pRb2/p130, Vascular Endothelial Growth Factor, p27$^{(KIP1)}$, and Proliferating Cell Nuclear Antigen Expression in Hepatocellular Carcinoma: Their Clinical Significance

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ABSTRACT

Hepatocarcinoma (HCC) is the fifth most common cancer, with more than one million fatalities occurring annually worldwide. Multiple risk factors are associated with HCC disease etiology, the highest incidence being in patients with chronic hepatitis B virus and hepatitis C virus, although other factors such as genetic makeup and environmental exposure are involved. Multiple genetic alterations including the activation of oncogenes and inactivation of tumor suppressor genes are required for malignancy in human cancers and are correlated with increased stages of carcinogenesis and further tumor progression. In this study of 21 HCC patients, we analyzed pRb2/p130, vascular endothelial growth factor (VEGF), p27$^{(KIP1)}$, and proliferating cell nuclear antigen as potential HCC molecular biomarkers. In our sample set, we found that p27$^{(KIP1)}$ was absent. Univariate survival analysis showed that proliferating cell nuclear antigen expression (diffuse staining >50% of positive cells in tumor) was confirmed as a significant HCC prognostic biomarker for determining patient survival agreeing with previous studies ($P = 0.0126$, log-rank test). Lower pRb2/p130 expression was associated to a borderline *P* value of inverse correlation with tumor malignancy and to a positive correlation with respect to the time from HCC diagnosis (Spearman coefficient = 0.568; $P < 0.05$). Conversely, higher VEGF expression was associated with a poor survival ($P = 0.0257$, log-rank test). We demonstrate for the first time that pRb2/p130 is inversely correlated with VEGF expression and tumor aggressiveness ($P < 0.05$) in p27$^{(KIP1)}$-negative HCC patients. pRb2/p130 and VEGF expression are independent from tumor staging, suggesting their possible role as independent prognostic molecular biomarkers in HCC. Furthermore, we have evidence that VEGF together with pRb2/p130 may act as new HCC biomarkers in a p27$^{(KIP1)}$-independent manner. Additional studies with larger numbers of patient data would allow the use of multivariable techniques and would be able to further identify patients with poorer survival.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies worldwide, being the third largest cause of cancer deaths. HCC is considered a multistage disease whose occurrence is caused by the interaction between genetic and environmental factors (1). Several studies demonstrated that HCC is more frequent in patients with hepatitis B virus (HBV)- and hepatitis C virus (HCV)-related chronic hepatitis and cirrhosis as well as in patients with a history of aflatoxin B1 exposure, chronic alcohol abuse, and cigarette smoking (2). Moreover, a higher incidence of HCC has been shown in certain genetic defects such as porphyria, α-1 antitrypsin deficiency, and Wilson disease (2). Usually, HCC arises from an adenomatous hyperplasia in an already diseased liver and progresses from a well-differentiated stage to less-differentiated forms (3). Ordinary HCCs formed by progression show highly increased cellular proliferation, neovascularization, production of basic fibroblast growth factor, and aneuploidy in some tumors (4). Corresponding to its malignant progression, HCCs show loss of heterozygosity for multiple chromosomes (5).

The long-term prognosis of HCC patients is typically quite poor. In one study involving 245 HCC cases, the median survival from the point of diagnosis was only 8 months (6). In another study of 336 patients, survival was as little as 0.7 months for stage III HCC *versus* 11.5 months for stage I tumors, with a median survival rate of 2.1 months (7). However, the 5-year survival rate increases with medical treatments. Unfortunately, shortly after curative hepatic resection, HCC tumor recurrence remains the rule, with the recurrence rates ranging from 75% to 100% at 5 years (8–10). Second liver resections

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might in fact afford a 5-year survival rate for HCC at 40% with a median survival time of 40 months (11). For nonresectable HCC, clinical trials involving 545 patients indicate that chemoembolization with cisplatin or doxorubicin may in fact increase patient survival (12). Liver transplant is one potentially curative HCC treatment. In a study of 42 HCC cases, patient survival at 6 months, 1 year, and 4 years was 88, 80 and 60%, respectively. This treatment was not effective for patients with stage IV HCC. Although liver transplants do offer a safe and potentially effective treatment for patients in stage I, II, and III HCC, unfortunately, the availability of useable organs remains an issue (13).

The molecular mechanisms behind HCC malignancies are complex and currently not well understood; however, it is known that both cirrhosis and long-term viral infection with both HBV and HCV can ultimately result in HCC. It has been proposed that inflammation of the liver may lead to aberrant cell death and/or the deviant stimulation of mitosis, resulting in an accumulation of molecular and cellular events necessary for oncogenic hepatocyte cell transformation. Furthermore, there may be an increase in hepatocyte chromosomal instability that is mediated by abnormally expressed protein(s) capable of inducing genetic recombination during chronic hepatitis infection (14).

Additionally, whereas the gene expression profiles of liver biopsies from patients affected by cirrhosis or various grades of primary and metastatic HCC have been largely used to assess the molecular events involved in the hepatocarcinogenic process, a clear sequence of genetic steps has not yet been identified. Loss of the retinoblastoma protein, high hepatocyte proliferative activity, and vascular endothelial growth factor (VEGF)-mediated angiogenesis has been associated with tumor progression and has been widely studied to provide new independent prognostic factors (15–17). A number of molecular factors have been shown to be associated with HCC invasiveness, and the analysis of molecular markers for the HCC cellular malignancy phenotype remains important for patient prognosis, morbidity, and mortality (18).

In this study, we examined 21 cases of hepatocellular carcinoma to analyze potential molecular biomarkers useful as prognostic indicators for HCC. We performed immunohistochemical analysis of various HCC-graded tumor biopsies from patients to determine the expression patterns of several cell cycle-regulated proteins as well as other proteins known to be involved in carcinogenesis. Specifically, we examined the $p27^{(KIP1)}$ protein [cyclin-dependent kinase (cdk) inhibitor], VEGF, the "bona fide" tumor suppressor protein pRb2/p130, and the proliferation marker proliferating cell nuclear antigen (PCNA).

The retinoblastoma gene family (*RB/p105*, *p107*, and *RB2/p130*) regulate cell cycle progression through the G_1 phase of the cell cycle. Retinoblastoma family members are nuclear proteins, known also as pocket proteins for their unique structure, which are phosphorylated in a cell cycle-dependent manner and exhibit growth-suppressive properties in a cell type-dependent manner. Whereas Rb/p105 is found in both cycling and quiescent cells, Rb2/p130 and p107 act exclusively in a cell cycle-dependent fashion to regulate several cellular transcription factors such as E2Fs (19).

$p27^{(KIP1)}$ is a cdk inhibitor that binds to cyclin-CDK complexes and facilitates the inhibition of the catalytic activity of cdk, ultimately inducing G_1 cell cycle arrest (20, 21). Inactivation of $p27^{(KIP1)}$ is believed to be a fundamental step in carcinogenesis, and decreased $p27^{(KIP1)}$ protein expression correlates with poor prognosis in many human malignancies (22, 23). Recent studies demonstrated that $p27^{(KIP1)}$ is expressed at lower levels in normal hepatocytes (24). On the other hand, other studies showed that under-expression of $p27^{(KIP1)}$ in HCC was associated with a poor prognosis, high-grade tumors, and early recurrence (25). Furthermore, HCC patients with higher expression levels of the $p27^{(KIP1)}$ protein experienced longer disease-free survival (26). Acquired $p27^{(KIP1)}$ expression is considered to be a favorable independent prognostic parameter for HCC (27).

VEGF is a secreted homodimeric cytokine that positively regulates tumor neovascularization (28). Solid tumors require a constant vascular supply for their survival as do metastasis. Recent studies suggest that angiogenesis, which is a highly orchestrated and multistep process, is essential in tumor growth and progression, including that of HCC, which are typically characterized by a high level of vascularization (29–31). In fact, it has been shown that VEGF expression in HCC tumors is significantly higher than in normal liver tissue (32).

PCNA is a nuclear protein that is synthesized in G_1-S-phase of the cell cycle. It is an accessory factor for DNA polymerases Δ and ϵ and functions as a DNA sliding clamp. PCNA is required for eukaryotic DNA synthesis, replication, and repair and is expressed at high levels in cycling cells (33). Other PCNA cellular functions include Okazaki fragment joining, DNA methylation, and chromatin assembly (34, 35). Because liver disease progresses from chronic hepatitis infection to HCC, PCNA expression levels increase dramatically (33).

In the present study, we demonstrate that VEGF expression is markedly up-regulated in high-grade HCC tumors and that higher VEGF expression was associated to a poor survival (P = 0.0257, log-rank test). Inversely, the negative cell cycle regulator pRb2/p130 was undetectable in advanced HCC. In fact, lower pRb2/p130 expression was associated to a borderline P and to a positive correlation with respect to the time from HCC diagnosis (Spearman coefficient = 0.568; $P < 0.05$). Furthermore, we found that the time from HCC diagnosis is negatively correlated with VEGF expression levels. Additionally, for the first time, we demonstrate that pRb2/p130 is inversely correlated with VEGF expression and tumor aggressiveness ($P < 0.05$) in $p27^{(KIP1)}$-negative HCC patients. pRb2/p130 and VEGF expression are independent from tumor staging, suggesting their possible role as independent prognostic molecular biomarkers in HCC. Moreover, we have evidence that VEGF together with pRb2/p130 may act as new HCC biomarkers in a $p27^{(KIP1)}$-independent manner.

MATERIALS AND METHODS

Patient Populations and Clinicopathological Data. Twenty-one cases of HCC were enrolled in the present study. Sections from paraffin-embedded liver tumors were obtained from patients who underwent hepatic surgical resection as a treatment for hepatocellular carcinoma at the Surgery Department of the Università Cattolica del Sacro Cuore, Roma, Italy.

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Tumor tissues were submitted for routine histopathological examination. The grade of HCC differentiation was estimated according to the Edmondson-Steiner classification. All patients were resected before undergoing any other treatments in this study. The median age of patients was 67 (range, 32–78). The Edmondson score was between I and IV, with a higher prevalence of grade III type tumors. Two (9.5%) of 21 tumors were not confined to the liver, involving the intestines as well. One (4.7%) case had an atypical resection performed. Median tumor size was 5.7 cm (range 0.5–15 cm). Two (9.5%) patients had more than one nodule present in the liver at the time of surgical resection. All patients in our study were not exposed to aflatoxin and were infected with either HBV and/or HCV.

Immunohistochemistry. A total of 21 formalin-fixed and paraffin-embedded HCC specimens were processed. Sections of each specimen were cut at 3 μm, mounted on glass, and dried overnight at 37°C. All sections were dewaxed, rehydrated, quenched in 0.5% hydrogen peroxide, and microwave pretreated in 10 mM citrate buffer (pH 6.0), as follows: 15 min at 650 W for p107; 15 min at 650 W for pRB2/p130 and VEGF; and 10 min at 650 W for p27^{kip1} and PCNA. After blocking with normal serum for 1 h at room temperature, our rabbit polyclonal antibody against p107 was incubated with tissue sections at a 1:200 dilution at 4°C overnight (36). The purified monoclonal antibody against the NH_2-terminal region of pRB2/p130 (Signal Transduction Laboratories, Lexington, KY) was used instead at 4°C overnight at a 1:100 dilution. The mouse monoclonal antibody against p27^{kip1} (clone DCS-72.F6; Neomarkers, Inc., Union City, CA) was incubated with tissue sections at a 1:150 dilution at 4°C overnight. The mouse monoclonal antibody against VEGF (clone JH121; Upstate, Lake Placid, NY) was incubated with tissue sections at a 1:50 dilution at 4°C, overnight. The mouse monoclonal antibody against PCNA (clone PC-10; Dako, Hamburg, Germany) was incubated with tissue sections at a 1:200 dilution for 3 h at room temperature. Negative control slides were also processed in parallel using a nonspecific immunoglobulin IgG (Sigma Chemical Co., St. Louis, MO) at the same concentration as the primary antibody. The positive immunostaining of infiltrating lymphocytes represented an internal-positive control for preservation of antigenicity in the sections examined. All slides were processed using the peroxidase-antiperoxidase method (Dako, Hamburg, Germany). Diaminobenzidine was used as the final chromogen, and Gill's hematoxylin was used for counterstaining. Additionally, to assess overall tumor morphology, tissue biopsies were deparaffinized as described above, stained with Gill's hematoxylin, and counterstained with eosin.

pRb2/p130, p27$^{(KIP1)}$, VEGF, and PCNA Staining Evaluation. Immunohistochemical staining in sample sections was categorized as negative, weakly positive, positive, or strongly positive. A strongly positive result was recorded when >50% of the cells exhibited strong staining. The sections were initially scanned at low power to determine the areas that were evenly labeled. The cases were evaluated independently by two different pathologists (C. M. and F. M. V.), and discrepancies in estimation were reconciled by concurrent review using a multiheaded microscope. At least 10 high-power fields were chosen randomly, and 2000 cells were counted. Tumor sections were considered negative if staining was absent or present in <5% of the tumor cells.

Statistical Analysis. Nonparametric statistics were used for all of the analyses, because data failed to show a normal distribution. Rank correlation of marker expression was performed by using four levels (negative, low positive, medium positive, and high positive). A matrix of correlation (Spearman coefficient) was performed among the available variables to detect possible paired correlations. Mann-Whitney *U* test and the exact significance of the Pearson χ^2 test were used to detect significant statistical value for dichotomous variables. A two-sided $P < 0.05$ was taken to indicate statistical significance.

In a follow-up of 36 months, univariate-survival analysis was performed by the Kaplan-Meier algorithm. Cases that were lost or deceased for causes not related to the HCC were considered censored. Data were stratified for the variables of interest to detect association with the survival rate. A matrix of correlation (Spearman coefficient) was performed among the available variables to detect possible paired correlations. Statistics were performed by SPSS (SPSS Inc. Chicago, IL) and SAS 9.1 software (SAS Institute Inc., Cary, NC).

RESULTS

Expression Levels of pRb2/p130, p27^{Kip1}, VEGF, and PCNA in HCC Specimens. The expression levels of pRb2/p130, p27$^{(Kip1)}$, VEGF, and PCNA in hepatocellular carcinoma tumors were determined immunohistochemically (Fig. 1).

The descriptive statistics for the overall series are shown in Table 1. No differences have been observed in variable distributions according to the Mann-Whitney *U* test.

Survival analysis for the overall series yielded a crude cumulative survival rate of 36 months (70%) with a mean survival time of 28 months as reported in Fig. 2. A stratification of survival rate, based on pRb2/p130, VEGF, and PCNA was performed by using cutoff values able to maximize differences between groups as reported in Table 2 and Figs. 3–5. Lack of pRb2/p130 expression, even if associated with a trend of lower survival, did not reach a statistical *P* (Table 2; Fig. 3). Higher expression of VEGF was instead associated with a poorer survival (Table 2; Fig. 4). A significant-negative correlation was found, by Spearman coefficient, between VEGF and pRb2/p130 expression levels (-0.446, $P = 0.049$), and a positive correlation was found between VEGF and PCNA expression levels (0.472, $P = 0.048$). Interestingly, grading (well-, moderately, and poorly differentiated), did not statistically correlate (by rank correlation) to the VEGF and pRb2/p130 expression levels. Additionally, we found that VGEF and Rb2/p130 expression did not correlate with tumor staging ($P > 0.05$). VEGF expression showed a borderline correlation with tumor size ($P = 0.055$). These results open up new prospectives to include markers in a screening model for HCC aggressiveness.

We also found that PCNA expression was significantly increased in poorly differentiated HCC specimens and progressively decreased in moderately and well-differentiated ones, in accordance with previously published literature (18, 33, 37–39). PCNA positively stained cells were <10% in low-grade HCC specimens. In moderately differentiated malignancies, PCNA staining was detected in approximately 30% of the nuclei.

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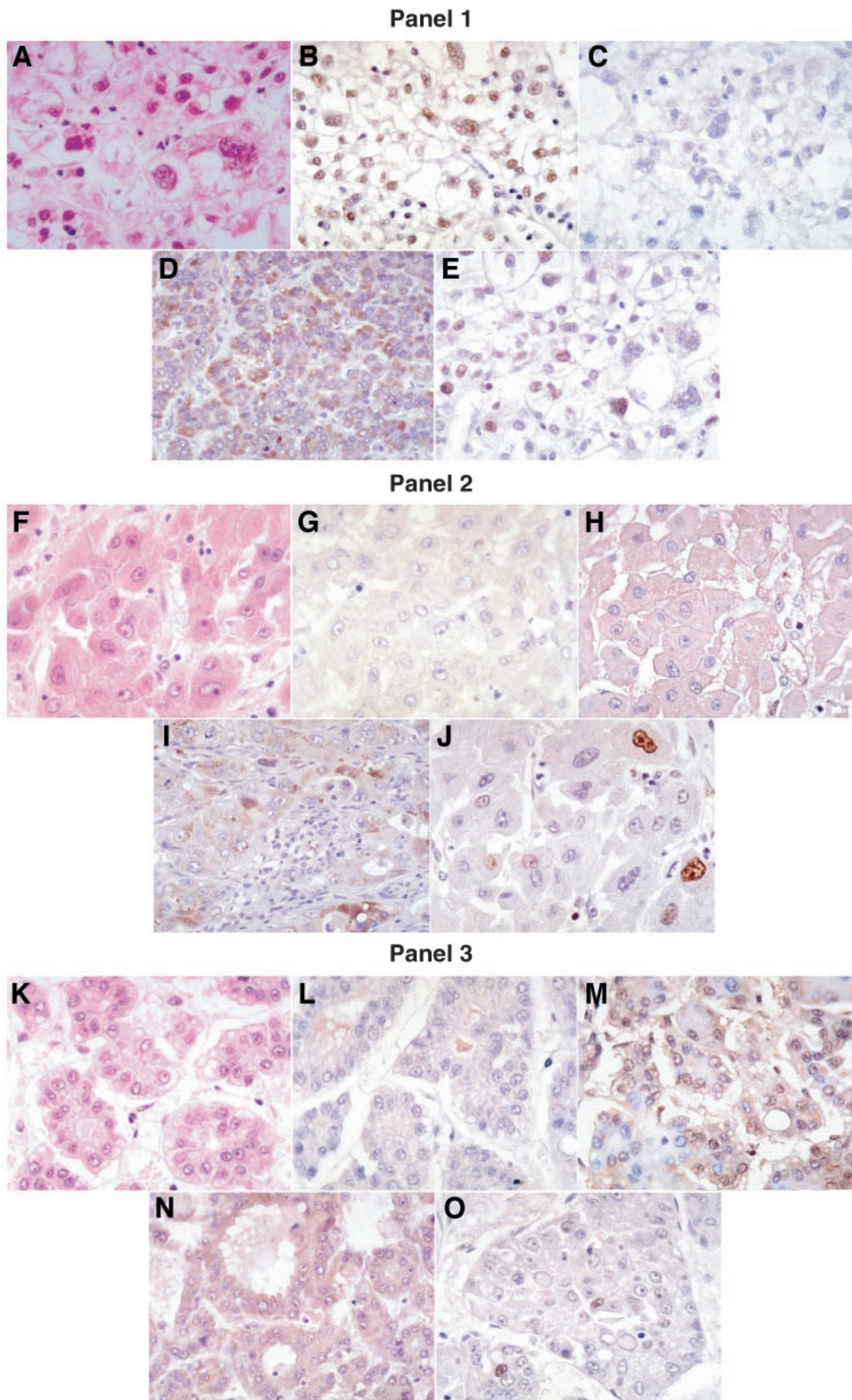

Fig. 1 Immunohistochemical analysis of cell cycle proteins in malignant hepatocellular carcinoma (HCC) liver tissues. All original magnification is ×60 unless noted otherwise. *Panel 1,* high-grade, undifferentiated HCC. *A,* H&E. Marked nuclear pleomorphism and anisonucleosis. Note chromatin clumping, margination, and prominent nucleoli. Malignant hepatocytes are in *solid sheets*. *B,* $p27^{(KIP1)}$. Note strong and diffuse nuclear positivity. *C,* pRb2/p130. No expression is noted. *D,* VGEF. Most tumor cells show strong cytoplasmic expression. This aspect is present throughout the entire tumor. Original magnification ×40. *E,* proliferating cell nuclear antigen (PCNA). Approximately 70% of nuclei show strong nuclear positivity. *Panel 2,* moderately differentiated HCC. *F,* H&E. Moderate nuclear pleomorphism and anisonucleosis; clearing of chromatin and prominent eosinophilic nucleoli are noted. The malignant hepatocytes tend to aggregate in disorganized laminae. *G,* $p27^{(KIP1)}$. No expression is noted. *H,* Rb2/p130. No nuclear expression is noted. *I,* vascular endothelial growth factor (VEGF). Some groups of tumor cells show coarse and strong cytoplasmic expression whereas the remaining cells show faint expression. Original magnification ×40. *J,* PCNA. Approximately 30% of nuclei show nuclear positivity. *Panel 3,* low-grade, well-differentiated HCC with tubular features. *K,* H&E. Nuclei are *round to oval* with centrally placed eosinophilic nucleoli. There is mild pleomorphism and anisonucleosis. The malignant hepatocytes are arranged in *tubular structures* surrounded by well-defined sinusoidal spaces lined by histiocytic and endothelial cells. *L,* $p27^{(KIP1)}$. No expression is noted. *M,* Rb2/p130. Most of the nuclei show positivity with variable intensity. Note the clearing of the chromatin that may give a false-negative aspect of nuclear staining if not carefully examined. *N,* VEGF. Some diffuse but weak cytoplasmic expression is present. In other areas, there is no VEGF expression. Original magnification ×40. *O,* PCNA. Approximately 10% of the nuclei are positive.

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Table 1 Descriptive statistics of the overall series

Variable	Valid cases	Category	%	Missing	Median	Minimum	Maximum
Age	20			1	67	32	78
FA[a]	20			1	249.50	124	739
GGT	18			3	60	18	363
BILTOT	20			1	0.60	0.40	4.70
BILDIR	18			3	0.20	0.10	2.10
AST	20			1	52.50	14	281
ALT	20			1	32	13	248
PT	20			1	84.05	47.70	105
PTT	19			2	31.20	20	49.70
FIBR	20			1	259.20	121	645
PLT	20			1	149.50	33.70	726
HB	20			1	13.10	8.50	15.60
CREATI	16			5	1	0.60	1.20
GGLOB	17			4	23.50	12.40	42.70
AFP	11			10	21.80	2.50	1500
Gender male	14		66.7	0			
HbsAg pos	11		52.4	0			
HbeAg pos	10		47.6	0			
HbcAg pos	6		28.6	0			
HCV pos	11		52.4	0			
HAV pos	3		14.3	0			
p27							
	17	*n*	94.4				
	2	+++	5.6				
	3	ND					
VEGF							
	5	*n*	27.8				
	7	+	38.9				
	2	++	11.1				
	4	+++	22.2				
pRb2/p130							
	11	*n*	61.1				
	2	+	11.1				
	3	++	16.7				
	2	+++	11.1				
PCNA staining distribution							
	11	Diffuse	61.1				
	7	Patchy	38.9				
PCNA staining intensity							
	3	Low	16.7				
	5	Medium	27.8				
	4	Strong	22.2				
	6	Variable	33.3				
PCNA % of (+) cells in tumor							
	2	1	11.1				
	2	5	11.1				
	1	10	5.6				
	1	15	5.6				
	2	20	11.1				
	2	30	11.1				
	3	40	16.7				
	2	70	11.1				
	2	80	11.1				
	1	100	5.6				

[a] FA, fibroadenoma; GGT, γ-glutamyltransferase; BILTOT, total bilirubin; BILDIR, direct bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PT, prothrombin time; PTT, partial thromboplastin time; FIBR, fibrinogen; PLT, platelets; HB, hemoglobin; CREATI, creatinine; GGLOB, γ-globulin; AFP, α-fetoprotein; HbsAg, hepatitis B surface antigen; HbeAg, hepatitis B envelope antigen; HbcAg, hepatitis B capsid antigen; HCV, hepatitis C virus; HAV, hepatitis A virus; VEGF, vascular endothelial growth factor; PCNA, proliferating cell nuclear antigen; ND, not determined.

High-grade malignancies showed >70% of tumor cells positive to PCNA.

PCNA staining was also scored as the total percentage of tumor cells stained. PCNA staining as a percentage of total tumor cells that were positive was scored using the exact Pearson χ^2 test analysis with a significant P of 0.007.

Again, we found pRb2/p130 expressed in low-grade, highly differentiated HCC tumor specimens. pRb2/p130 expres-

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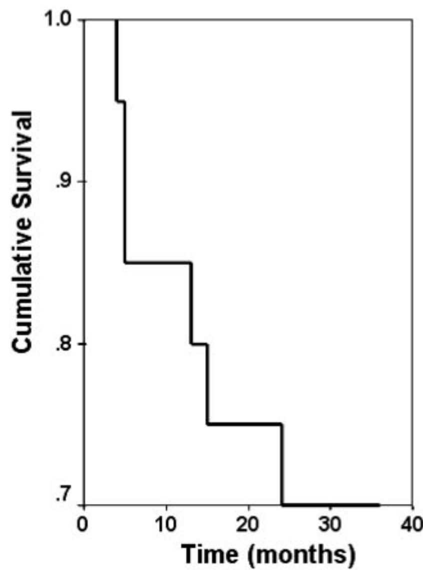

Fig. 2 Crude survival probability of the overall hepatocellular carcinoma series.

Table 2 Stratified survival analysis

Variable	Cut-off		P
pRb2/p130	+,++,+++	Negative	
Survival rate	85%	60%	0.2500
Median survival time (months)	32	25	
VEGF[a]	Negative	+,++,+++	
Survival rate	90%	33%	0.0091
Median survival time (months)	34	18	
PCNA	P and <50%	P and ≥50%	
Survival rate	84%	25%	0.2015
Median survival time (months)	32	15	
Grading	3	<3	
Survival rate	50%	23%	0.2602
Median survival time (months)	61	34	

[a] VEGF, vascular endothelial growth factor; PCNA, proliferating cell nuclear antigen.

sion occurs in the nuclei and shows variable degrees of staining intensity. Furthermore, it is important to point out that chromatin clearing may cause a false-negative staining aspect unless one carefully examines the nuclei. Although tumor malignancy increases and the tumor cells become less differentiated, pRb2/p130 expression levels decrease. In high-grade undifferentiated HCC tumor samples, pRb2/p130 expression is not detectable.

It has been reported previously that in normal liver cells, the level and intensity of VEGF is not detectable (4, 18, 30, 32). We found that VEGF was undetectable in the low-grade HCC tumors that we assayed. However, we discovered that VEGF expression increases in advanced tumor malignancy, and it is found in the cytoplasm of HCC cells, being highly up-regulated in high-grade, undifferentiated HCC specimens. We found an inverse statistically significant correlation between VEGF and pRb2/p130 expression ($P < 0.05$). In fact, high-grade tumors having low expression levels of pRb2/p130 show intense staining for VEGF and *vice versa* ($P = 0.048$).

The statistical and clinical findings of our study reside in the fact that VEGF and pRb2/p130 expressions are both associated with different survival rates. Multivariate techniques as well as Cox regression would be useful in a wider series of data, to quantify the marker-associated risk and to calculate a predictive value for the mortality associated with the marker

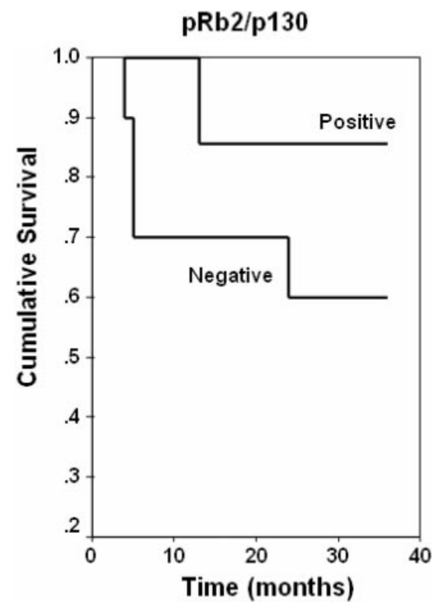

Fig. 3 Survival probability stratified according to the pRb2/p130 staining (negative *versus* positive).

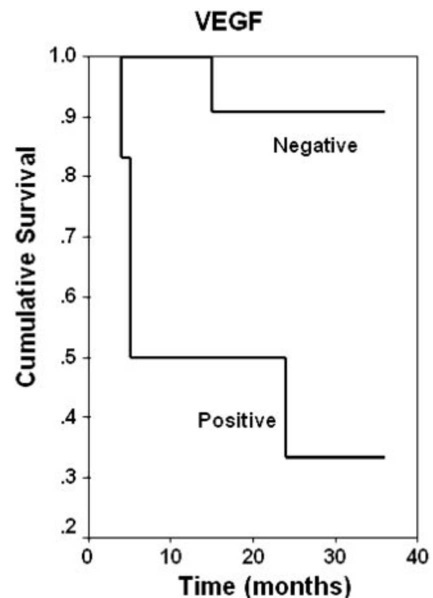

Fig. 4 Survival probability stratified according to the vascular endothelial growth factor (VEGF) staining (positive *versus* negative).

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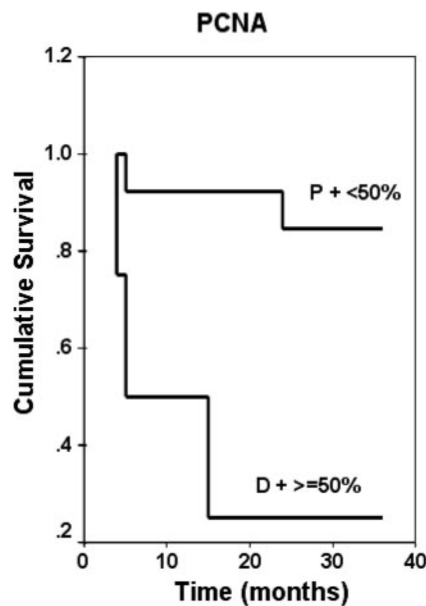

Fig. 5 Survival probability stratified according to the proliferating cell nuclear antigen (PCNA) staining (patchy and <50% positive cells in tumor *versus* diffuse and ≥50% positive cells in tumor).

expression. Furthermore, confirming our previously published studies demonstrating that pRb2/p130 expression down-regulates VEGF expression *in vitro* and *in vivo* (40), we have established, for the first time, the clinical possibility of a mutual exclusivity of pRb2/p130 and VEGF expression in HCC specimens ($P = 0.049$).

DISCUSSION

The prognosis of HCC-suffering patients still remains poor, although by studying them in the clinical setting, many advances have been made (41). With new emerging technologies in cancer biology, the pathological and biological prognostic factors of HCC have been studied quite extensively. It is universally accepted that biomarkers can be measured and evaluated as indicators of normal biological or pathological processes or as pharmacological response to a therapeutic intervention. Prognostic molecular biomarkers are invaluable for the clinician to evaluate patients and to aid in tumor control. Molecular indicators for the HCC malignancy phenotype include alterations in DNA ploidy, nuclear morphology, and the expression levels of proteins involved in cellular proliferation such as tumor suppressors and cell cycle regulators, apoptotic factors, telomerase activity, adhesion molecules, extracellular matrix proteinases, and angiogenic factors (17, 18, 24, 25, 27, 30, 37, 39, 42). Molecular surveillance of at-risk patients, those who are chronically infected with HBV or HCV or those who have cirrhosis, can result in earlier detection and more favorable treatment outcomes including increased survival. Cases with advanced tumors, impaired liver function, and generally poor health do not respond positively to treatments and typically receive palliative treatments only, whereas those with more favorable prognostic indicators receive an aggressive treatment regimen including surgical resection, *trans*-arterial chemo-embolization with various chemotherapeutic agents, and chemo-lipiodol treatments (43–46).

Liver transplant is a potentially curative HCC treatment. Although liver transplants do offer a safe and potentially effective treatment for patients in stage I, II, and III HCC, unfortunately, the availability of useable organs remains an issue (13).

In modern medicine, biomarkers are critical key elements for optimizing clinical treatment and drug discovery. Previous studies clearly demonstrated that PCNA expression is a reliable molecular biomarker for HCC. In fact, a progressive increase in the PCNA-labeling index from regenerative to dysplastic nodules to HCC has been observed (39). Additionally, an increase in the DNA index was correlated with an increase in PCNA labeling, and both were correlated with pathological changes in HCC tumors (47). PCNA staining in low-grade HCC tumors is typically <10%. However, in moderately differentiated malignancies, PCNA staining is observed in approximately 30% of the nuclei. Staining increases to ≥70% of tumor cells in high-grade malignancies (33, 39, 47). In our study, we scored PCNA staining according to its intensity from 0 to 4 and also scored the total percentage of PCNA-positive tumor cells. Exact Pearson χ^2-test analysis yielded a significant P of 0.007 for PCNA staining as a percentage of total tumor cells stained. Cumulative survival curves (Fig. 5) generated from these data showed that when a diffuse intensity and 50% of tumor cells were positively stained for PCNA, the cumulative survival rate decreased over time. These data served us as an internal quality control because PCNA expression has been shown to be a significant HCC prognostic biomarker.

Another established HCC biomarker is the cdk inhibitor $p27^{(KIP1)}$. $p27^{(KIP1)}$ has been emerging as a predictor of survival and tumor behavior. It has been suggested that $p27^{(KIP1)}$ loss occurs early in the carcinogenesis process (24). The more aggressive, metastasizing cancers tend to lack $p27^{(KIP1)}$ expression as well (18, 24). High $p27^{(KIP1)}$ expression, correlated with prolonged survival, is a favorable independent prognostic parameter for HCC (24, 27). Furthermore, it is known that HCC is formed by a heterogeneous cell population, and hence, protein expression may vary within the same tumor. Recently, it has been demonstrated that $p27^{(KIP1)}$ protein was frequently overexpressed in primary HCC and that longer disease-free survival rates were seen in patients whose tumors had higher $p27^{(KIP1)}$ expression (26). On the other hand, in a recent study it was shown that $p27^{(KIP1)}$ expression is reduced at relatively early evolutionary stages of HCC, and it was not associated with tumor stage (25). In our study, lack of p27 expression was observed in >90% of our cases (17 of our HCC samples), and it was independent from tumor stage, in accordance with Armengol *et al.* (25).

Those samples that stained positively for $p27^{(KIP1)}$ were not considered statistically significant ($P > 0.05$); therefore, we deduced that $p27^{(KIP1)}$ expression was not correlated in this cohort. It is possible that in our study group, hepatocarcinoma is independent from $p27^{(KIP1)}$ expression levels, suggesting another possible molecular scenario in which other protein products could be involved, leaving the following question open: are there any other biomarkers involved in HCC etiology? Recently, in our laboratory we were able for the first time to identify new

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target genes, the expression of which is modulated by pRb2/p130 (48). Moreover, we have demonstrated previously that RB2/p130 inhibits angiogenesis *in vitro* and *in vivo* by down-regulating VEGF expression at the transcriptional and translational levels (40). Our findings that higher VEGF and lower pRB2/p130 expressions correlated with survival are in agreement with our previous results (40). In fact we have shown, for the first time, a mutual exclusivity of pRb2/p130 and VEGF expressions.

Multiple risk factors are associated with HCC disease etiology, the highest incidence being in patients with chronic HBV and HCV, although other factors such as genetic makeup and environmental exposure such as aflatoxins are involved. Although it is conceivable to hypothesize that some of these agents could induce, either directly or indirectly, pRb2/p130 or VEGF expression, it is important to point out that to date, the relationship between HBV/HCV infection and VEGF expression is still unclear. In fact, in the last 4 years, different studies have highlighted some interesting but conflicting scenarios. One of these studies suggested that the presence of HCV infection increased *de facto* the expression of VEGF (49, 50). The second scenario suggested that in HCC samples infected by HCV, VEGF is down-regulated (51). A further complication is presented by the study of Shimoda *et al.* (52), which demonstrated that VEGF mRNA expression is not related at all to HBV and HCV infection in HCC patients.

Furthermore in HCC tissues, the mechanisms of HBV infection involved in VEGF modulation remain unknown. In fact, a study by Lee *et al.* (53) demonstrated that HBV-X protein can stimulate VEGF transcription. Nevertheless, Iizuka *et al.* (51) reported a minimal up-regulation of VEGF in HBV-positive samples by using a high-density microarray analysis of 45 HCC samples.

Moreover, to our knowledge, no *in vivo* study has been published showing the presence of HBV and HCV infection in HCC tissues and pRb2/p130 regulation. In light of this conflicting or absent evidence, there are no definitive data that HBV and HCV infections induce directly and/or indirectly VEGF and/or pRb2/p130 expression at this time. Additionally, very recently another paper reported that expression of the entire HCV genome in the primary liver cancer cell line HepG2 up-regulates the CDK-Rb-E2F pathway, causing pRb/p105 hyperphosphorylation and its subsequent protein degradation via the ubiquitin pathway (54). Our study demonstrating low expression of the retinoblastoma family member pRb2/p130 in either HBV- and/or HCV-positive HCC patients could be in part explained by a change in the phosphorylation status in Rb2/p130 similar to that which occurs *in vitro* with pRb/p105. More studies regarding pRb2/p130 phosphorylation status both *in vitro* and *in vivo* are needed to fully comprehend the potential prognostic power of the Rb family members together with VEGF expression in HCC.

To our knowledge, this is the first investigation in which VEGF and pRb2/p130 are suggested to be independent novel prognostic markers in HCC. More studies are needed to confirm the clinical significance of the inverse correlation between VEGF and pRb2/p130 expression patterns that together could be "*de facto"* new biomarkers in hepatocarcinoma and to confirm the role of HBV and HCV infection and VEGF/pRb2 gene regulation in hepatocarcinogenesis.

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