

Acquired Expression of p27 Is a Favorable Prognostic Indicator in Patients with Hepatocellular Carcinoma¹

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ABSTRACT

The p27 cyclin-dependent kinase inhibitor is a negative regulator of cell-cycle progression. In many human epithelial malignancies, decreased expression of p27 correlates with high grade, early recurrence, and poor prognosis. To evaluate the prognostic significance of p27 in hepatocellular carcinoma (HCC), we studied 54 HCCs along with corresponding nontumoral tissue. Immunohistochemistry (IHC) and Western blot (WB) analysis before and after immunoprecipitation with Cdk2 were performed on paraffin-embedded tissues and protein homogenates, respectively, to compare localization and expression of the p27 protein and to determine the total and active (Cdk2-bound) fractions of p27. Correlations were analyzed between IHC-assessed levels of p27, survival, and major clinical and pathological variables. IHC revealed no p27 expression in the majority of hepatocytes from normal and cirrhotic liver, whereas 14 HCCs (26%) were high p27 expressers (>50% positive cells), 26 (48%) low expressers (<50% positive cells), and 14 (26%) negative. High IHC signals of p27 correlated with Cdk2-bound p27 as assessed by immunoprecipitation-WB; by contrast, WB alone displayed similar levels of p27 protein in all normal and tumoral samples. High IHC p27 expression correlated with prolonged survival ($P = 0.027$), whereas the presence of cirrhosis was associated with poor outcome ($P = 0.029$). We conclude that with respect to their nonneoplastic counterparts, a subset of HCCs acquire significant p27 expression and that high expression of p27 is a favorable independent prognostic parameter for HCC.

Received 1/24/00; revised 6/20/00; accepted 7/14/00.

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¹ This research was supported by the Ministero della Ricerca Scientifica e Tecnologica (MURST) and Università degli Studi di Bologna.

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INTRODUCTION

Cell-cycle progression is regulated by sequential activation and inactivation of a series of Cdks.³ In association with various cyclins, different Cdks regulate progression through various stages of the cell cycle (1, 2). Cyclin-Cdk complexes are in turn regulated by phosphorylation events and Cdk inhibitors (2). Cdk inhibitors can be divided into two structurally related families: the Ink4 group of proteins (p15, p16, p18, and p19), which inhibit cyclin D/Cdk4–6 complexes (3–5), and the Cip/Kip group (p21, p27, and p57), which share partial structural homology and preferentially inhibit cyclin-Cdk complexes that contain Cdk2 (2).

The p27 gene was first identified in G₁ cells arrested by transforming growth factor- β (6–8). 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 (9). The function of p27 as a negative regulator of the cell cycle, suggests its putative role as a tumor suppressor gene. *In vivo*, p27 knockout mice develop generalized hyperplasia and pituitary tumors (10–12). In addition, heterozygous mice that are haploinsufficient for p27 have increased susceptibility for tumors (13). However, no homozygous deletions and only rare point mutations have been found in the p27 gene in humans (14–16). Reduced p27 levels in colon and lung carcinomas result from an accelerated proteolytic degradation via the ubiquitin-proteasome pathway (17). Lost or decreased p27 protein expression has been correlated to aggressive behavior in human cancers of the breast, gastrointestinal tract, prostate, and lungs (18–29).

The progression of human HCC has been putatively related with various cell cycle regulators (30, 31). In particular, overexpression of the p27 protein has been observed in confluent differentiating cells of the HBG hepatoma cell line and during experimental hepatic regeneration, providing further evidence that p27 may play a regulatory role in the liver *in vivo* (32, 33). It recently has been reported that p27 expression could be an independent prognostic marker for disease-free survival in HCC (34).

The present study analyzed the expression of p27 in 54 human HCC specimens and in corresponding nonneoplastic tissues. Tissues from 17 cirrhotoses with different etiologies were also included as nonneoplastic controls. We show that p27 is not expressed by normal hepatocytes, but that a subset of HCCs are high expressers of p27; the patients bearing these tumors fared better, independently of other variables.

³ The abbreviations used are: Cdk, cyclin-dependent kinase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HBV, hepatitis B virus; IHC, immunohistochemistry; WB, Western blot; IP, immunoprecipitation; AU, arbitrary units; RR, risk ratio.

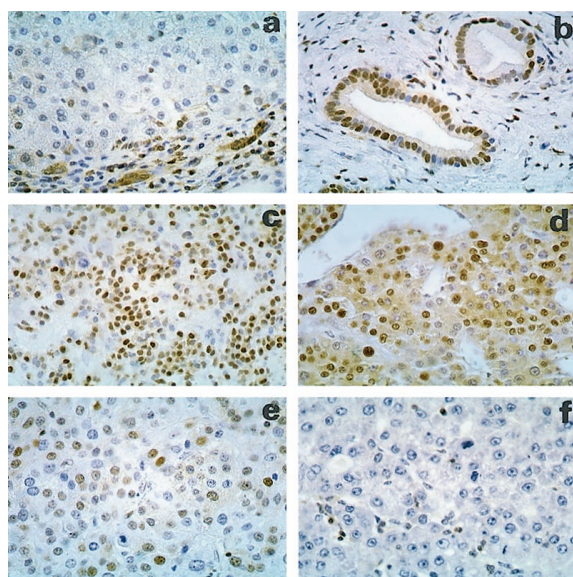


Fig. 1 IHC detection of the p27 protein in different benign and malignant liver tissues. *a*, cirrhotic liver shows positive immunostaining for p27 in proliferating bile ducts and in most inflammatory cells but only rarely in hepatocytes. *b*, p27 is also expressed in most nuclei of differentiated bile-duct cells. Solid type, grade III (*c*) and trabecular type, grade III (*d*) HCCs display, respectively, strong nuclear and mixed nuclear/cytoplasmic positivity for p27 in >50% of the neoplastic cells (high p27 expressers; IHC score 2). *e*, solid type, grade II HCC with <50% p27-positive nuclei (low p27 expresser; IHC score 1). *f*, no p27 IHC signal is detectable in the neoplastic hepatocytes of a grade III HCC (IHC score 0). Inflammatory cells represent the internal positive control (3,3'-diaminobenzidine-hematoxylin; original magnification, $\times 25$).

MATERIALS AND METHODS

Patient Population. We performed a retrospective study on 54 patients (44 men, 10 women; mean age, 63 years) who underwent liver resection for HCC between 1985 and 1997 in our hospital for whom complete survival data were available. Pathological, demographic, and survival data of the patients were retrieved from the files of the Pathology Unit at the "Addarii" Institute of Oncology, University of Bologna. Thirty-seven of 54 HCCs arose in cirrhotic liver. All tumors were histologically diagnosed and graded according to the Edmondson's scale (35) and grouped as well differentiated (grade I-II; $n = 17$) or poorly differentiated (grade III-IV; $n = 37$). Tumors were also grouped according to size as < 5 cm ($n = 35$) and ≥ 5 cm ($n = 19$). Twenty-nine patients (54%) had HCV infection, 13 (24%) HBV infection, and 5 (9%) had a history of alcohol abuse. The remaining 7 (13%) had chronic liver diseases unrelated to HCV, HBV, or alcohol. The follow-up interval ranged from 4 to 154 months from the date of surgery (mean, 50 months). None of the patients received postoperative adjuvant therapy. Cirrhotic tissues from 17 patients with no concomitant HCC were also selected as nonneoplastic controls. Among these cirrhotoses six were HCV correlated, six were HBV correlated, and five were associated with alcohol abuse. After surgical resection, specimens from neoplastic and nonneoplastic surrounding tissue were fixed in 10% buffered formalin. In 20 patients, snap-frozen immediately adjacent tissue (normal and

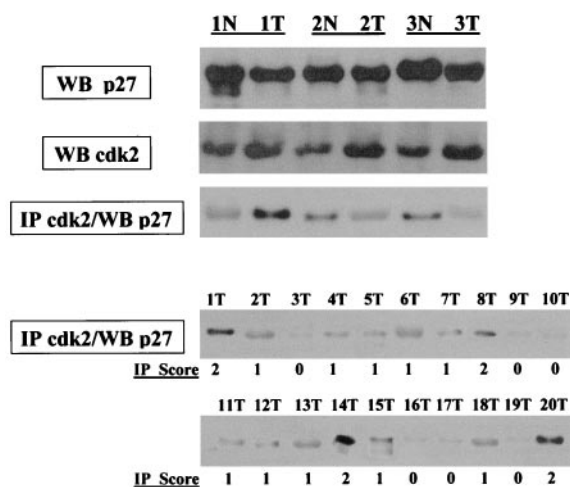


Fig. 2 Comparison among WB for p27, WB for Cdk2, and IP for Cdk2 plus WB for p27 (*IP cdk2/WB p27*) of protein homogenates from three normal-tumor liver pairs and 20 HCC tumor cell lysates. WB before IP shows p27 and Cdk2 bands with similar intensity in normal/tumor pairs. Conversely, p27 bands after IP for Cdk2 are absent (IP score 0), weak (IP score 1), or strong (IP score 2) in tumor samples. IP score based on densitometric values is provided for all 20 HCC samples. Products of p27 degradation are detectable in some samples. N, normal; T, tumor.

tumoral) was available for protein extraction. Informed consent was obtained from all patients.

IHC. Tissue sections (4 μ m) were cut, placed on silane-pretreated slides, deparaffinized, and rehydrated through graded alcohol. Antigen retrieval was performed by microwave heating at high power (750 W) in 10 mM sodium citrate buffer (pH 6) for four cycles of 5 min each. Slides were then allowed to cool for 30 min prior to incubation for 1 h at room temperature with the p27 monoclonal antibody (Transduction Laboratories, Lexington, KY) diluted 1:200. Staining was performed with the Envision monoclonal System (Dako, Carpinteria, CA). The reaction was developed with 3,3'-diaminobenzidine. Slides were counterstained with Mayer's hematoxylin and mounted. Nonimmune mouse serum was used as negative control. Lymphocytes were used as the internal positive control for p27. Identical reaction times allowed accurate comparison of all samples. IHC staining was evaluated independently by two pathologists (M. F. and A. D.).

p27 immunostaining was scored as the ratio of strongly positive nuclei to the total number of cells as described previously (18–21): score 0, no staining; score 1, <50% positive nuclei; score 2, $\geq 50\%$ positive nuclei. At least 20 high-power fields were randomly chosen, and 2000 cells were counted.

WB Analysis. In the 20 cases where snap-frozen tissue was available, histological analysis of the tissues used for WB confirmed the presence of only a minimal stromal or necrotic component in the tumor samples. Two-hundred mg of each sample were promptly homogenized at 15,000 rpm with a Polytron homogenizer (PT 3000; Brinkman, Westbury, NY) in 1 ml of ice-cold lysis buffer containing PBS, 0.1% Triton X-100, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 5 μ g/ml leupeptin, 1 μ g/ml aprotinin, 5 μ g/ml antipain, 1 μ g/ml

Table 1 p27 expression and clinicopathological parameters in 54 HCC specimens

	Total	p27 expression		<i>P</i> ^a
		High (%)	Low or no (%)	
Age (yr)				
<65	28	7	21	0.87
≥65	26	7	19	
Sex				
M	44	12	32	0.64
F	10	2	8	
Tumor grade (Edmonson's)				
I, II	17	5	12	0.69
III, IV	37	9	28	
Tumor size (cm)				
<5	35	11	24	0.21
≥5	19	3	16	
Liver status				
Noncirrhotic	17	5	12	0.69
Cirrhotic	37	9	28	

^a Statistical analyses were performed by the Pearson χ^2 test. *P* < 0.05 was considered significant.

pepstatin, and 10 μ g/ml trypsin-chymotrypsin inhibitor (all from Sigma, St. Louis, MO). Lysates were kept on ice for 30 min and then centrifuged at 13,000 rpm for 5 min at 4°C. Supernatants were collected, and protein quantification was performed using the DC (detergent compatible) protein assay (Bio-Rad, Hercules, CA). One hundred μ g of extracted proteins were heated at 95°C for 5 min in lysis buffer containing 1:5 bromophenol blue (Sigma) and electrophoresed in a 10% SDS-PAGE gel for 2 h at 45 mA. Proteins were then transferred to Hybond 0.45 μ m nitrocellulose membrane (Amersham, Buckinghamshire, England) at constant voltage (40 V) overnight at 4°C in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol). Blots were then incubated with either the polyclonal anti-Cdk2 antibody (Santa Cruz Laboratories, Santa Cruz, CA) or the anti-p27 monoclonal antibody, diluted 1:1000 and 1:2500, respectively, for 1 h at room temperature. Signals were revealed with the Envision system (Dako), the reagent of which was diluted 1:500 for 1 h and developed using the ECL detection kit (Amersham).

IP-WB. For each sample, 300 μ g of protein homogenate was diluted in 1 ml of lysis buffer and precleared with 50 μ L of a 1:1 slurry of protein A-Sepharose (Sigma) in PBS on ice for 30 min. Samples were centrifuged for 2 min at 2000 rpm at 4°C, and the supernatants were incubated with the polyclonal anti-Cdk2 antibody, diluted 1:50, overnight at 4°C. After IP, complexes were recovered on protein A-Sepharose for 2 h in ice. Proteins were subsequently collected by brief centrifugation and then submitted to WB analysis for the detection with the anti-p27 monoclonal antibody. Control for the specificity of the IP method was performed by immunoprecipitating all of the cell lysates with the anti-p27 monoclonal antibody followed by probing of the blots with the anti-Cdk2 polyclonal antibody.

Densitometry. The intensities of each band on WB and IP-WB were quantitated on a RBR Altair (Florence, Italy) densitometer using the Autogel image analysis software program. The values of p27 bands were evaluated after normalization for the intensity of a reference sample that was added to

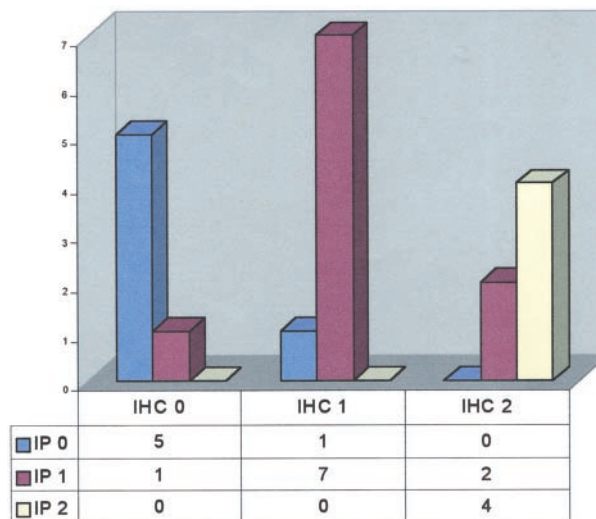


Fig. 3 Comparison between p27 IHC score and p27 band intensity score after IP with the anti-Cdk2 antibody. High p27 IHC scores ($\geq 2+$) correlate significantly with increased densitometric values of the p27 bands at IP-WB (score 2; *P* = 0.00).

each run and scored in AU. IP-WB band intensities were grouped as 0 (0–0.3 AU), 1+ (0.3–1 AU), and 2+ (>1 AU) using a non-IP WB p27 band as reference (2+) standard.

Statistical Analysis. Contingency table methods were used to analyze the univariate association between p27 and other variables (age, sex, tumor grade, tumor size, and liver status) and the univariate correlation between survival status and all of the above-mentioned variables. Significance was evaluated by Pearson's χ^2 test and confirmed by Fisher's exact test. Logistic regression was also performed to assess survival with respect to the variables. Survival curves were calculated using the Kaplan-Meier method, and the log-rank test was used for analysis. Patients who died of other causes during the follow-up period were treated as censored data in the survival analysis. Univariate and multivariate RRs were calculated using Cox proportional hazards regression. The RRs for age are represented as age ≥ 65 versus <65 years. For sex, the RRs are given as male versus female. For grade and dimensions, Edmondson's grade I-II and tumor size <5 cm were used as baselines, respectively. All calculations were performed using the SPSS 8.0 statistical software package, and the results were considered statistically significant at *P* < 0.05.

RESULTS

Low Expression of the p27 Protein in Nonneoplastic Liver Tissues. IHC staining for p27 was found in <5% of the hepatocytes from cirrhotic and normal liver tissues. Conversely, inflammatory cells, mature bile-duct cells, and the neo-ductules at the periphery of the cirrhotic nodules strongly stained with the p27 antibody in the same sections (Fig. 1, *a* and *b*). The localization of the p27 protein was nuclear in the rare positive hepatocytes and in all of the mature bile-duct cells, whereas it was mixed nuclear/cytoplasmic in the neo-ductules and inflammatory cells. No difference was found in the cirrhotic tissues

Table 2 Survival status and clinicopathological parameters in 54 HCC specimens

	Total	Survival status		<i>P</i> ^a
		Alive	Dead	
Age (yr)				
<65	28	11	17	0.013 ^b
≥65	26	19	7	
Sex				0.69
M	44	25	19	
F	10	5	5	
Tumor grade (Edmondson's)				0.36
I, II	17	11	6	
III, IV	37	19	18	
Tumor size (cm)				0.80
<5	35	19	16	
≥5	19	11	8	
Liver status				0.007 ^b
Noncirrhotic	17	14	3	
Cirrhotic	37	16	21	
p27				0.008 ^b
High expression	14	12	2	
Low or no expression	40	18	22	

^a Statistical analyses were performed by the Pearson χ^2 test. *P* < 0.05 was considered significant.

^b The same value was also obtained when the Fisher's exact test was used for the analysis.

adjacent to HCC compared with cirrhotoses without HCC. Foci of both small- and large-cell hepatocellular dysplasia showed p27 expression comparable to the other cirrhotic nodules. WB for p27 was positive in all normal liver samples (Fig. 2).

p27 Protein Expression in HCC. The 54 HCC samples showed a heterogeneous pattern of positivity for p27 both in terms of the percentage of positive cells and subcellular localization. p27 was scored as positive only when strong nuclear immunostaining was seen. In 14 of 54 (26%) HCCs there was no p27 (score 0; Fig. 1f). Twenty-six of 54 (48%) were low expressers (score 1; Fig. 1e) and 14 of 54 (26%) expressed high levels of p27 (score 2; Fig. 1, c and d). Diffuse cytoplasmic immunostaining was also noted in 24 cases. Of these, 8 were high nuclear expressers and 14 were low nuclear expressers. In two cases, the p27 immunostaining was exclusively cytoplasmic. Among the 14 high p27 expressers, 5 were grade I-II and 9 were grade III-IV HCCs. Among the low expressers, 13 were grade I-II and 27 were grade III-IV (Table 1). Twenty HCC samples showed either p27 or Cdk2 bands with similar intensity by WB (Fig. 2).

Cdk2-bound p27 Levels Correlate with Detectable p27 in Tumors. To determine the amount of the Cdk2-bound fraction of p27, WB analysis was performed after IP with the anti-Cdk2 antibody on the same cell lysates used previously for WB (Fig. 2). IP-WB revealed the presence of a p27 band in all 20 normal samples examined, with similar densitometric values ranging from 0.6 to 1.9 AU. Conversely, samples from the tumor counterparts in the same cases displayed different intensities of the p27 bands (see "Materials and Methods" for AU intervals). In particular, 6 of 20 HCC (30%) samples showed no signal (densitometric value, 0), 10 of 20 (50%) displayed a weak band (densitometric value, 1+), and the remaining 4 (20%) had a strong p27 band (densitometric value, 2+; Fig. 2). Detection

of the p27 band after IP with Cdk2 revealed a positive highly statistically significant correlation (*P* = 0.00) to high expression of p27 by IHC (Fig. 3). In particular, the four cases that displayed a strong band (densitometric value, 2+) in IP-WB analysis were all high p27 expressers in IHC (score 2). In addition, the six cases that displayed a densitometric value of 0 after IP were low p27 expressers by IHC (score for five cases, 0; score for one case, 1). Control IP-WB performed by immunoprecipitating tumor cell lysates with p27 followed by probing with Cdk2 revealed bands with similar intensities in all cases (data not shown).

p27 Is an Independent Prognostic Parameter for HCC.

Based on IHC positivity, patients were divided into two groups: high p27 expressers (IHC score 2) and low p27 expressers (IHC score 0–1). p27 expression did not correlate significantly with gender, age, tumor grade, tumor size, or liver status (background of normal liver *versus* cirrhosis; Table 1). No significant correlation was found between viral or alcoholic etiology (data not shown). Concerning survival, only 2 of 14 (14%) patients in the high-expresser group died of disease *versus* 22 of 40 (55%) in the low-expresser group (Table 2). When all variables were compared separately to survival status, only p27 (*P* = 0.008), the presence of cirrhosis (*P* = 0.007), and age (*P* = 0.013) significantly influenced survival (Table 2). Logistic regression confirmed that cirrhotic status implies a 5.9-fold higher risk of mortality with respect to noncirrhotic status. Low or absent p27 expression had a 10:1 higher risk of mortality than high expression.

In univariate analysis, the Kaplan-Meier survival curves did not show any significant relationship between tumor grade (*P* = 0.313, log-rank test) or size (*P* = 0.913, log-rank test) and survival. Conversely, the Kaplan-Meier survival curves of low *versus* high expressers of p27 showed a highly significant separation (*P* = 0.012; Fig. 4), as did those of cirrhosis *versus* non-cirrhosis (*P* = 0.015) and age ≥65 years *versus* <65 years (*P* = 0.021). When a multivariate Cox proportional hazard model was constructed (including gender, age, tumor grade, tumor size, liver status, and p27 expression), p27 was the strongest independent predictor of survival (*P* = 0.027; RR = 5.313), the second predictor being liver status (Table 3).

DISCUSSION

The worldwide incidence of HCC is ~1 million cases a year, and its prognosis is poor (36). Pathological features such as tumor grade and size are the most frequently used prognostic parameters for HCC (37–39). Proliferative index, p53 mutations, and the expression of cell cycle-related genes have also been proposed as negative predictors of biological behavior, and recently p27 has been proposed for independent prediction of clinical recurrence (31, 34, 40, 41).

Our data confirmed that HCC patients who express p27 fare better than those who do not. This is in keeping with recent data suggesting a strong predictive value for p27 in HCC and other human epithelial tumors (18–29, 34). Univariate analysis showed that p27 expression does not correlate with any of the clinicopathological parameters analyzed in this study. In multivariate analysis, p27 expression was the strongest predictor of survival for HCC patients, independent of the other variables.

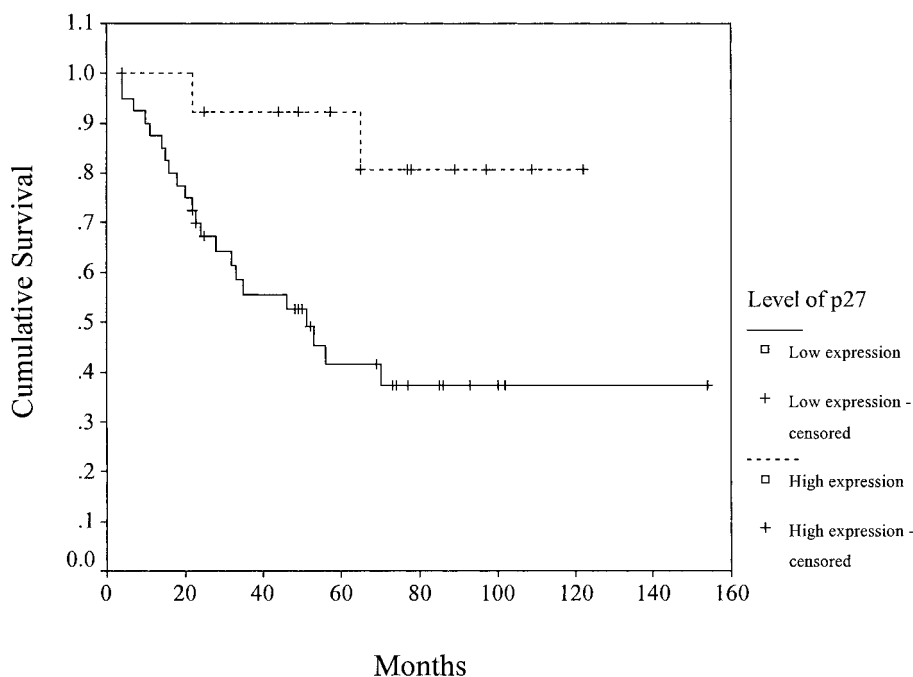


Fig. 4 Kaplan-Meier survival curves for low IHC expressers versus high IHC expressers of p27 in 54 patients with HCC showed a highly significant separation ($P = 0.012$, log-rank test).

Table 3 Contribution of various potential prognostic factors to survival by Cox regression analysis in 54 HCC specimens

	RR	95% confidence interval	P^a
Age (yr)	0.359	0.142–0.909	0.031
Sex	1.294	0.446–3.753	0.636
Tumor size	1.703	0.677–4.285	0.258
Liver status	4.203	1.152–15.330	0.029
p27 ^b	5.313	1.214–23.250	0.027

^a Statistical analyses were performed by the log-rank test. $P < 0.05$ was considered significant.

^b The RR is given as low or no vs. high expression.

Not unexpectedly, a significant association between presence of cirrhosis and poor prognosis was also found (42). In fact, in cirrhotic patients a more extensive surgical resection increases the risk of liver failure, and development of a further HCC is also more likely.

High expression of nuclear p27 was generally found in bile-duct cells of both cirrhotic and noncirrhotic livers but only rarely in hepatocytes. Despite the fact that hepatocytes have a very low proliferative rate, only low levels of p27 were observed by IHC in normal tissues. Lack of correlation between p27 protein levels and proliferative rates has been extensively demonstrated in a variety of tumors. Quiescent cells in normal tissues generally express nuclear p27. However, it is unclear whether p27 is expressed to maintain cells in G_0 or as a result of the induction/maintenance of differentiation. In fact, colonic epithelial cells express p27 in superficial terminally differentiated cells, whereas benign breast epithelial cells commonly express high levels of p27 (18, 21). It is possible that hepatocytes are kept in the resting state by other cell-cycle inhibitory

proteins. One possible explanation is that similarly to what happens in esophageal dysplasia, up-regulation of p27 in HCC occurs in the presence of increased proliferative signals (27). Its up-regulation may then result in a more controlled growth pattern and consequently in less aggressive behavior.

Induction of p27 in HCC results in its binding to Cdk2 (2). This further underscores the hypothesis that increased expression of p27 occurs as an attempt to block Cdk2 activity in tumor cells. Our results showed that high p27 expression in tumor cells by IHC correlated with increased p27 binding to Cdk2 on tumor samples by IP-WB. The failure to show differences in the p27 levels by WB without IP among different normal and tumor samples was probably attributable to the presence of inflammatory, stromal, and bile-duct elements contaminating the cell lysates, as seen previously (20). Whereas the variable levels of Cdk2-bound p27 observed at IP-WB among our tumor samples may reflect an increase of p27 in the smaller proportion of cells that are truly malignant. On technical grounds, our data revealed that IHC is the most reliable technique for the detection of p27. Indeed, IHC was particularly sensitive for cellular localization of the p27 protein and assessment of its active fraction.

The significance of cytoplasmic p27 ICH localization is still a matter of debate and generally is not considered in the assessment of the p27 IHC score. However, in adenocarcinomas associated with Barrett's esophagus, the presence of cytoplasmic p27 was found to correlate with poor prognosis (27). These clinical data were corroborated recently by experimental studies on thyroid and liver cells showing that cytoplasmic sequestration of the p27 protein by cyclins D1 and D3 prevents it from penetrating the nucleus and exerting its inhibitory effect on the G_1 -S transition (43, 44). In our series of HCCs, reliable cytoplasmic p27 staining was observed in 44% of cases, mostly in association with the nuclear signal. Biochemical studies using

subcellular fractionation of protein lysates are needed to confirm the role of cytoplasmic localization in preventing p27 function in HCC.

The life expectancy of patients with HCC is generally poor because of a lack of truly effective therapeutic agents and the rarity of early diagnosis. Therapy consists of either surgery (liver resection or transplantation) or local treatments (chemo-embolization, alcoholization). Predictive studies recently have shown that the flavonoid antioxidant silibinin induces G₁ arrest in prostate cancer cells via an increased binding of p27 to Cdk2 (45). Furthermore, the anticancer agent indole-3-carbinol in combination with tamoxifen was found to arrest breast cancer cells in the G₁ phase via a decrease in Cdk2-specific enzymatic activity (46). In the meantime, we recommend IHC detection of p27 on routine tissue sections as a reliable new method to help predict survival of individual HCC patients and possibly to help direct future therapeutic strategies.

ACKNOWLEDGMENTS

We are grateful to Giulia Cavrini for statistical analysis and to Robin M. T. Cooke for editing.

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Clin Cancer Res 2000;6:3966-3972.

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