

# P53 immunohistochemical scoring: an independent prognostic marker for patients after hepatocellular carcinoma resection

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## Abstract

**AIM: To confirm if p53 mutation could be a routine predictive marker for the prognosis of hepatocellular carcinoma (HCC) patients.**

**METHODS: Two hundreds and forty-four formalin-fixed paraffin-embedded tumor samples of the patients with HCC receiving liver resection were detected for nuclear accumulation of p53. The percent of P53 immunoreactive tumor cells was scored as 0 to 3+ in P53 positive region (<10% -, 10-30% +, 31-50% ++, >50% +++). Proliferating cell nuclear antigen (PCNA) and some clinicopathological characteristics, including patients' sex, preoperative serum AFP level, tumor size, capsule, vascular invasion (both visual and microscopic), and Edmondson grade were also evaluated.**

**RESULTS: In univariate COX hazard regression model analysis, tumor size, capsule status, vascular invasion, and p53 expression were independent factors that were closely related to the overall survival (OS) rates of HCC patients. The survival rates of patients with 3+ for P53 expression were much lower than those with 2+ or + for P53 expression. Only vascular invasion ( $P<0.05$ ) and capsule ( $P<0.01$ ) were closely related to the disease-free survival (DFS) of HCC patients. In multivariate analysis, p53 overexpression (RI 0.5456,  $P<0.01$ ) was the most significant factor associated with the OS rates of patients after HCC resection, while tumor size (RI 0.5209,  $P<0.01$ ), vascular invasion (RI 0.5271,  $P<0.01$ ) and capsule (RI-0.8691,  $P<0.01$ ) were also related to the OS. However, only tumor capsular status was an independent predictive factor ( $P<0.05$ ) for the DFS. No significant prognostic value was found in PCNA-I, Edmondson's grade, patients' sex and preoperative serum AFP level.**

**CONCLUSION: Accumulation of p53 expression, as well as tumor size, capsule and vascular invasion, could be valuable markers for predicting the prognosis of HCC patients after resection. The quantitative immunohistochemical scoring for P53 nuclear accumulation**

**might be more valuable for predicting prognosis of patients after HCC resection than the common qualitative analysis.**

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## INTRODUCTION

Liver cancer has been ranked the 2<sup>nd</sup> cancer killer in China since 1990s. The age-standardized mortality rate in China is as high as 34.7/10<sup>5</sup>, which accounts for 53% of all liver cancer deaths worldwide<sup>[1]</sup>. Although many advances in its clinical study have been made, a definitive subset is cured by surgery only, and encouraging long-term survival of patients have been obtained in some clinical centers, the overall dismal outcome of patients with hepatocellular carcinoma (HCC) have not been completely changed<sup>[2,3]</sup>. Lack of control of metastatic foci and recurrence is the most prevalent cause of death in patients with HCC, and it is important to identify the factors that predispose patients to death. Much effort has been made to predict HCC behavior, but there is still lack of specific prognostic indicators.

Prognostic factors in HCC conventionally consist of staging with the tumor node metastasis system (TNM) and grading by tumor cellular differentiation. With new discoveries in the cancer biology, pathological and biological factors of HCC in relation to prognosis have been studied quite extensively. Morphological features of the tumor, both gross and histological, are found to be significantly associated with tumor recurrence and patient survival. A complementary approach is to analyze the HCC for molecular markers with prognostic significance with reference to the recurring possibility and survival time. A large number of molecular biological factors have been shown to be associated with the invasiveness of HCC, and have potential prognostic significance, e.g. c-erbB-2, uPA, PAI-I, VEGF, CDKN2 and p53 mutations, p53 antibody, H-ras, mdm-2, TGF  $\alpha$ , EGFR, VEGF, bFGF, PD-ECGF, MMP-2, ICAM-1 are positively related to HCC invasiveness, whereas nm23-H1, Kai-1, TIMP-2, Integrin  $\alpha$ 5 and E-cadherin are negatively relative factors<sup>[4]</sup>. Although a great amount of markers have been tried, a routine biomarker for prediction of prognosis of HCC is not yet available.

As early as in 1995, we found that p53 mutations were related to the invasiveness of HCC. Thereafter, we performed immunohistochemical staining for p53 overexpression in the surgical specimen of HCC patients treated in our institute, to see if p53 mutation could be a routine predictive marker for the prognosis of HCC patients. In the present study, we reviewed the results of the studies over the past 5 years, and evaluated whether nuclear accumulation of p53 would be a feasible prognostic marker in the routine diagnostic evaluation of HCC, in particular, to analyze the relationship between p53 overexpression and survival of patients with HCC.

## MATERIALS AND METHODS

### Tumor samples and HCC patients

Totally, 256 consecutive patients with HCC were enrolled in this study. All of them were surgically treated in the Liver Cancer Institute of Fudan University (former Liver Cancer Institute of Shanghai Medical University), Shanghai, China in the years 1996-1999. Except for 12 cases without informative follow-up data, 244 cases were reviewed, which included 197 male (80.7%) and 47 female (19.3%). The mean age was 50.4 (15-76) years. All specimens were formalin-fixed, paraffin-embedded, which were proved to be hepatocellular carcinoma (HCC). Some histological characteristics including the states of capsule, vascular invasion (both visual and microscopic), cell differentiation were reviewed by one pathologist. The mean tumor size was 5.9 (1-16)cm,  $\leq 5$ cm in 131 cases (53.7%),  $>5$ cm but  $\leq 8$ cm 56 cases (23.0%), and  $>8$ cm 57 cases (23.3%). The tumor of 128 cases (52.5%) had no capsule, and 116 cases (47.5%) were well-capsulated. The Edmondson grade distribution was grade I in 7 (2.9%) cases, Grade II-III in 235 (96.3%) cases, and grade IV in 2 (0.8%) cases. Vascular invasion was found in 69 cases (28.3%), which included visual tumor thrombi in portal vein in 31 cases and microvascular invasion in 38 cases. Obvious evidence of liver cirrhosis was found in 217 (88.9%) cases, no cirrhosis in 27 (11.1%) cases. Two hundred and twenty-two cases (91%, 222/244) were followed up. The mean follow-up time was 21.6 (2.2-49) months.

### Immunohistochemistry

A standard indirect immunoperoxidase protocol was used for immunohistochemistry (ABC-Elite; Vector Laboratories, Inc., Burlingame, CA). Monoclonal anti-p53, PCNA antibodies (Dako) were used for detection of p53 and PCNA, respectively (1:200 dilution in PBS containing 1% bovine serum albumin and 0.1% Triton X-100). A high-temperature (20 minutes in a pressure cooker) treatment procedure with antigen unmasking solution (Vector Laboratories, Inc.) was used to enhance the staining. The primary antibody was omitted for negative controls.

We used the percentage of p53-positive tumor nuclei in all major foci of cancer as p53 immunohistochemical scoring system. The percent of p53 immunoreactive tumor cells was scored as 0 to 3+ in p53 positive regions. Nuclear p53 expression in  $\geq 10\%$  of tumor cells was scored as aberrant overexpression,  $<10\%$  -, 10%-30% +, 31%-50% ++, and  $>50\%$  +++.

The patients' sex, serum AFP level, and the pathological characteristics of tumor, including tumor size, capsule, vascular invasion (both visual and microscopic), Edmondson grade, etc. were also evaluated.

### Statistical analyses

The log-rank test, Kaplan-Meier analysis and univariate and multivariate Cox regression modeling were used for evaluation of contribution of the variables to relapse and disease-specific survival. Overall and disease-free survival rates were calculated with the Life-Table method, and the survival time was calculated from the operative date. The survival curves were estimated by Kaplan-Meier analysis. The prognostic significance of these markers was analyzed using the log-rank test. A Cox regression analysis was performed to show the relationship of the markers studied with the overall and disease-free survival rates of the HCC patients, and to identify the prognostic factors of the HCC patient after operation. The results were correlated with clinicopathological parameters and the prognosis evaluated by uni- and multi-variate analysis using local control, freedom from distant metastasis, disease-free survival, and overall survival as endpoints.

## RESULTS

The 1-, 3- and 5-year overall survival rates of the 244 cases of HCC patients studied were 81.9%, 57.6% and 48.7%, respectively. And, the 1-, 3- and 5-year disease-free survival rates were 55.2%, 38.3%, and 32.2%, respectively. p53 immunohistochemical staining was heterogeneous in the HCC tissues, and nuclear staining for p53 were found in 112 of the 222 (50.5%) cases. The 1-, 3- and 5-year overall survival rates of the HCC patients with positive p53 nuclear accumulation were 81.2% (91/112), 50.9% (57/112), and 33.0% (37/112), while those of the HCC patients with negative p53 expression were 88.8%, 66.3% and 60.6%, respectively. Furthermore, among the patients with positive P53 expression, those with 3+ ( $>50\%$ ) for P53 immunohistochemical scoring had a poorest prognosis, their 1-, and 3-year overall survival rates were only 38.5%, and 12.3%, which were much lower than those with 2+ (60.0% and 46.7%, respectively) and those with + (83.5% and 57.3%, respectively). Therefore, the score of P53 overexpression was adversely related to the survival rates of HCC patients (Table 1).

**Table 1** Relationship between some clinicopathological parameters and overall survival rates of HCC patients (a univariate analysis)

Parameters	n	Overall survival rates %		
		1-year	3-year	5-year
<b>P53 expression</b>				
-	110	88.8	66.3	60.6
+	86	83.5	57.3	42.9
++	13	60.0	46.7	
+++ <sup>b</sup>	13	38.5	12.3	
<b>Tumors size (cm)</b>				
$\leq 5$ cm	122	92.6	69.0	55.8
$>5$ , $\leq 8$ cm	50	78.0	63.0	55.1
$>8$ cm <sup>b</sup>	50	60.0	23.9	
<b>Vascular invasion</b>				
No	160	88.7	68.5	60.7
Microscopic	36	80.6	47.0	
Visual	26	42.3	7.5	
<b>Capsule</b>				
No	112	74.0	42.0	26.4
Well <sup>b</sup>	110	90.5	75.9	75.9

<sup>b</sup>P<0.01

The overall survival rates of patients after radical resection of small HCC ( $\leq 5$ cm) were higher than those  $>5$ cm and  $\leq 8$ cm in diameter. The 3-year survival rate of those with tumor  $>8$ cm in diameter (23.9%) was much lower than those with tumor  $\leq 8$ cm in diameter (63.0%) (Table 1). The 1- and 3-year overall survival rates of the HCC patients with vascular invasion (both visual and microscopic) were 64.5% (42/62) and 30.6% (19/62), respectively. The patients with visual tumor thrombi in portal vein had a poorer prognosis, their 1- and 3-year overall survival rates were only 42.3% (11/26) and 7.5% (2/26), respectively. No 5-year survival was found. The 1-year, 3-year, and 5-year overall survival rates of patients without vascular invasion were 88.7%, 68.5%, and 60.7%, respectively, which were much higher than those with vascular invasion (P<0.01). A similar situation was also found with disease-free survival (Table 1). The 1-, 3-, and 5-year overall survival rates of patients with well-capsulated HCC were 90.5%, 75.9%, and 75.9%, respectively, while those of the patients without tumor capsule were 74.0%, 42.0%, and 26.4% Table. No significant relationship

between the PCNA-LI, Edmondson grade, patients' sex, or serum AFP level, and overall or disease-free survival rates was found ( $P>0.05$ ).

In univariate Cox hazard regression model analysis, tumor size, capsule status, vascular invasion, p53 expression were independent factors that were closely related to the overall survival rates of HCC patients, while no obvious relationship was found between the PCNA ( $P>0.05$ ) expression and the overall survival. Only vascular invasion ( $P=0.0187$ ) and tumor capsule ( $P=0.0059$ ) were closely related to the disease-free survival of HCC patients, no obvious relationship was found between p53, PCNA status and the disease-free survival (Tables 1, 2).

Similarly, in multivariate analysis, the p53 (RI 0.5456,  $P<0.01$ ) was the most significant factor associated with the overall survival rates of HCC patients after resection. Tumor size (RI 0.5209,  $P<0.01$ ), vessel invasion (RI 0.5271,  $P<0.01$ ) and capsule (RI-0.8691,  $P<0.01$ ) were also related to the overall survival. For the disease-free survival, tumor capsule status remained the only independent predictive factor ( $P<0.05$ , Table 3). No significant prognostic value was found in the PCNA-LI, Edmondson grade, or patients' sex, serum AFP level for the overall or disease-free survival both in univariate and multivariate Cox analyses.

**Table 2** Parameters affecting the disease-free survival rates of HCC patients (a univariate analysis)

Parameters	n	Disease-free survival rates (%)		
		1-year	3-year	5-year
Vascular invasion				
No	116	93.9	64.6	54.2
Yes <sup>a</sup>	22	72.7	37.0	
Capsule				
No	57	82.3	44.9	44.9
Yes <sup>b</sup>	81	96.3	73.2	

<sup>a</sup> $P<0.05$ , <sup>b</sup> $P<0.01$ , vs No.

**Table 3** Relationship between some clinicopathological parameters and overall survival rates of HCC patients (a multivariate analysis)

	Correlation coefficient	Wald	Standard error	P
P53 expression	0.55	18.88	0.13	<0.01
Tumor size	0.52	14.97	0.13	<0.01
Vascular invasion	0.53	11.85	0.15	<0.01
Tumor capsule	-0.87	10.30	-0.10	<0.01

## DISCUSSION

It is difficult to predict the prognosis of patients with HCC, because so far, there is no any specific marker for that yet. Assessment of the clinicopathological and biological malignancy of HCC may help predict outcome. Some pathological features, such as size of the tumor, vascular invasion, fibrous capsule infiltration, and intrahepatic metastasis are thought as prognostic factors for HCC<sup>[5]</sup>. New invasiveness scoring system has been proposed based on the items such as venous invasion, tumor capsule, intrahepatic spreading, etc. In our previous report, tumor size was the most important factor for the prognosis of HCC patients and postoperative recurrence possibility<sup>[3]</sup>. In this study, in univariate Cox hazard regression model analysis, tumor size, capsule status, and vascular invasion were independent factors which were closely related to both of the overall and disease-free survival rates of HCC patients. Similarly, in multivariate analysis, they were also independent prognostic factors for the overall survival, and the tumor capsule status and vascular invasion were predictive factors for disease-free survival. All these further confirmed

the significance of pathological characteristics of tumor itself in the survival of HCC patients, and the importance of early detection, early diagnosis and early treatment of HCC.

Some biomarkers such as the tumor DNA content, P53 protein expression, proliferating cell nuclear antigen (PCNA) labeling index, and argyrophilic proteins of nuclear organizer regions were used as markers of biological malignancy. P53 protein plays a central role in cellular responses, including cell-cycle arrest and cell death in response to DNA damage. p53 dysfunction can induce abnormal cell growth, increased cell survival, genetic instability, and drug resistance. p53 mutations occur in approximately half of human cancers. Associations of p53 mutation or positive immunohistochemical stain with higher grade and more advanced stage are common. p53 mutation has been found related to advanced tumor stage in cancers of endometrium, cervix, ovary, liver, prostate and bladder, indicating that for these tumors p53 mutation may be a late event contributing to tumor progression<sup>[6]</sup>. And p53 mutation has been reported to be a strong marker predicting an increased risk of local relapse, treatment failure, and overall and disease-free survival in many kinds of human carcinomas, such as breast<sup>[7-11]</sup>, colon-recta<sup>[12,13]</sup>, esophagus<sup>[14]</sup>, head and neck<sup>[15]</sup>, lung<sup>[16-18]</sup>, ovarian<sup>[19]</sup>, as well as sarcoma<sup>[20]</sup>. An increased intracellular concentration of the P53 protein, although not identical to, is sometimes seen in tumors with p53 mutation and correlated with poor prognosis in some tumors. Several studies have shown a relationship between the nuclear accumulation of p53 protein and poor disease-free and overall survival of prostate cancer<sup>[21,22]</sup>, and oral cancer<sup>[23]</sup>. Detection of micrometastasis of the regional lymph nodes of ovarian cancer by immunohistochemical staining of P53 protein may be useful in predicting the prognosis of patients with stage I or II epithelial ovarian cancer<sup>[24]</sup>. The presence of serum anti-p53 antibody has also been found to be associated with survival of patients with breast cancer, ovarian cancer, and hepatocellular carcinoma, and colorectal cancer<sup>[25,26]</sup>. However, there is still a great controversy as to whether alteration of the p53 gene adversely affects the survival of cancer patients. Many reports failed to show the independent prognostic value of p53 in the carcinomas of tongue<sup>[27]</sup>, breast<sup>[28-30]</sup>, stomach<sup>[31]</sup>, lung<sup>[32]</sup>, ovarian<sup>[33]</sup>, bladder<sup>[34,35]</sup>, colorectal<sup>[36]</sup>, and non-Hodgkin's lymphoma<sup>[37]</sup>.

In a similar situation, there are controversial results about the relationship between the p53 overexpression or p53 gene mutation and the prognosis of HCC patients. It was shown that p53 mutation was involved in determining the dedifferentiation, the proliferative activity, tumor progression<sup>[38]</sup>, and closely related to the invasiveness of HCC<sup>[4]</sup>, which might also influence the postoperative course. Mutations of p53 gene or positive immunostaining for mutant P53 protein expression could be used as a significant indicator of poor prognosis<sup>[39-41]</sup>. Serum anti-p53 antibody could also be a useful prognostic factor for patients with HCC<sup>[42]</sup>. However, many studies showed that neither the immunohistochemical detection of p53 expression, nor the serum anti-p53 antibodies had a significant prognostic value for outcome of patients with HCC<sup>[43-45]</sup>.

In this study, nuclear staining for P53 was found in 112 of the 222 (50.5%) cases, which was similar to the previous study. The 3-year and 5-year overall survival rates of the HCC patients with positive P53 nuclear accumulation were much lower than those of the HCC patients with negative P53 expression. Its significance was even better than that of the factors such as tumor size, vascular invasion and tumor capsule, though they were also related to the overall survival. Therefore, the score of P53 overexpression was adversely related to the survival rates of HCC patients. These indicated that p53 mutation or nuclear accumulation of p53 expression could be a valuable marker for predicting the prognosis of HCC patients after resection. Among the patients with positive P53 expression, those

with 3+ (>50%) for P53 immunohistochemical scoring had a poorest prognosis, their 1-, and 3-year overall survival rates were only 38.5% and 12.3%, respectively, which were much lower than those with 2+ (60.0% and 46.7%) and those with + (83.5% and 57.3%). Therefore, the quantitative immunohistochemical scoring for P53 expression might be more valuable than the common qualitative analysis for P53 expression for predicting the prognosis of HCC patients.

Proliferating cell nuclear antigen (PCNA) labeling index has been thought as another marker of biological malignancy. A correlation between PCNA-LI and recurrent time and rate was reported. PCNA-LI could be a valuable prognostic marker for HCC. However, in this study, no correlation between PCNA-LI and overall or disease-free survival was found.

In summary, HCC is one of the most common cancers in China. Although great advances in its clinical study have been made, metastatic recurrence is the most prevalent cause of death in patients with HCC. Over the past few years, much effort has been made on this target, including predicting HCC behavior<sup>[46-60]</sup>. In this study, through the retrospective review of the 244 HCC patients, we found that accumulation of p53 expression as well as tumor size, capsule or vascular invasion could be a valuable marker for predicting the prognosis of HCC patients after resection. The quantitative immunohistochemical scoring for P53 nuclear accumulation might be more valuable than the common qualitative analysis for P53 expression for predicting prognosis of patients after HCC resection.

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