Is the p53 Gene Mutation of Prognostic Value in Hepatocellular Carcinoma After Resection?

Kuo-Shyang Jeng, MD; I-Shyan Sheen, MD; Be-Fong Chen, MD; Ju-Yann Wu

**Hypothesis:** Mutant p53 gene has lost its tumor suppression function and is considered to be a very important step in hepatocellular carcinoma development. We propose that the mutant p53 gene plays a role in its invasiveness and prognosis after resection.

**Design:** A case-controlled study.

**Setting:** A referral center.

**Patients:** Seventy-nine consecutive patients who underwent surgical resection for hepatocellular carcinoma entered this study.

**Intervention:** Tissue sections of resected hepatocellular carcinoma (deparaffinized and rehydrated from formalin-fixed and paraffin-embedded sections) were incubated with antihuman p53 monoclonal antibody and immunostained. The p53 result was scored without prior knowledge of the patients’ status. A 10% immunopositivity was regarded as the threshold value.

**Main Outcome Measure:** The immunopositive rate of p53 was 69.6% (55 of 79 patients). The clinical variables (age, sex, associated liver cirrhosis, hepatitis B virus infection, hepatitis C virus infection, serum α-fetoprotein, and Child-Pugh class); the histological variables (size, capsule, vascular permeation; grade of differentiation, and multinodularity); and postoperative course (recurrence, tumor-free interval, death, and survival period) were correlated with p53 immunopositivity.

**Results:** From univariate analysis, more patients with p53 positivity were male (92.7 vs 0%) ($P < .001$); had vascular permeation (80% vs 50%) ($P = .007$) (odds ratio [OR], 4.0); no complete capsule (83.6% vs 62.5%) ($P = .04$) (OR, 3.1); and daughter nodules (90.9% vs 70.8%) ($P = .04$) (OR, 4.1) than patients with negative p53 staining. From multivariate analysis, only sex and vascular permeation remained significant ($P = .001$ and $P = .008$, respectively). Although more patients with p53 positivity had tumor recurrence (78% vs 50%) ($P = .01$) and death (64% vs 33%) ($P = .01$), the Cox proportional hazards model showed that p53 overexpression had only weak correlations with tumor-free interval and survival time ($P = .09$ and $P = .08$, respectively).

**Conclusions:** Our results show that the biological behavior of the mutant p53 gene is strongly related to the invasiveness of hepatocellular carcinoma and may also influence the postoperative course. We suggest that the immunopositivity of the mutant p53 gene has a predictive role in the prognosis of patients with resected hepatocellular carcinoma.

Arch Surg. 2000;135:1329-1333

From the Departments of Surgery (Dr Jeng), Pathology (Dr Chen), and Medical Research (Ms Wu), Mackay Memorial Hospital; and the Liver Research Unit, Chang Gung Memorial Hospital (Dr Sheen), Taipei, Taiwan.

The cellular wild-type p53 gene on chromosome 17p is an established tumor suppressor gene.1,2 It regulates the cell cycle of DNA repair and synthesis, as well as programmed cell death. Once it mutates, loss of the normal function leads to the evolution of neoplasms; the speed of tumor growth and invasion may also be enhanced. When mutated, this gene may have transforming properties and be stained immunohistochemically.3-9 Its prognostic significance in some types of human cancer has been reported. The relationship between hepatocellular carcinoma (HCC) and overexpression of the mutant p53 gene have been studied in different countries.3-7 9-11 The results have varied. In addition, structural anomalies in the p53 gene were found in advanced but not in early HCC.18,19

During the last 10 years, efforts have been made worldwide toward earlier detection and safer surgical resection of HCC. However, despite these recent diagnostic and therapeutic advances, postoperative recurrence is still common.20-22 How to predict the prognosis before resection remains a challenging problem for surgeons. Certain characteristics related to HCC recurrence have been reported widely and variably in the literature.20-30 Risk factors, including vascular permeation, absence of capsule, presence of daughter nodules, histological grade of tumor differentiation, tumor size, associated cirrhosis, hepatitis B virus infection, multicentric HCC, and adequate section margin, have been mentioned.
PATIENTS AND METHODS

Formalin-fixed, paraffin-embedded material was available for study from 100 consecutive cases of HCC collected from surgical resections between January 1993 and December 1997 at Mackay Memorial Hospital, Taipei, Taiwan.

IMMUNOHISTOCHEMICAL STAINING FOR p53

The 5-µm-thick, formalin-fixed, paraffin-embedded sections were cut, deparaffinized, and rehydrated with graded alcohol and xylene. Endogenous peroxidase was blocked using 3% hydrogen peroxide for 5 minutes, followed by a brief wash in Tris buffer (pH, 7.2). Sections were rehydrated and heated in citrate buffer (pH, 6.0) in a microwave oven at 500 W for 10 minutes to retrieve the antigen. The tissues were stained with a monoclonal mouse antihuman p53 antibody (DAKO-p33, clone DO-7; Dako Corp, Carpinteria, Calif) and a labeled streptavidin-biotin staining kit (DAKO LSAB kit, alkaline phosphatase system 40, Dako Corp). They were incubated with the antibody at a dilution of 1:100 (in Tris buffer) for 1 hour at room temperature. The peroxidase reaction used 3,3-diaminobenzidine tetrahydrochloride as chromogen and the slides were counterstained with hematoxylin-eosin. All tissue sections were evaluated by 2 independent, blinded observers. Only nuclear staining was regarded as positive. Cases were scored as negative when no one cell was stained even at a concentration as high as 1:10 in a triplicate study. A known colon adenocarcinoma with diffuse p53 nuclear accumulation was stained in parallel as the positive control. For negative controls, we used buffer instead of the primary antibody.

SCORING OF p53

We used light microscopy to search for the highest concentration of reactive staining nuclei in each p53 staining section and counted 1000 cells from the most aggressive area of the tumor to represent the tumor’s behavior and reduce count variability. Specific staining was identified by the presence of a red reaction product in the nuclei and the percentage of nuclei that were immunostained was estimated and was scored without knowledge of the grade of tumor differentiation. A 10% immunopositivity was regarded as the threshold value regardless of the intensity of staining.

Clinical details were available from medical records on all patients. Seventy-nine patients entered this study; 21 cases were excluded for the following reasons: immediate operative mortality, failure to obtain p53 results because of severe, extensive tumor necrosis, some of which probably resulted from preoperative transcatheter hepatic arterial chemoembolization, incomplete follow-up, and causes of death not related to liver disease. The mean SD age of patients was 52.4±16.6 years (range, 16-82 years) with a male-to-female ratio of 2:1 (52:27). The surgical procedures included major resections (15 partial lobectomies, 31 lobectomies, and 9 extended lobectomies) and minor resections (19 segmentectomies, 3 subsegmentectomies, and 2 wedge resections). After resection, all these patients were followed up at our outpatient clinic and received regular clinical assessment; periodic abdominal ultrasonography (every 2-3 months during the first 3 years, then every 4-6 months thereafter) to detect tumor recurrence; and α-fetoprotein and liver biochemistry (every 2 months during the first 2 years, then every 4 months during the following 3 years, and every 6 months thereafter). Abdominal computed tomographic scans were also done (every 6 months during the first year, then every year).

The differences of p53 expression in diverse clinicopathologic parameters were evaluated. Parameters (Table 1) included the presence of liver cirrhosis (confirmed from the operative findings and also from the pathology examination of the specimen); hepatitis B virus (hepatitis B surface antigen); hepatitis C virus infection (anti–hepatitis C virus); Child-Pugh classification of liver reserve (A vs B); serum α-fetoprotein titer (α-fetoprotein, ≤20 ng/mL vs 20-1000 ng/mL vs >1000 ng/mL); tumor size (≤3 cm, 3-10 cm, and >10 cm); cell differentiation grade (Edmondson and Steiner grade 1 vs 2-4); encapsulation (complete, infiltration by HCC, or absent); vascular permeation (including vascular invasion and/or tumor thrombi within the portal or hepatic vein); presence of daughter nodules; and resection margin (safety margin, ≥1 cm vs <1 cm). No one in this study had incomplete resection of HCC. During the follow-up (median, 3 years; range, 2-5 years), 55 patients had tumor recurrence and 43 patients died. We also correlated the p53 overexpression with the outcomes.

We used statistical programs (BMDP, t test, or Mann-Whitney U test for continuous variables, χ² test or Fisher exact test for categorical variables, and logistic regression and Cox proportional hazards model for multivariate analysis. A value of P<.05 was defined as a significant difference.

RESULTS

Among the 79 patients, 55 patients (70%) had a p53-positive result. Among the 55 patients with immunopositivity, the intensity of immunostaining was slight (+) in 15 patients (27%), moderate (+++) in 15 patients (27%), and strong (++++) in 15 patients (27%). There was no correlation between the intensity of immunostaining with either pathologic parameters or outcomes (earlier recurrence or death) (Table 2). The correlation between a positive oncoprotein p53 and patient characteristics is shown in Table 3. Age, positivity of hepatitis B surface antigen or hepatitis C antibody, levels of α-fetoprotein, liver cirrhosis, Child-Pugh class A or B, size of the HCC, grade of differentiation, and adequate margin of resection showed no significant differences between p53 positive and negative groups. From the univariate analysis, a significant correlation was found between p53 expres-
The p53 gene mutation has been identified in more than half of human tumors, including HCC, and is the most common genetic abnormality in human cancers.5,8,13,19,31-38 Its inactivation by mutation is thought to be a fundamentally important step in carcinogenesis.2,5,8,13,19,31-38 Wild-type p53 protein regulates at least 2 important events in cells. First, it induces the cyclin-dependent kinase inhibitor p21Waf1/Cip1, which negatively regulates the cell cycle. Second, it induces apoptosis through the regulation of genes, such as BAX. In addition, correlation with metastasis, recurrence, and prognosis have been reported in p53 gene mutation.12,16,37-43

COMMENT

sion and men (93% vs 0%) (P < .001); vascular permeation (80% vs 50%) (P = .007; odds ratio [OR], 4.0); complete capsule vs infiltration or absent capsule (16.4% vs 37.5%) (P = .04; OR, 3.1); and daughter nodules (90.9% vs 70.8%) (P = .04; OR, 4.1) (Table 3). From multivariate analysis, only sex and vascular permeation remained significant (P = .001 and P = .008, respectively).

Table 4 shows that more patients with p53 positivity had tumor recurrence (78% vs 50%) (P = .01) and death (64% vs 33%) (P = .01). After analysis with the Cox proportional hazards model, p53 overexpression had only a weak correlation with tumor-free interval and survival time (P = .09 and P = .08, respectively). Factors influencing HCC recurrence and time lapse of recurrence (Table 5) were vascular permeation (P < .001), complete capsule (P = .01),
The high recurrence rate after resection is one of the main factors in the poor outcome for patients with HCC. Invasiveness has been the most consistent reported contributing factor. In our study, p53 protein overexpression correlated well with tumor recurrence (P = .01). To analyze the factors relating to HCC recurrence and death, vascular invasion, complete capsule, and safety margin correlated well with tumor-free interval, and only vascular permeation correlated with duration of survival. A weak association with both tumor-free interval and duration of survival with mutation of the p53 gene was found. The weak correlation may be attributed to the short duration of follow-up (range, 2-5 years; median, 3 years). Tumor size has been emphasized as one of the significant prognostic factors because vascular invasion and daughter lesions may increasingly develop as the tumor grows. In our study, no correlation between p53 positivity and tumor size was found. In addition, tumor size also had no correlation with histological grade, vascular invasion, disease-free interval, or survival in our patients. From our experience, some large HCCs were the result of expansive growth and had slow intraportal or distant spread. Vascular permeation, indicating tumor invasiveness, consists of tumor invasion of the hepatic vein, portal vein and/or hepatic artery or tumor thrombi within the vessels. It may be detected preoperatively by ultrasonography, arteriography, or portography, intraoperatively by ultrasonography or direct observation, or postoperatively by pathological examination of surgical specimens. Vascular permeation is the most consistent significant prognostic factor of postoperative tumor recurrence. In our univariate analysis, the positive p53 status significantly related to vascular permeation; in the Cox model, patients with vascular permeation had significantly shorter tumor-free intervals and survival periods. Whether the grade of differentiation of HCC is a determinant of recurrence after resection has been debated for a long time. The association of grade of anaplasia (Edmondson and Steiner's classification) with p53 positivity also varied in reports. In our series, less overexpression of p53 and less recurrence were found in patients with well-differentiated tumors (Edmondson and Steiner grade 1) than in those with grade 2 to 4 tumors. The exact mechanism of capsular formation is not known. A tumor capsule may act as a barricade preventing the spread of cancer cells and has a positive role in the prognosis of HCC. The invaded capsule was regarded as incomplete in our series. We found the overexpression rate of p53 was similar in patients with no capsule and incomplete capsule (87% vs 86%), but was significantly lower in those with a complete capsule (61%) (P = .03). Other authors had different findings.

It is clear that the functional loss of p53 is important in multistep carcinogenesis. The data summarized in this report indicate that p53 inactivation is important in subsets of HCC. Not all HCCs contain p53 mutations, suggesting that there may be both p53-dependent and p53-independent pathways leading to liver cancer. Inactivation of p53 by other mechanisms, such as binding to the hepatitis B virus X protein, remains an important possibility. The selective advantage conferred on a liver cell by a mutant p53 gene seems to be significant in later steps of tumorigenesis, after the accumulation of additional genetic changes.

### Table 5. Multivariate Analysis of Factors Influencing Tumor Recurrence and Death of Patients in Cox Proportional Hazards Model

<table>
<thead>
<tr>
<th></th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recurrence</td>
<td></td>
</tr>
<tr>
<td>Vascular permeation</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Resection margin ≥1 cm</td>
<td>.004</td>
</tr>
<tr>
<td>Complete capsule</td>
<td>.01</td>
</tr>
<tr>
<td>p53 Positivity</td>
<td>.09</td>
</tr>
<tr>
<td>Death</td>
<td></td>
</tr>
<tr>
<td>Vascular permeation</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>p53 Positivity</td>
<td>.08</td>
</tr>
</tbody>
</table>

The antibody DO-7 used in this study recognizes both the protein of the wild-type and mutant forms of the p53 gene. Unlike the mutant form, the wild type is too short-lived to be detected by the immunohistochemical method. If immunoreactivity for p53 is detected in the tissue, it suggests that reactivity is because of the mutant form. Thus, tumors with immunohistochemically undetectable p53 might represent tumors with no mutation of the p53 gene or with the mutant form under the detectability. A considerable heterogeneity in the local distribution of immune reactive tumor cells has already been recognized in many tumors, including ours. It may represent true tumor heterogeneity but may also be attributed to specific types of p53 mutation or cell cycle variations in the p53 level.

Immunohistological assessment of p53 may provide clinical relevance with regard to diagnosis, prognosis, or assessment of tumor progression. The correlation between p53 gene alteration and cancer prognosis has been investigated mostly by immunohistochemical staining of p53 protein. A significant association was noted in breast, gastric, and other types of cancer. We adopted 10% immunopositivity as the threshold value and above this it was considered to be positive. The positive rate of the mutant p53 gene in our patients with HCC was 69%. In the literature, positive rates have ranged from 0% to more than 70%. Factors related to the wide variation in positivity may include different thresholds of positivity adopted, different anti-p53 antibodies used, geographical variations and differences in the molecular mechanisms of hepatocarcinogenesis, such as aflatoxin exposure. Some authors have raised a question of whether p53 protein overexpression can represent p53 gene mutation in neoplasms. Hall and Lane mentioned a very close correlation between p53 expression and mutations of the p53 gene and found that most antibodies give the same results.

Overexpression of p53 protein in nonuntumor regenerative nodules, adjacent to HCC tissue, or in dysplastic hepatocytes in cirrhotic livers before the development of HCC has been demonstrated. A role in carcinogenesis was thus proposed. However, the cirrhosis itself may contribute to the hepatocarcinogenesis. Other authors, including us, have not detected the mutant p53 gene in nontumor liver tissue, in either cirrhotic or noncirrhotic livers. In general, mutations of p53 have been considered relatively late events in HCC development and correlate with tumor progression.


©2000 American Medical Association. All rights reserved.
The identification of contributing genetic alterations related to long-term hepatitis B virus infection remains a considerable challenge in the field of liver cancer research. There is some discrepancy between our results and the findings of previous studies on the role of p53 expression in determining the prognosis of patients with HCC. However, these discrepancies might reflect important variables of selection, such as number of patients, histological types of tumors, tumor stages, periods of follow-up, and type of antibody used. The patients entering our study had received previous hepatic resection. The implications of our results, nevertheless, are that the immunohistochemical detection of p53 is a valuable tool for prediction of recurrence in patients after resection or for identifying subgroups of patients who may be at higher risk.

Tumor recurrence is well correlated with tumor invasiveness. Tumor invasiveness and prognosis may be determined from vascular permeation, the grade of cell differentiation, infiltration or absence of capsule, multinodular lesions, and tumor-free interval. According to our study, they are also all compatible with oncprotein p53 positivity. From the results of our data, we suggest that the biological behaviors of p53 gene mutation be strongly related to the invasiveness of HCC. We recommend that oncprotein p53 positivity may play a significant role in predicting the prognosis of HCC in patients after resection.

Supported by grants from the Department of Health, National Science Council, Executive Yuan, Taiwan (Dr Jeng).

Corresponding author: Kuo-Shyang Jeng, MD, Department of Surgery, Mackay Memorial Hospital, No. 92, Section 2, Chung-San North Road, Taipei, Taiwan, Republic of China (e-mail: issheen@gcn.net.tw).

REFERENCES