression in chronic hepatitis B when compared with normal liver, whereas positive staining was found in 5 (66.7%) cases of steatosis; in 8 (66.7%) of steatohepatitis; in 3 (50%) of chronic hepatitis B; in 7 (38.9%) of cirrhosis; in 7 (77.8%) cases of HCC (Figure 8), and 2 (13.3%) cases of metastasis (Table II). The overall percentage of P27-positive cells ranged from 1-100% (median 10%). A statistically significant higher frequency of P27 expression was found in cirrhosis compared to HCC (p=0.001) as well between cirrhosis and HCC (p=0.001).

**Results**

**P21WAF/Cip1 protein expression.** P21 immunoreactivity was always nuclear; no cytoplasmic staining of P21 was seen in any specimen. No positive P21 staining was observed in normal liver tissue. The epithelium of bile ducts was also negative. Nuclear staining was found in 3 (50%) cases of patients with steatosis; in 7 (58.3%) of steatohepatitis; in 3 (50%) cases of chronic hepatitis B; in 10 (55.5%) of cirrhosis; in 7 (77.8%) cases of HCC (Figures 1-3); and in 5 (33.3%) cases of metastasis (Table II). The overall percentage of P21-positive cells ranged from 0% to 50% (median 3%) in all cases studied. P21 expression was more frequent in metastasis when compared with chronic hepatitis (p=0.010), and more frequent expression was found in cirrhosis (p=0.001) when compared with chronic hepatitis. Statistically significantly higher frequency of P21 expression was found in cirrhosis compared to HCC. No statistically difference was found between HCC and metastasis.

**P27Kip1 protein expression.** Immunohistochemistry revealed no P27 expression in the majority of hepatocytes from normal liver, whereas positive staining was found in 5 (66.7%) cases of steatosis; in 8 (66.7%) of steatohepatitis; in 3 (50%) of chronic hepatitis B; in 7 (38.9%) of cirrhosis, in 3 (33.3%) of HCC (Figures 4-6), and in 6 (40%) cases of metastasis (Table II). The overall percentage of P27-positive cells in all the cases ranged from 1-50% (median 3%). An increased frequency of P27 protein expression was found in chronic hepatitis B when compared with steatohepatitis (p=0.005). P27 protein expression was more frequent in steatohepatitis than in HCC (p=0.003). Statistically significant differences were found between the frequency of P27 expression in chronic hepatitis B when compared with cirrhosis (p=0.001), as well between cirrhosis and HCC (p=0.001).

**BCL-2 protein expression.** BCL-2 immunostaining was cytoplasmic in all cases. There was no evidence of BCL-2 expression by hepatocytes in normal liver tissue and steatosis cases. In portal tracts, the BCL-2 protein was expressed by epithelial cell lining bile ductules and small interlobular bile ducts. BCL-2 was also found in lymphocytes. BCL-2 protein was noted in 3 (25%) cases of steatohepatitis, 4 (66.7%) cases of chronic hepatitis B; 5 (27.8%) cases of cirrhosis; 6 (66.7%) cases of HCC (Figure 7), and 2 (13.3%) cases of metastasis (Table II). The overall percentage of BCL-2 positive cells ranged from 1-100% (median 10%). Apoptotic hepatocytes were markedly increased in steatohepatitis (p<0.05) as compared to normal liver tissue. BCL-2 protein expression was more frequent in hepatitis than in cirrhosis (p=0.002) and in HCC than in metastases (p=0.033).

**BAX protein expression.** The positive immunoreaction of BAX protein showed as brown color of the cytoplasm. BAX protein expression was absent from hepatocytes of normal liver tissue. BAX protein staining was observed in 2 (33.3%) cases of steatosis; 7 (58.3%) cases of steatohepatitis; 5 (83.3%) cases of viral hepatitis B; 4 (22.2%) cases of cirrhosis; and 5 (55.6%) cases of HCC (Figure 8), and 3 (20%) cases of metastasis (Table II). The percentage of overall BAX protein expression ranged from 1-50% (median 3%). The samples from patients with steatohepatitis showed increased BAX protein expression (p=0.001) when compared with normal liver tissue. The expression of BAX protein was more frequent in hepatitis (p=0.001) when compared with steatohepatitis and cirrhosis (p<0.001). No correlation was found between cirrhosis and HCC (p=0.526).

**Expression of P53 protein.** No positive p53 staining was observed in hepatocytes of normal liver tissue. Positive P53 immunostaining was seen in nuclei of the cells, but the intensity was quite variable from cell to cell. P53 expression was observed in 2 (41.7%) cases of steatosis; 5 (41.7%)

### Table II. Immunohistochemical expression of cell cycle regulators, proliferation markers and apoptosis-related proteins in liver pathologies.

<table>
<thead>
<tr>
<th>Cases (n)</th>
<th>P21 (%)</th>
<th>P27 (%)</th>
<th>P53 (%)</th>
<th>Ki-67 (%)</th>
<th>BCL-2 (%)</th>
<th>BAX (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steatosis</td>
<td>6</td>
<td>3 (50)</td>
<td>5 (83.3)</td>
<td>2 (33.3)</td>
<td>2 (33.3)</td>
<td>0</td>
</tr>
<tr>
<td>Steatohepatitis</td>
<td>12</td>
<td>7 (58.3)</td>
<td>8 (66.7)</td>
<td>5 (41.7)</td>
<td>5 (41.7)</td>
<td>3 (21)</td>
</tr>
<tr>
<td>HBV</td>
<td>6</td>
<td>3 (50)</td>
<td>3 (50)</td>
<td>3 (50)</td>
<td>4 (66.7)</td>
<td>4 (66.7)</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>18</td>
<td>10 (55.5)</td>
<td>7 (38.9)</td>
<td>8 (44.4)</td>
<td>8 (44.4)</td>
<td>5 (27.8)</td>
</tr>
<tr>
<td>HCC</td>
<td>9</td>
<td>7 (77.8)</td>
<td>3 (33.3)</td>
<td>7 (77.8)</td>
<td>8 (88.9)</td>
<td>6 (66.7)</td>
</tr>
<tr>
<td>Metastasis</td>
<td>15</td>
<td>5 (33.3)</td>
<td>6 (40)</td>
<td>11 (73.3)</td>
<td>14 (93.3)</td>
<td>2 (13.3)</td>
</tr>
</tbody>
</table>

Numbers in brackets represent percentage of positive cases; HBV, hepatitis B; HCC, hepatocellular carcinoma.

**Statistical analysis.** Data analyses were performed using the Statistical Package for Social Sciences (SPSS, 13.0, Chicago IL, USA). Fisher’s exact and χ2 tests were used for the analysis of categorical data, whereas for correlation analysis, the Kruskal-Wallis test was used for continuous data. Tests were considered significant when p-values were less than 0.05.
Figure 1. Immunohistochemical expression of P21/WAF1 in steatohepatitis (×400).

Figure 2. Immunohistochemical expression of P21/WAF1 in well-differentiated hepatocellular carcinoma (×400).

Figure 3. Immunohistochemical expression of P21/WAF1 in another case of well-differentiated hepatocellular carcinoma (×400).

Figure 4. Immunohistochemical staining of P27 in chronic hepatitis B (hepatocytes show nuclear staining) (×400).
Figure 5. Immunohistochemical staining of P27 in regenerative nodule in cirrhosis (×400).

Figure 6. Immunohistochemical staining of P27 in well-differentiated hepatocellular carcinoma (×400).

Figure 7. Cyttoplasmic BCL-2 expression (moderate to strong) in well-differentiated hepatocellular carcinoma (×400).

Figure 8. Cyttoplasmic BAX expression (moderate to strong) in well-differentiated hepatocellular carcinoma (×400).
cases of steatohepatitis; 3 (50%) cases of hepatitis; 8 (44.4%) cases of cirrhosis; 7 (77.8%) cases of well-differentiated hepatocellular carcinoma, and 11 (73.3%) cases of metastasis (Table II). The overall percentage of p53-positive cells ranged from 0-70% (median 1%). The samples from patients with cirrhosis showed increased frequency of P53 expression as compared with steatohepatitis (p=0.007). Statistically, an increased frequency of expression of p53 was found in cirrhosis and HCC (p=0.001 and p=0.029 respectively) when compared with hepatitis.

Expression of MIB1 (Ki-67) protein. Ki-67 expression was noted in the nucleus of hepatocytes. In the present series of samples, the Ki-67 expression was observed in 2 (33.3%) cases of steatosis, in 5 (41.7%) of steatohepatitis, in 4 (66.7%) of chronic hepatitis, in 8 (44.4%) of liver cirrhosis, in 8 (88.8%) of HCC, and 14 (93.3%) cases of metastases (Table II). The epithelium of bile ducts was consistently negative. The proliferating marker Ki-67 was markedly increased in cirrhosis (p=0.001) as compared to steatohepatitis. Statistically, we found increased frequency of expression of Ki-67 in cirrhosis and HCC (p<0.001 and p=0.012 respectively) when compared with hepatitis.

Discussion

HCC is one of the most frequent types of human cancer, and is mainly caused by infection with hepatitis B (HBV) or C virus (HCV). HCC incidence is still increasing in civilized countries despite considerable progress in diagnostic and therapeutic modalities, and the prognosis of HCC remains poor for reasons of its frequent recurrence after surgery (20). Because hepatocarcinogenesis is unique in that most HCCs emerge from cirrhotic livers, elucidating its molecular mechanism may help to establish an efficient strategy for improving the prognostic modality of individuals with a high risk of HCC development. Many studies have suggested that genetic or epigenetic alterations may play important roles in the progress of HCC via oncogene activation or tumor suppressor gene inactivation (21).

P21 was first isolated as one of CDK-interacting proteins (CIP1), and is a potent and tight binding inhibitor of CDKs (5). The discovery of p21 gene has provided not only a lucid access to negative regulation of the cell cycle as an inhibitor of cyclin–CDK complex, but also a direct link between the tumor suppressor protein and the cell cycle because a gene, named WAF1, which has an identical cDNA sequence, was identified simultaneously and was induced by wild-type p53 but not mutant p53 gene expression (10). In addition to cell cycle regulation and tumor suppression mediated by p53, P21 is involved in many other functions including differentiation and apoptosis. Albrecht et al. (22) reported that P21 expression is regulated by P53-dependent and -independent pathways in the liver and is up-regulated in a biphasic manner with enhanced mRNA expression during G1-phase and after S-phase, according to hepatic regeneration after partial hepatectomy.

In the current study, we evaluated P21 expression in human tissue from liver diseases, using immunohistochemical methods. P21 was not only expressed in patients with HCC, but also in patients with chronic liver diseases such as steatosis, steatohepatitis, chronic HBV infection and cirrhosis. The P21 antigen was apparent in the nucleus of hepatocyte, especially in cells near the portal area and around areas of necrosis, suggesting that P21 expression was up-regulated in response to hepatic injury, in agreement with other reports (23, 24).

Recently another important role of P21 in the protection of cells against apoptosis has been proposed, namely an antiapoptotic effect in certain cell types. This function of P21 as an inhibitor of cell cycle progression or apoptosis is most likely determined by subcellular localization (25). P21 localized in the nucleus induces cell cycle arrest, whereas cytoplasmic P21 prevents Fas-mediated apoptosis (25). However, from our observations, P21 was stored primarily in the hepatocyte nucleus, in contrast with the study of Shiraki and Wagayama, who found that in most HCCs, P21 is expressed in the cytoplasm rather than the nucleus (26).

The p27 gene was first identified in G1-phase cells arrested by transforming growth factor-β. Its protein is highly expressed in quiescent cells, where it preferentially binds to and inactivates cyclin A-E/CDK2 complexes, thereby preventing cell entry into the S-phase (27). The function of P27 as a negative regulator of the cell cycle, suggests its putative role as a tumor suppressor gene. Reduced P27 levels in colon and lung carcinomas result from accelerated proteolytic degradation via the ubiquitin-proteasome pathway (28). Lost or reduced P27 protein expression has been correlated to aggressive behavior in human cancer of the breast, gastrointestinal tract, prostate, lung and larynx (29-33).

Despite the fact that hepatocytes have a very low proliferative rate, only low levels of P27 were observed by immunohistochemistry in normal tissues. Quiescent cells in normal tissues generally express nuclear P27. However, it is unclear whether P27 is expressed to maintain cells in the G0-phase or as a result of the condition/maintenance of differentiation. In fact benign breast epithelial cells commonly express high levels of P27 (29). It is possible that hepatocytes are kept in the resting state by other cell-cycle inhibitory proteins. Our results revealed that P27 protein was expressed in a few hepatocytes of normal liver, with increased positive expression in steatosis steatohepatitis, chronic hepatitis B infection, liver cirrhosis; but the positive expression rate of P27 protein significantly decreased in well-differentiated HCC compared that in the other groups.
(Table II). The loss or decrease of P27 protein may lead to reduction or disappearance of its cell cycle negative regulation, thus cells pass from the G1-phase into the S-phase, resulting in division and autonomous program. Previous studies have demonstrated that P27 is expressed at various levels in classical HCC, becoming significantly decreased in cases with biologically aggressive phenotypes featuring portal invasion, poor differentiation, large tumor size and intrahepatic metastasis (34).

To our knowledge, this is the second study showing that P27 protein is expressed in very early stages of hepatocarcinogenesis. The possible mechanisms remain to be elucidated; in very early stages of hepatocarcinogenesis P27 could act as a negative regulator of the cell cycle to prevent cell proliferation. Early HCC generally shows a very well-differentiated histology with minimal atypia and lack definite invasive or destructive growth (34). Therefore, it is often difficult, even for an experienced hepatopathologist, to distinguish precancerous lesions and early HCC from regenerative nodules. For this reason, the discovery of an objective molecular marker that could help to standardize the histological diagnosis of precancerous lesions and early HCC and lead to appropriate treatment is eagerly anticipated.

Apoptosis is an important physiological mechanism of cell death, which depends on the expression of genes capable of inducing or inhibiting the process. It has a decisive role in the homeostasis and development of tissues (18). A few oncogenes and tumor suppressor genes control apoptosis. The BCL-2 gene family involved in apoptosis consists of two subgroups with different functions. BCL-2 and others (BCL-XL, MCL1, MBCL-X, CED-9, BHRFI, LMW5-HL) as cell death suppressors and BAX, BCL-XS, BAK and BAD, as cell death promoters, control programmed cell death (18). It is well known that BCL-2 protein is not expressed in hepatocytes in normal human liver (19, 36, 37), in contrast with other normal tissues (38, 39). Surprisingly, we found up-regulation of BCL-2 in steatohapatitis, HBV infection (p=0.07), cirrhosis (p=0.019), and HCC (p=0.02), contrary to normal liver, where the expression was absent. Studies in patients with cirrhosis demonstrated that under pathological conditions, hepatocytes can induce de novo expression of BCL-2 (40). Nevertheless, the activation of the liver-protective pathway appears to be associated with the severity of the disease. The BCL-2 family is tightly involved in this pathway, and thus we sought to investigate the expression of BAX, a pro-apoptotic protein. After an apoptotic stimulus, BAX protein increases and is translocated to the mitochondria, where it promotes cytochrome c release (41). This event in turn leads to caspase-3 activation, with consequent cell death (18). We found an up-regulation of BAX in steatohapatitis and cirrhosis in accordance with the reports of Panasiuk et al. and Ramalho et al. (42, 43), suggesting that these cells may be susceptible to apoptosis.

The pathogenesis of steatohepatitis is not entirely clear. It has been suggested that free fatty acids can play a crucial role in steatosis intensification and necrotic-inflammatory processes. Damage of lysosome integrity, which results in cathepsin B and tumor necrosis factor-α release, is due to the effect of lipotoxicity.

Studies reported to date have assessed the expression of BCL-2 and BAX levels in HCCs by using an immunohistochemical approach: BCL-2 positivity was reported to range from 14 to 70%, whereas BAX positivity was reported to range from 46 to 70% according to different cut-off values (44). In our series, BCL-2 and BAX protein levels were not differently distributed in well-differentiated HCC, confirming previously reported results (44).

p53 is a major tumor suppressor gene located on the short arm of chromosome 17. Wild-type P53 protein is involved in negative regulation of cell growth by controlling cell entry into the S-phase. An interesting finding in the present study, is the expression of P53 in samples of chronic liver disease, *e.g.* steatosis (41.7%), steatohepatitis (41.7%), chronic hepatitis B (50%), and cirrhosis (44.4%), in accordance with other studies (46-48). One possible explanation for this finding may be reduced P53 protein breakdown, resulting in an accumulation of the wild-type P53 protein. Another possibility is that other unknown gene products somehow modulate the *p53* gene, enhancing P53 synthesis to a level that can be detected by immunohistochemistry.

Positive P53 immunostaining was seen in nuclei of tumor cells, but the intensity was quite variable from cell to cell. No immunostaining was seen in tumor cells undergoing mitosis. In the current study we showed that P53 expression was detected immunohistochemically in 77.8% of patients with well-differentiated HCC a difference with the report of Qin et al. (45). This discrepancy could be accounted for by differences in criteria regarding the classification of positive and negative cases (46). We must keep in mind that immunohistochemistry is not a strictly quantitative method, as there is no uniform scoring system and the interpretation of staining intensity is highly subjective. In addition, variations in protocols, such as in fixation procedures, antibodies and storage time of tissue samples are likely to affect sensitivity of these assays, making comparison of results from different laboratories difficult.

The evaluation of the hepatocyte proliferative index has been suggested as a useful tool for: (i) analyzing liver regeneration and carcinogenesis processes; (ii) identifying cirrhotic patients at risk of developing HCC; (iii) discriminating between normal, regenerative, and neoplastic liver in cytological or microhistological samples; and (iv) predicting survival in hepatocemized patients with HCC. Hepatocyte proliferative activity has rarely been assessed in patients with chronic liver damage as a tool to investigate its pathophysiology (49). Nonetheless, information on the
proliferative activity of the liver in patients with chronic liver damage might be relevant in evaluating liver regenerative capacity in response to different noxious agents. It might also be relevant because chronic hepatitis and cirrhosis might be viewed as precancerous steps in the long-lasting natural history of chronic liver damage toward HCC (50, 51). With respect to the above, it has been suggested that, even in the case of HBV, persistent hepatocellular necrosis and consequent irregular regeneration are more important than viral integration in terms of hepatocarcinogenesis (51).

In summary, the etiology of HCC is unique in that most HCC cases are associated with liver cirrhosis or chronic hepatitis attributable to HBV or HCV infection, chronic alcohol abuse and more recently, non alcoholic steatohepatitis. Since the prognosis of HCC patients remains poor, identification of useful molecular prognostic markers for HCC is required. The present study has some limitations. Although we are aware only a limited number of cases were investigated here and we did not follow up patients, this is an attempt to compare a set of markers that might be of significance in progression in liver diseases. We demonstrated the expression of P21, P27, P53, MIB1 (Ki-67), BCL-2, and BAX expression at different levels in liver pathologies in our study. Such observations strongly suggest that dysregulation of cell cycle and apoptotic proteins may play a unique role in the initiation and progression of HCC. Further studies of cell-cycle regulators will provide better insights into the mechanism of hepatocarcinogenesis.

References


