

Concurrent Evaluation of p53, β -Catenin, and α -Fetoprotein Expression in Human Hepatocellular Carcinoma

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Abstract

Recent models suggest that hepatocellular carcinoma (HCC) develops through several independent pathways marked by key mutations in the β -catenin or p53 gene. An additional pathway potentially is marked by aberrant expression of α -fetoprotein (AFP). To see whether these potential markers are expressed independently, we immunostained sequential sections from 55 HCCs. Of the cases, 30 (55%) were positive for 1 or more proteins: AFP, 19 cases (35%); p53, 12 cases (22%); and β -catenin, 9 cases (16%). Seven tumors (13%) were positive for more than 1 protein, with 4 of 7 positive in the same area of tumor and 3 of 7 positive in different areas of the carcinomas. By statistical analysis, expression of the markers was independent of one another and of tumor size. Concurrent evaluation of p53, β -catenin, and AFP protein expression showed no associations, supporting models in which these proteins might serve as markers of independent pathways in the development of HCC.

Hepatocellular carcinoma (HCC) is one of the 10 most frequent cancers worldwide, yet much remains unknown about the molecular pathways leading to tumor development. In part this reflects the lack of inherited cancer syndromes and the lack of readily available preneoplastic lesions. Nevertheless, results from recent cytogenetic and gene expression studies have suggested models of HCC in which tumors develop through one of several possible pathways: a chromosomal stable pathway marked by β -catenin mutations and a chromosomal unstable pathway marked by p53 dysregulation.^{1,2} Another potential subgroup of HCCs seems to be marked by aberrant expression of fetal proteins that normally are expressed only during liver organogenesis, such as α -fetoprotein (AFP).³

Models of HCC development are important because they provide testable hypotheses on the pathways involved in HCC and might help guide the development of future therapeutics. The antibodies used to detect these proteins have been available for many years, and many studies have examined the frequency of positivity for these proteins individually or in pairs, yet there is no information available on concurrent expression of all 3 markers in HCC. Because of this, it is unclear whether protein expression data support the potentially independent pathways marked by AFP, p53, and β -catenin or whether they represent steps in a shared HCC developmental pathway. In addition to its use in models of HCC development, information on the prevalence and coexpression of these proteins also is important for the development of rational strategies for HCC screening, which historically have been based on expression of AFP but eventually might add p53.⁴

Previous studies have shown that nuclear accumulation of p53^{5,6} and β -catenin⁷ proteins can be detected reliably by immunohistochemical analysis and can serve as markers for mutations in their respective genes or dysregulation of gene expression. Thus, we examined the prevalence and clinical and pathologic associations of AFP, p53, and β -catenin by immunohistochemical analysis in a large series of HCCs from a tertiary care center in the United States. In addition, we correlated immunostain results with tumor size to study the possibility that positivity represented a later event common to many HCC development pathways, in which case positivity would be expected to increase with tumor size.

Materials and Methods

Tissue Samples Examined

After obtaining institutional review board approval, serial sections from 55 surgically resected HCCs from the Johns Hopkins Hospital, Baltimore, MD, were immunostained for p53, β -catenin, and AFP. Cases were chosen solely on the basis of tissue availability. Only typical primary HCCs were included in this study. Hepatoblastomas, fibrolamellar carcinomas, and metastatic hepatocellular carcinomas were excluded. Sections of tumor and adjacent nontumor tissue were stained in each case. Tumor positivity also was analyzed in relation to tumor size after grouping tumors into 3 categories: 1 to 3 cm, 3.1 to 5 cm, and greater than 5 cm.

Immunostaining

We immunostained 5- μ m sections from paraffin-embedded tissue samples following heat antigen retrieval. The primary antibodies were as follows: α -fetoprotein (polyclonal rabbit, dilution 1:1,000; DAKO, Carpinteria, CA), p53 (monoclonal mouse, clone DO-7, dilution 1:1,000; DAKO), and β -catenin (monoclonal mouse, clone

14, dilution 1:1,000; Transduction Laboratories, Lexington, KY). The DAKO EnVision+ Peroxidase kit was used for all immunostaining. This non-biotin-based system relies on a labeled dextran polymer to help control background staining in the biotin-rich liver. Separate positive and negative control results were appropriate in all cases. Cases were scored as positive when at least 5% of the neoplastic hepatocytes were immunolabeled.

While the number of cases with chronic hepatitis B virus (HBV) infection as an underlying cause of liver disease was small (n = 12), further subanalysis was performed because of reports of associations between HBV infection and p53 mutations.^{1,8}

Statistical Methods

Student *t* tests were used to compare metric variables and χ^2 tests to compare frequencies. Log-linear analysis then was used to assess for more complex associations between expression of β -catenin, p53, and AFP proteins. SYSTAT version 10 (SPSS 2000, SPSS, Chicago, IL) was used for data analysis.

Results

Demographics

The study included samples from 44 men and 11 women with a mean \pm SD age at resection of 57 \pm 14 years (range, 23-85 years). Of these patients, 20 underwent orthotopic liver transplantation; the remainder underwent surgical resection. The underlying liver diseases were known in 51 of 55 cases: chronic hepatitis C virus (HCV) infection, 26; chronic HBV infection, 11; HCV and HBV coinfection, 1; cryptogenic cirrhosis, 4; alcohol abuse, 3; hemochromatosis, 1; and no known liver disease, 5 (Table 1). Overall, the mean \pm SD tumor size was 4.9 \pm 3.4 cm (range, 0.8-18 cm). There was no significant difference in tumor size for HCV vs HBV or for sex (*P* > .05 for each test), but tumors in explanted livers were

Table 1
Prevalence of Immunostaining Results by Underlying Liver Disease*

Underlying Liver Disease	No. of Cases	Positive Results		
		α -Fetoprotein	p53	β -Catenin
Hepatitis C virus	26	9 (35)	6 (23)	5 (19)
Hepatitis B virus†	12	7 (58)	5 (42)	2 (17)
Cryptogenic	4	1 (25)	1 (25)	1 (25)
No known liver disease	5	0 (0)	0 (0)	1 (20)
Other or unknown	8	2 (25)	0 (0)	0 (0)
Total	55	19 (35)	12 (22)	9 (16)

* Data are given as number (percentage).

† Includes 1 case of coinfection with hepatitis B and C viruses.

smaller on average (mean \pm SD, 3.3 \pm 1.6 cm) than those from hepatic resections (mean \pm SD, 5.8 \pm 3.8 cm) ($P = .001$).

Immunohistochemical Results

A total of 30 cases (55%) were positive for 1 or more of the proteins: AFP, 19 (35%); p53 nuclear accumulation, 12 (22%); and β -catenin nuclear accumulation, 9 (16%). Four HCCs were positive for 2 immunostains, with the same histologic areas of the tumors positive in 3 of 4 cases, while distinctly different areas were positive in the remaining case (Image 1 Table 2). An additional 3 cases were positive for all 3 immunostains, with 1 showing positivity in the same area of tumor, while in the remaining 2 tumors, β -catenin positivity was in areas separate from areas positive for both p53 and AFP (Figure 1). By log-linear analysis, there was no association in expression of any of these proteins.

Tumor Size and Immunostaining Results

Next, we examined the association between tumor size and immunostaining. Tumors were grouped into 3 categories: 1 to 3 cm ($n = 21$), 3.1 to 5 cm ($n = 16$), and greater than 5 cm ($n = 18$). Although smaller tumors tended to have a higher percentage of AFP positivity, there was no correlation between AFP and tumor size ($P = .42$) or p53 and β -catenin and tumor size ($P = .79$ and $P = .85$, respectively) (Figure 2).

HBV Subgroup Analysis

In this cohort, there was a nonsignificant trend toward increased p53 immunostaining in cases with HBV infection (5/12 [42%]) vs cases with non-HBV causes of liver disease (7/43 [16%]) ($P = .11$; Fisher exact test). AFP positivity, however, was correlated with HBV as an underlying cause of liver disease ($P = .035$; Fisher exact test). The small number of HBV cases does not permit further formal analysis for correlations between p53 and AFP positivity within this subgroup, but no strong clustering was seen, with 4 of 12 cases negative for both markers, 4 of 12 positive for both markers, and 4 of 12 positive for one marker but not the other. No association was seen between HBV infection and β -catenin immunostaining ($P = .9$).

Discussion

Of the 55 HCCs in this series, 30 (55%) were positive for AFP, β -catenin, or p53 immunostaining, and each protein was expressed independently. The lack of association in expression of these 3 proteins supports previously developed models suggesting that these proteins might serve as markers of separate pathways in HCC development.^{1,2}

Because these models were developed largely using messenger RNA expression data and cytogenetic analysis, these results provide important independent supporting evidence based on protein expression. The antibodies used in this study are well characterized and have been used for many years for diagnostic and research purposes, increasing the confidence in the immunohistochemical findings. In addition, the results from this study found no association between positivity and tumor size, suggesting that these markers do not represent a later event common to many tumor development pathways. Because HCC tumor development models are new, they undoubtedly will be refined as additional data accumulate. In particular, there are other pathways not examined in this study that clearly are important in the development of HCC, as evidenced by other data² and by the observation that 45% of the cases ($n = 25$) in this study did not express any of these markers.

Overall, AFP was the most commonly expressed (19/55 [35%]), with a frequency similar to the 24% to 50% reported previously for HCCs in the United States.⁹⁻¹¹ The prevalence of p53 staining in this series (12/55 [22%]) is similar to that reported in areas with low aflatoxin exposure^{12,13} and lower than the 35% to 51%¹⁴ seen in areas with high aflatoxin exposure. In contrast with the AFP and p53 results, the percentage of cases with β -catenin positivity in this study (9/55 [16%]) is less than the weighted average of 32% β -catenin positivity for 684 previously reported HCCs, most of which were in Asia.¹⁵ One study from the Mayo Clinic (Rochester, MN), however, reported a similar prevalence of β -catenin mutations.¹⁶

We observed an association between AFP positivity and HBV as an underlying cause of liver disease and a trend toward increased p53 positivity in cases with HBV infection, findings similar to those reported by others.^{13,17} In this regard, the X-gene product of HBV has been reported to functionally disrupt p53-mediated repression of AFP and provide a potential mechanism for the association.¹⁸ Nevertheless, there must be other important factors involved in AFP expression because most AFP-positive cases in the overall series were negative for p53, as were 3 of 7 AFP-positive cases in the HBV subgroup. One of the limitations of this study is the small HBV subgroup. While no associations were seen between AFP, p53, or β -catenin expression in the overall group of HCCs, our findings do not exclude the possibility of an association between AFP and p53 in patients with chronic HBV infection.

Of the 55 HCCs in this US cohort, 30 (55%) were positive for at least one of the studied markers, with AFP expression most common (19/55 [35%]), followed by p53 (12/55 [22%]) and β -catenin (9/55 [16%]). AFP positivity was associated with HBV as an underlying cause of liver disease. Expression of p53, β -catenin, and

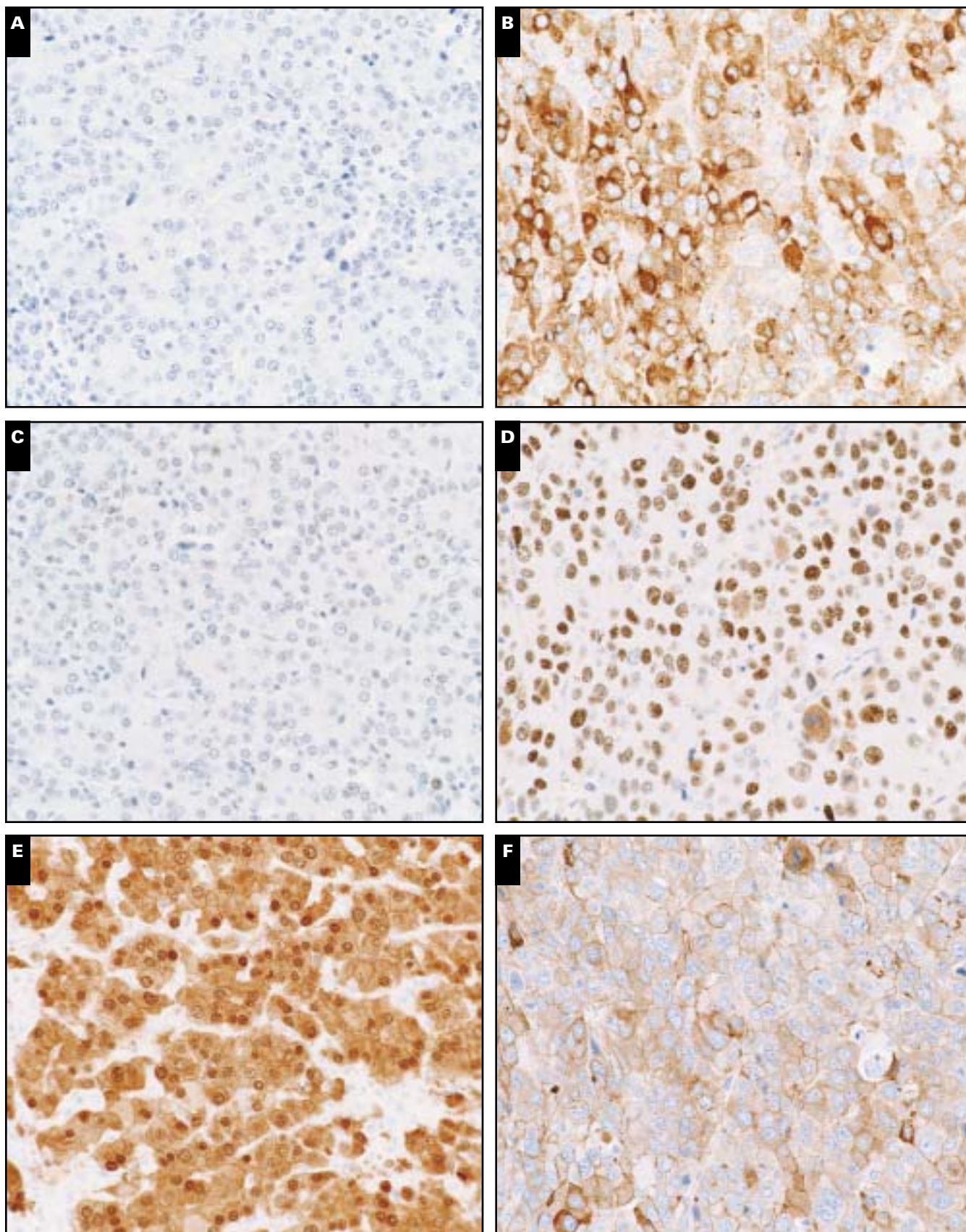


Image 1 Hepatocellular carcinoma (HCC) with multiple nodules showing different staining patterns. **A, C, and E**, From the same area of 1 nodule of HCC. **B, D, and F**, From the same area of a different HCC nodule. Immunostains for α -fetoprotein (**A**, $\times 100$; **B**, $\times 100$) show positivity in the same areas that are positive for p53 (**C**, $\times 100$; **D**, $\times 100$) but are negative in the areas positive for β -catenin nuclear accumulation (**E**, $\times 100$; **F**, $\times 100$).

Table 2
Patterns of Positivity in Seven Hepatocellular Carcinomas Positive for More Than One Immunostain

Immunostain Positivity	Same Area of Tumor	Different Areas of Tumor
α -Fetoprotein/p53	1	1
β -Catenin/ α -fetoprotein	0	0
β -Catenin/p53	2	0
α -Fetoprotein/p53/ β -catenin	1	2

AFP protein showed no associations, supporting models in which these proteins serve as markers of independent pathways in the development of HCC. However, associations between AFP expression and p53 dysregulation in the setting of chronic HBV infection are not excluded by our results.

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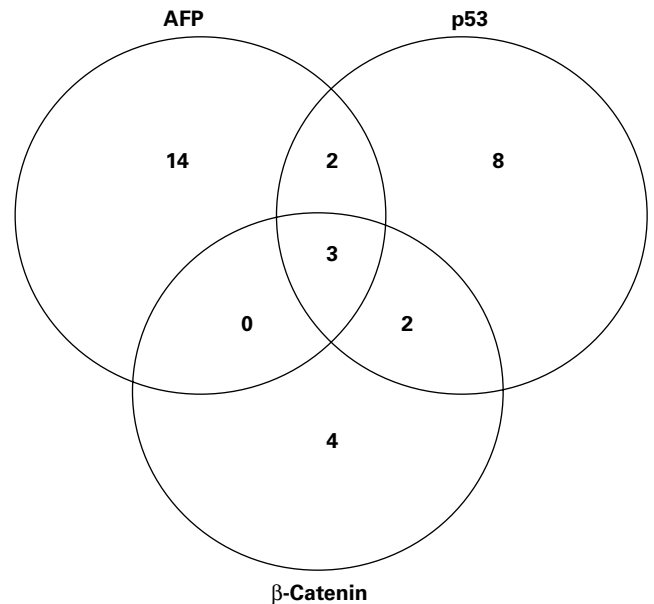


Figure 1 A Venn diagram showing the lack of shared positivity in hepatocellular carcinomas for α -fetoprotein (AFP), p53, and β -catenin immunostaining.

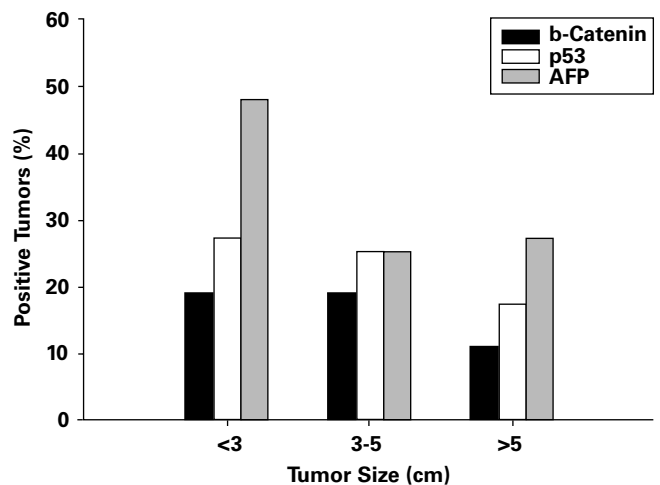


Figure 2 No association is seen between tumor size and frequency of positivity for α -fetoprotein (AFP), p53, and β -catenin.

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