

Expression of p53, Bcl-2, VEGF, Ki67 and PCNA and Prognostic Significance in Hepatocellular Carcinoma

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Abstract

Background. Hepatocellular carcinoma is one of the most common malignant tumors that carry a poor prognosis. To improve the long-term outlook for HCC, an accurate prognosis is important. **Aims.** To study the immunohistochemical expressions of p53, Ki67, Bcl-2, VEGF and PCNA and their potential role as prognostic factors in patients with radical resection of hepatocellular carcinoma. **Patients and methods.** Forty-seven formalin-fixed paraffin-embedded tumor samples from patients with HCC receiving liver resection were investigated immunohistochemically for the expression of cellular proliferation markers PCNA, Ki67, p53, Bcl-2 and VEGF and their correlation with tumor characteristics and survival time after resection. **Results.** p53 was expressed in a higher percentage (85.7 vs. 42.1%) in undifferentiated histological tumor grades (Edmondson Steiner G3/G4 vs. G1/G2). Patients with p53 accumulating tumors showed a worse survival than patients with p53 non-accumulating tumors (median 9.5 vs. 16.5 months). Over-expression of VEGF was found in 38.3% of all HCCs. VEGF expression was significantly correlated with p53 expression and recurrence rates. The results showed that the labeling index of PCNA and expression of p53 are correlated. The high labeling index of PCNA or over-expression of p53 resulted in high risk of tumor recurrence, more aggressive growth and poor survival. **Conclusion.** High labeling index of PCNA, p53 nuclear accumulation and VEGF high expression are associated with poor survival in patients with HCC.

Key words

Immunohistochemistry – p53 – PCNA – VEGF – prognosis – hepatocellular carcinoma.

Introduction

With the development of diagnostic techniques many primary liver cancers can benefit from resection at an early stage [1, 2]. Because of the biological behavior of HCC, the recurrence rate after hepatectomy is very high [3-5]. Some authors described influencing factors such as tumor size, capsule formation, differentiation of tumor cells, clinico-pathological stage, proliferating cell nuclear antigen (PCNA), mutation of p53 [5-10].

The most commonly observed genetic alteration in human cancers has been reported to be the mutation of the p53 gene [1]. This gene has the features of a recessive oncosuppressor in its wild-type (wt) form and can be a dominant oncogene in its mutated form. The p53 gene product acts as a genetic sentinel, initiating cell cycle arrest or apoptosis. A defective copy of p53 behaves as a dominant negative, resulting in a cell that is resistant to undergoing apoptosis. Indeed, cancers presenting mutations of p53 tend to be more aggressive and resist chemotherapy [9, 10].

The vascular endothelial growth factor (VEGF) is the most important angiogenesis factors and a hot spot for study at present. It has been reported that VEGF, a dimeric glycoprotein with a structural homology with PDGF [7, 8] is a highly specific mitogen for blood vessel endothelial cells that can stimulate endothelial cells of microvessels to proliferate and increase permeability, resulting in tumor angiogenesis [9].

The most widely used proliferation-associated marker is Ki-67, which is a nuclear antigen present only in proliferating cells. A detailed cell cycle analysis showed that the Ki-67 antigen is expressed in cells during the G1, S, and G1-M phases but not during the G0 phase of the cell cycle [10, 11]. In HCC, Ki-67 expression was found to be in close relation to tumor growth rate [12] and was an independent prognostic indicator of patient disease-free and overall survival rates [13].

Proliferating cell nuclear antigen (PCNA) is a highly conserved nuclear protein that is expressed during cell replication and DNA repair [11, 14].

The protein encoded by the Bcl-2 (B cell lymphoma/

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leukemia 2) proto-oncogene has been implicated in the prolongation of cell survival by blocking the programmed cell death, i.e. apoptosis [1]. Expression of the Bcl-2 protein has mainly been studied in lymphoid tissue [2-5] and Bcl-2 has also been occasionally detected in several non-lymphoid fetal and adult tissues [7-9], some of which are characterized by apoptotic cell turnover e. g. epidermal and digestive epithelia [9]. As the normal adult human liver is a slow self-renewing tissue that probably harbors long-lived progenitor cells [10], we undertook an immunohistochemical study of the cellular distribution of the Bcl-2 protein in this tissue.

We studied the individual differences and prognosis of HCCs after hepatectomy using 47 specimens obtained from resected HCCs and examined them immuno-histochemically using anti-p53, anti-VEGF, anti Bcl-2, anti-PCNA and anti-Ki67 monoclonal antibodies.

Patients and methods

Patients

From the database of the Department of Pathology of the Fundeni Clinical Institute, 47 cases of radically resected HCCs operated at the Department of General Surgery and Liver Transplantation of the same hospital from January 2006 to January 2008 were recruited for this study. Forty patients were males and 7 were females with ages ranging from 36 to 72. Clinical information was reviewed retrospectively through medical records. The mean age of the patients was 57.5 years. Regarding the basic liver disease preceding HCC development, 23 cases of liver cirrhosis and 17 cases of chronic hepatitis were recognized. There were 7 patients without an underlying liver disease. The median follow-up period was 17 months (range 3–29 months). No patient received neo-adjuvant therapy in this cohort. Also, no patient died immediately after surgery because of surgical complications. After operation, all patients were followed up routinely every 3 months. The cases with palliative liver resections (R1 or R2 resections) were excluded from this study.

Immunohistochemical staining

Formalin-fixed, paraffin-embedded sections of tumor tissue obtained from the resected liver specimens of patients with HCC were cut into 4 microns thick sections. The sections were deparaffinized in xylene and the xylene was subsequently removed with absolute ethanol. Endogenous peroxidase was blocked by treatment with 0.3% hydrogen peroxide in methanol for 20 minutes. Antigen retrieval was performed

by autoclave. The slides were then incubated with goat polyclonal anti-VEGF antibody (Dako Carpinteria) followed by biotin-conjugated goat anti-mouse immunoglobulin and horseradish peroxidase-conjugated streptavidin (Dako, Glostrup, Denmark). Aminoethylcarbazole was used as the chromogenic substrate. The red precipitate was identified as positive staining. The specimens were counterstained with hematoxylin and mounted with glycerol gelatin. The immunoactivity for VEGF was evaluated independently by two of the authors without any prior information about each slide. For a negative control, secondary antibody was applied to the tissue without the primary antibody. The formalin-fixed and paraffin-embedded specimens were examined immunohistochemically using anti-PCNA monoclonal antibody Pc-10 and anti-p53 monoclonal antibody DO-7 (LSAB kit Dako). Ki-67 was detected immunohistochemically by the streptavidin-biotin peroxidase method using anti-Ki-67 (MIB-1, DakoCytomation). The deparaffinized tissue sections had to be treated in a microwave oven before the immunohistochemical staining procedure for the p53 [10, 11]. The immunostaining p53 protein and PCNA appeared red-brown in the nucleus of the tumor or peritumor cells with various densities and were distributed unevenly (Fig. 1 a, b). The Bcl-2 protein was detected by the immunoenzymatic alkaline phosphatase method, as previously described [13], using an anti-human Bcl-2 monoclonal antibody (Dako A/S, Glostrup, Denmark).

The criteria of nuclear labeling index for PCNA and expression of p53 in this study were as follows: the positive nuclei number was semiquantitatively evaluated by counting the number of positive nuclei in 8-10 randomly-chosen medium power ($\times 160$ magnification) fields; four degrees of positive nuclei for PCNA and Ki67 were identified: “-” $< 24\%$, “+” = $25\%-50\%$, “+++” = $51\%-74\%$, “++++” $> 75\%$; four degrees for P53, VEGF and Bcl-2 were identified and used for simplicity: “-” no positive cell, “+” $< 30\%$, “++” = $31-70\%$, “+++” $> 71\%$.

After the completion of the immunohistochemistry tests, the markers' expression was correlated with clinicopathological data, recurrence rate and survival, in order to ascertain the real prognostic value of these variables. For this purpose the patients were divided into two groups in relation with value of certain parameters, i.e. small (\leq with 5 cm) vs. big tumor (tumor size > 5 cm), high (++, +++) vs. low (-, +) over-expression of molecular markers, alive or dead at the end of the follow-up, with or without recurrence.



Fig 1. a) HCC poorly differentiated - PCNA 98 % positive (x100); b) HCC moderately differentiated – P53 positive (x100); c) HCC moderately differentiated – VEGF (x100).

Statistics

Fisher exact test was used to examine the association between clinico-pathological features and the biomarkers expression. Cumulative overall and disease-free survival curves were constructed using the Kaplan-Meier method, and clinico-pathological variables were tested by the log-rank test. Factors identified as statistically significant were included in subsequent multivariate analysis by using the Cox multiple regression model. Enter method was used for multivariate analysis and Chi-square test to compare frequencies. A p value of ≤ 0.05 was considered significant.

Results

Immunohistochemical expression of the biomarkers

The p53 protein expression was positive in 32 of 47 cases (68%): in 24 from 28 (85.7%) of the high grade HCCs (Edmonson-Steiner III-IV), versus 8 from 19 cases (42.1%) of low grade HCCs (Edmonson-Steiner I-II). Moreover, most deaths during the follow-up (89.5%) pertained to the group of high expression p53, and the median survival of the high p53 expression group was significantly lower (9.5 months) than the median survival of the low p53 expression patients (16.5 months). We found a very strong correlation between p53 expression and PCNA expression; their high expression values were associated in 30 of all HCCs (63.8%). Comparatively, the expression of Ki67 evolved in the same direction like PCNA expression pattern, but without the utterance of the PCNA (tendency to a weaker expression than PCNA). High and low labeling index of PCNA, slight and over expression of p53 as well as VEGF expression were significantly different between the small and the large tumors (Tables I-III). The labeling index of PCNA corresponded to p53 expression.

Table I. PCNA labeling index and tumor size (p=0.0003, OR=10.68)

	Cases	PCNA labeling index	
		Low index (-, +)	High index (++, +++)
Small tumor	20	13	7
Large tumor	27	4	23

Table II. p53 expression and tumor size (p=0.0055, OR=8.36)

	Cases	p53 expression	
		Slight expression (-, ++)	Over expression (++, +++)
Small tumor	20	18	2
Large tumor	27	14	13

Immunohistochemical staining for VEGF

The expression of VEGF was observed in the cytoplasm and the staining pattern was homogeneous. In some cases almost all cancer cells were immunopositive for VEGF (Fig. 1c), whereas in others, the cancer cells were immunonegative. No VEGF expression was identified in normal epithelia. According to the criteria for the VEGF immunostaining evaluation, 18 (38.3%) of the 47 cases

Table III. VEGF expression and tumor size (p=0.00005, OR=32.30)

	Cases	p53 expression	
		Slight expression (-, +)	Over expression (++, +++)
Small tumor	20	19	1
Large tumor	27	10	17

were strongly positive for VEGF in immunohistochemistry. Twenty cases (42.5%) were negative and 9 cases (19.2%) showed weak immunoreactivity. VEGF expression was very intense in patients with poorly differentiated tumors, and this pattern of staining for VEGF was correlated with a high rate of recurrence (Table IV) and short postoperative survival.

Recurrence and labeling index of PCNA, expression of p53 and VEGF

During the follow-up period tumor recurrence occurred in 24 of the 47 (51.06%) patients who underwent radical resection. PCNA high labeling index (++,+++) accounted for 83.33% (20/24). Over-expression of p53 accounted for 54.33% (14/24); in the remaining 23 cases who had no tumor recurrence, PCNA high labeling index accounted for 43.47% (10/23). Over-expression of p53 accounted for 4.34% (1/23) (p < 0.01) (Tables V-VI).

Table IV. VEGF expression and tumor recurrence (p=0.00004, OR=21)

	Cases	p53 expression	
		Slight expression (-, +)	Over expression (++, +++)
Recurrence	24	8	16
No recurrence	23	21	2

Table V. PCNA labeling index and tumor recurrence (p=0.0044, OR=6.50)

	Cases	PCNA labeling index	
		Low index (-, +)	High index (++, +++)
Recurrence	24	4	20
No recurrence	23	13	10

Table VI. p53 expression and tumor recurrence (p=0.00007, OR=30.80)

	Cases	p53 expression	
		Slight expression (-, ++)	Over expression (++, +++)
Recurrence	24	10	14
No recurrence	23	22	1

Survival rates and labeling index of PCNA, expression of p53 and VEGF

The different survival rates (Figs. 2, 3) of the patients with low or high labeling index of PCNA, mild or over expression of p53 who underwent curative hepatectomy are listed in Tables VII-IX. The Kaplan-Meier survival curves until 30 months of follow-up are shown in Figs. 2-5.

Table VII. Comparison of the survival rates of patients with low or high labeling index of PCNA after curative hepatectomy

PCNA index	Cases	Survival rates (%)	
		6 months	12 months
Low index (+, -)	17	94.2	94.1
High index (++, +++)	30	93.0	46.6

Table VIII. Comparison of the survival rates of patients with weak or over expression of p53 after curative hepatectomy

p53 index	Cases	Survival rates (%)	
		6 months	12 months
Low index (+, -)	32	93.7%	78.1%
High index (++, +++)	15	93.3%	33.3%

Table IX. Comparison of the survival rates of patients with weak or over expression of VEGF after curative hepatectomy

VEGF	Cases	Survival rates (%)	
		6 months	12 months
Low index (-, +)	29	100%	89.6%
High index (++, +++)	18	83.3%	22.2%

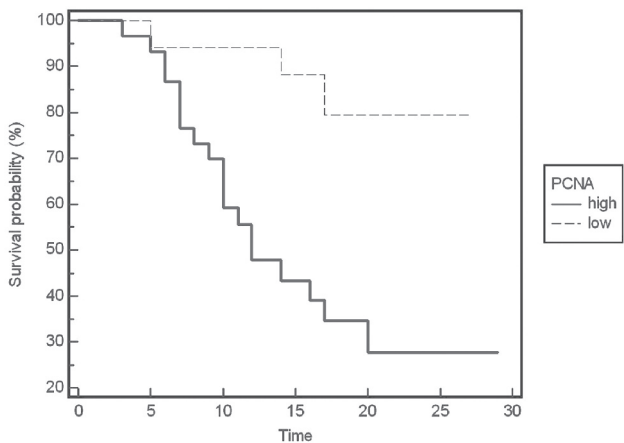


Fig 2. High vs low labeling index of PCNA after curative hepatectomy. n=47, n1=30 - high labeling index of PCNA, median survival 12 months; n2=17 - low labeling index of PCNA, median survival over 30 months (p=0.002).

The Kaplan Meier survival for patients with high vs low labeling index of Bcl-2 was not statistically significant (p=0.5869).

These results show that the levels of expression of PCNA, VEGF, Ki67, p53 in HCC have a prognostic value (p<0.0001) similar with Edmonson Steiner grading and tumor size. No statistic correlations were found between the immunohistochemical expression of Bcl-2 and the clinico-pathological parameters.

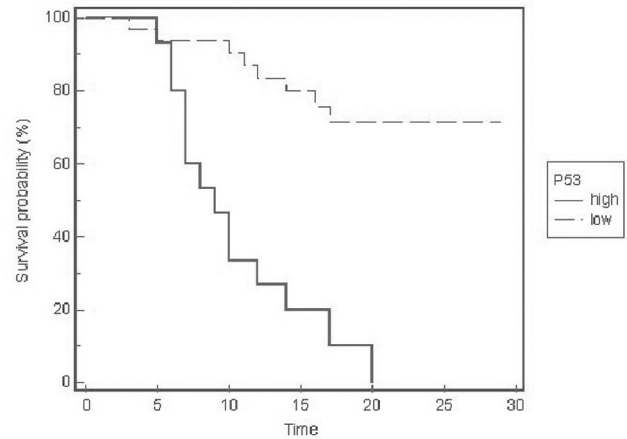


Fig 3. High vs low labeling index of p53 after curative hepatectomy, n=47, n1=15 - high labeling index of p53, median survival 9 months; n2 =32 - low labeling index of p53, median survival over 30 months (p<0.0001).

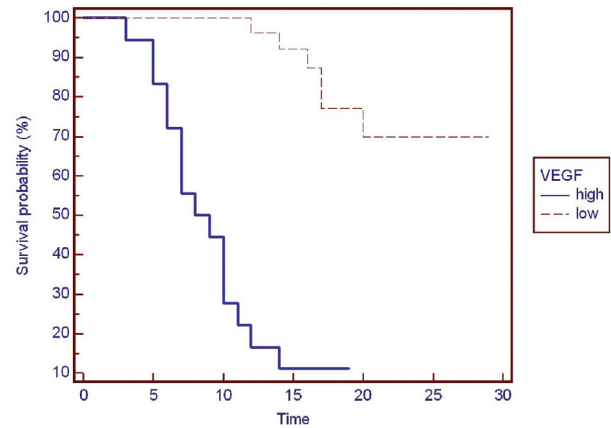


Fig 4. High vs low labeling index of VEGF after curative hepatectomy, n=47, n1=18 - high labeling index of VEGF, median survival 8 months; n2=29 - low labeling index of VEGF, median survival over 30 months (p<0.0001).

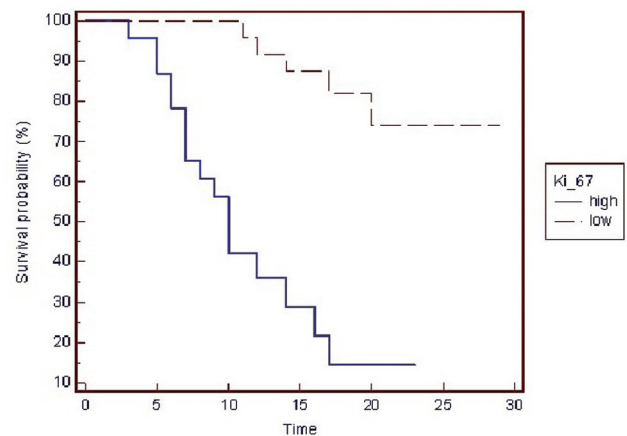


Fig 5. High vs low labeling index of Ki67 after curative hepatectomy, n=47, n1=23 - high labeling index of Ki67, median survival 10 months; n2 =24 - low labeling index of Ki67, median survival over 30 months (p<0.0001).

Table X. The multivariate analysis proving p53, Ki67 and VEGF as independent prognostic factors.

	B	SE	Wald	df	Sig	Exp(B)
Bcl-2	0.205	0.479	0.183	1	0.669	1.227
p53	1.301	0.531	5.998	1	0.014	3.672
PCNA	0.101	0.914	0.012	1	0.912	1.106
Ki67	1.729	0.796	4.722	1	0.030	5.636
VEGF	1.928	0.576	11.211	1	0.001	6.875

Discussion

The prognosis of patients with HCC after liver resection is mainly determined by HCC recurrence. The recurrence rate of HCC after resection is disappointingly high [9,10]. In our series, the recurrence rate was as high as 57.5% in those patients who underwent surgery and were discharged from the hospital. Liver was the most frequent site of recurrence. The prognosis of HCC after liver resection is grossly determined by three main factors: the functional and histological features of the liver bearing the HCC, the extent and characteristics of the tumor per se and the operative intervention or radicality of the procedure used. Many studies have shown that oncogenes play an important role in the growth, progression and metastasis of solid tumors. Recently, several oncogene factors have been identified.

The aim of this study was to investigate whether p53, VEGF, Bcl-2 and proliferation markers as well as parameters of cell loss could equally predict the malignant behavior of HCCs. Additionally, we assessed their prognostic value, their inter-correlation and their correlation to other histopathological parameters. Our positivity rates obtained by immunohistochemical assessment of Ki67, PCNA, Bcl-2 were in agreement with those in the literature [3, 15-18].

The finding that over-expression of Bcl-2 in follicular lymphomas, caused by a chromosomal translocation, inhibits apoptosis without increasing cell proliferation established a new mode of action for oncogenes [1, 2]. It is now accepted that inhibition of apoptosis is a component of some stages of oncogenesis and that Bcl-2 itself and anti- and pro-apoptotic Bcl-2 family members may play important roles in the process. Further studies revealed that Bcl-2 can block hepatocyte replication in the regenerating liver through an effect on cell cycle progression, acting at a stage beyond cyclin D1 expression [10]. We concluded from these and other studies [19] that Bcl-2 expression inhibits liver carcinogenesis by slowing down the replication of altered cells. In spite of a recording rate of Bcl-2 protein positivity (46.8%) similar with other researchers, we failed to discover any correlation of this marker with prognostic factors or survival.

Some authors reported that the labeling index of PCNA and over-expression of p53 might provide information about clinical outcomes. PCNA labeling index corresponded to the degrees of histological differentiation. Labeling index of PCNA was significantly lower in small tumors, encapsulated tumors and higher in the large tumors, and tumors without a

capsule [8-10]. But Ng et al [11] reported that the presence of p53 mutations did not show a significant association with tumor size, sex, age, tumor invasiveness, microsatellites and venous permeation, cirrhosis and encapsulation [8, 9]. This study showed that the high labeling index of PCNA and over-expression of p53 and VEGF were frequent in large tumors and those circumstances are associated with a high rate of post-resection recurrence and significantly lower survival.

Proliferating cell nuclear antigen (PCNA), as an index of the cellular proliferative status, was determined in various lesions. Waga et al [24] and Maeda et al [25] found that p53 tumor suppressor gene can control the cyclin-dependent kinases to regulate DNA replication involving PCNA interaction by p21 protein pathway. The over-expression of PCNA was usually used as a reliable marker for assessment of tumor progress, pre-malignant evolution and clinical prognosis of patients with various malignancies. Recurrence was closely related to the biological behavior. Labeling index of PCNA was correlated with the recurrence. The tumor with a high index of PCNA had a more aggressive growth and recurrence, resulting in low survival rates [10-12, 14]. This study indicated that the tumors with high labeling index of PCNA and over-expression of p53 and VEGF tended to have a high risk of recurrence.

Even in some small tumors a rapid tumor recurrence could appear. In this study, three cases of small tumors and 11 cases of large tumors had both high labeling index of PCNA and over-expression of p53. They underwent radical hepatectomy, but all of them experienced tumor recurrence 3-5 months after the operation. A low recurrence rate was found in the tumor with low labeling index, and even after recurrence, the tumor growth was slow and the patient had a good survival. However, our results demonstrate that the over-expression of p53 in advanced malignant lesions indicates that these abnormalities might occur at a late stage in HCC.

The observed correlation between p53 expression and PCNA labeling index is of clear interest. All cases positive for p53 expression exhibited compact and/or thick trabecular structures, with high proliferation. Out of the 5 cases, 4 exhibited the diffuse pattern of PCNA distribution characteristic to poorly differentiated HCC. Moreover, there was a significant relationship in location between cells expressing p53 and those positive for PCNA. Other authors [26] earlier reported that inhibition of cell-cycle progression into the S phase after induction of the wild-type p53 gene is accompanied by selective down-regulation of PCNA mRNA and protein expression. Fields and Jang [27] found that wild-type p53 protein activates the transcription of genes that negatively regulate cell proliferation. Therefore, it was suggested that p53 abnormalities could destroy the modulation system of cell proliferation in HCC. However, such a mechanism may not be a necessary event because p53 over-expression was not present in every case showing a high PCNA labeling index. This study showed that the immunohistochemical assessment of PCNA and p53 and VEGF could provide very useful information for the

prognosis of curatively resected HCCs.

The prognostic significance of VEGF expression has been reported in several cancers. The VEGF family is a group of growth factors that regulate endothelial cell proliferation. Although VEGF is known to play an important role in angiogenesis and portal tumor spread, its role in tumor progression has not been fully investigated [20, 21]. Several clinico-pathological factors, including tumor size, surgical margin, intrahepatic metastasis, lymph node metastasis, vascular invasion, lymphatic invasion, and perineural invasion, are known prognostic factors after surgical resection of HCC, as shown by multivariate or univariate analysis. In addition, survival curves determined by the Kaplan-Meier method showed that VEGF expression was associated with both overall survival and disease-free survival. Furthermore, VEGF expression, as well as vascular invasion, have a great impact on evolution and VEGF was confirmed to be a significant independent factor for poor prognosis [22] in the multivariate analysis in our study with a risk as high as 6.87 (Table X). Large cohort studies are needed to confirm our findings. In conclusion, VEGF expression serves as an independent and important prognostic factor in HCC patients. It may play an important role in the portal and systemic metastasis of HCC [23]. These findings suggest that VEGF could be useful as a prognostic marker and as a molecular target for the treatment of HCC.

The present study has some limitations. Although we are aware that only a limited number of cases have been investigated and the follow-up period is short, this is an attempt to compare a set of markers that might be of prognostic significance in HCC in a multivariate model. In our series, tumor grade was identified as the only independent prognostic factor in multivariate analysis. To identify and prove the actual prognostic value of p53 and of the proliferation markers, as well as of apoptosis in patients with HCC, further studies are needed on an adequate number of patients who receive standardized treatment procedures (R0 resection), who are within a specified stage of disease, and are assessed by identical methods.

Conclusions

This study demonstrated that VEGF was frequently over-expressed in pathologic specimens of HCC. VEGF over-expression was correlated with a high rate of recurrence and associated with poor prognosis, suggesting that VEGF expression might serve as a marker for disease progression and prognosis in patients with HCC. Our results also indicate that p53 over-expression may be related to late-stage progression of HCC and to high levels of cell proliferative activity.

The high labeling index of PCNA, p53 nuclear accumulation and VEGF high expression are associated with a poor survival in patients with HCC. This suggests that p53 and VEGF molecular diagnosis along with the expression of proliferating markers (Ki67 and PCNA) as prognostic markers could become further criteria in the selection of

adequate therapies for HCC and a target for future molecular therapies.

Conflicts of interest

None to declare.

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