Expression of p53 Gene in 184 Unifocal Hepatocellular Carcinomas: Association with Tumor Growth and Invasiveness

Hey-Chi Hsu,2 Hawai-Jung Tseng, Po-Lin Lai, Po-Huang Lee, and Shian-Yang Peng

Departments of Pathology [H-C. H., H-J. T., P-L. L.] and Surgery [H-H. L], College of Medicine, National Taiwan University, and National Taipei Nursing College [S-Y. P.], Taipei, Republic of China

ABSTRACT

To elucidate the biological significance of the p53 gene expression in human hepatocellular carcinoma (HCC), the p53 protein was studied in 184 resected unifocal primary HCCs, including 102 small (≤5 cm) and 82 large HCCs (>5 cm), using immunocytochemistry. The p53 mRNA expression was analyzed in 69 cases using Northern hybridization. The p53 protein, which was detected in 58 HCCs (31.5%), was overexpressed more frequently in HCC with elevated serum α-fetoprotein level (37.9 versus 25%, P < 0.04), in large HCC (39.0 versus 25.5%, P < 0.03), and in invasive HCC (35.1 versus 13.3%, P < 0.01). The overexpression of p53 protein closely correlated with p53 mRNA overexpression (75 versus 44.4%, P < 0.003), and p53 gene mutation (76.9 versus 19.2%, P < 1 × 10−6). HCCs with p53 protein expression (group A) and those negative for both p53 protein and mRNA expression (group B) had an unfavorable outcome, while HCC with no p53 protein but with p53 mRNA overexpression (group C) had the best outcome; the 4-year survival was 26.1, 26.3, and 62.5%, respectively. The p53 gene mutation was significantly higher in group A HCC (76.9%) than in groups B (27.3%) and C (23.5%), P < 0.0001. The results suggest that the p53 protein and mRNA expression patterns in HCC correlate with p53 gene mutation and tumor behavior and may serve as a molecular prognostic factor.

INTRODUCTION

HCC3 is one of the most common fatal malignancies in Taiwan and many other countries. Recently, mutations of the p53 gene have been reported in many human tumors (1–3), including HCC (4–7). The wild-type p53 can transactivate the transcription of a set of genes that may down regulate cellular growth-related genes (8, 9), inhibit cell cycle progression, cell division, and transformation (10, 11), and act as a tumor suppressor gene. In contrast, the mutant forms fail to do so and are oncogenic (8–10). Because of its very short half-life and minute amounts in normal cells, the wild-type p53 protein is almost undetectable by immunohistochemical stain. In contrast, the mutant p53 proteins have a much longer half-life (12, 13), are present in high concentrations, and become detectable by immunocytochemistry. The detection of p53 protein in tissue by immunohistochemistry is synonymous with mutant p53 (14–16) and has been reported in many human carcinomas (14–18). However, the validity of this suggestion has not been fully substantiated in a large series of human cancers, including HCC, by a complimentary complete DNA analysis of the p53 gene. Although p53 gene mutation is a common event in HCC, little is known regarding the biological significance of p53 gene expression in HCC (19). This investigation was conducted to study the prevalence and the clinical relevance of p53 protein and mRNA expression in 184 unifocal primary human HCCs.

MATERIALS AND METHODS

Subjects. Among 517 surgically resected HCCs collected during July 1980–August 1992 at the Department of Pathology, National Taiwan University Hospital at Taipei, 184 cases with unifocal primary HCC were randomly selected for this study. The tumor size was ≤5 cm (small HCC) in 102 cases and bigger (large HCC) in 82 cases. There were 152 males and 32 females. Hepatitis B surface antigen was positive in 3.6% of the cases (71.7%) and anti-hepatitis C virus antibody positive in 20 (31.7%) of 63 cases examined, including 5 cases positive for both hepatitis B surface antigen and anti-hepatitis C virus. Seven cases were negative for both.

Histological Study. HCC was divided into noninvasive and invasive HCC (20, 21). The tumor differentiation was classified into two groups: well-differentiated (grades I and II) and poorly differentiated (grades III and IV), as described previously (21, 22).

Detection of p53 Proteins in Tissues. The p53 proteins were detected in fresh frozen cryostat sections by ABC and peroxidase-anti-peroxidase methods, as described before (23), using three mouse monoclonal antibodies PAb1801 and PAb240 (Ab-2, Ab-3; Oncogene Science, Inc., Uniondale, NY) and DO-7 (DAKO-p53; Dakopatts, Glostrup, Denmark). PAb1801 and DO-7 recognize both wild-type and mutant p53 (24, 25), while PAb240 recognizes mutant p53 alone (26). A section of an HCC with mutation at codon 276 and abundant p53 product was included as a positive control in each run. The negative controls included the omission of the first antibody from the reaction and a negative reaction in the nontumorous liver part. The cytoplasmic staining was regarded as nonspecific. Sections were read as positive if nuclear p53 was detected in >10% of the tumor cells.

Northern Blot Analysis. Total cellular RNA was isolated by the guanidine isothiocyanate extraction method (27) and processed for the Northern blot experiment (28) using an [γ-32P]ATP-labeled 1.9-kilobase XhoI fragment of the human p53 cDNA covering all 11 exons recovered from the restriction fragment length polymorphism probe pflp53 plasmid (kindly provided by Professor Butel), as described previously (29).

Quantitation of p53 mRNA. Autoradiographs of Northern blots were scanned using a densitometer (Cliniscan 2; Helena Laboratories, Beaumont, TX). The 28S bands of ethidium bromide-stained gel were used as a signal intensity reference for RNA loading. p53 mRNA overexpression was defined as a ≥2.0-fold increase in signal intensity as compared with the paired nontumorous liver.

PCR-SSCP and DNA Sequencing. The PCR-SSCP analysis (30) was carried out to detect single-base changes using the oligonucleotide primers spanning exons 2–11 of the p53 gene (31–33). The PCR mixture containing the [γ-32P]ATP-labeled primers, 0.1–1 μg genomic DNA, and Taq polymerase (Promega, Madison, WI) were allowed to run for 30 cycles of the reaction at 94, 65–55, and 72°C for 30 s, 1 min, and 1 min, respectively. Then, 1/20–1/50 of the PCR products were electrophoresed in a 6% vertical polyacrylamide gel at 30 W for 6–22 h. The gel was dried and exposed to X-ray film at −0°C. DNA samples showing the mobility shift were then subjected to DNA sequencing by the dideoxy chain termination method with asymmetric PCR products (34). Purified single-stranded DNA products (1–4 μl) were used for chain elongation and termination with the United States Biochemical Corp. Sequenase kit. The sequencing reaction products were analyzed in a 6% polyacrylamide gel. The gel was dried and exposed to X-ray film.

Statistics. The X2 test was used for statistical analysis, and P < 0.05 was regarded as significant.

RESULTS

Detection of p53 Protein in HCC. The p53 protein was detected in the nuclei in 38 (31.5%) of 184 HCCs but in none of the paired...
nontumorous liver. The p53 protein was detected in >40% of the tumor cells in 86% of the cases, although the number of positive cells varied from focal to diffuse distribution (Figs. 1 and 2). HCC with increased p53 protein also showed intense nuclear staining for p53 in the tumor thrombi in the portal vein branches (Fig. 1B) and the satellite nodules. The tumor stroma, bile duct epithelium, and hepatocytes were negative. The cytoplasmic staining for both tumor and nontumorous liver cells was regarded as nonspecific. Occasionally, groups of degenerated and necrotic tumor cells with strong nuclear stain for p53 were found in the central necrotic zone of the tumor and were carefully excluded by Meyer’s hematoxylin counterstain of the paired section on the same slide.

Correlation of p53 Protein Expression with Clinical and Pathological Features. The p53 protein was detected more frequently in HCCs with elevated serum AFP levels (37.9 versus 24.7%, P < 0.03) (Table 1) but did not correlate with the age and sex of patients or hepatitis B or C virus infections (data not shown).

The p53 protein was found more commonly in large than in small HCC (39 versus 25.5%, P < 0.03), in poorly differentiated (grades III-IV) than in well-differentiated (grades I-II) HCC (34.8 versus 20%, P < 0.04), and in the invasive than in the noninvasive HCC (35.1 versus 13.3%, P < 0.01) (Table 1). p53 protein expression did not correlate with cirrhosis (data not shown).

Correlation of p53 Protein Expression with p53 mRNA Expression and Gene Mutation. The p53 protein expressed was significantly more often associated with p53 mRNA overexpression (75 versus 44.4%, P < 0.01) (Fig. 3), and p53 mutation (76.9 versus 19.2%, P < 1 × 10⁻⁹). The concordance rate between p53 protein and mRNA expression was only 60.8%. According to the p53 protein and mRNA expression, the p53 gene expression patterns were divided into three groups: group A, p53 protein expression; group B, no overexpression of p53 protein and mRNA; group C, no p53 protein but p53 mRNA overexpression. The p53 mutation detected by PCR-SSCP analysis was significantly higher in group A (76.9%) than in groups B (27.3%) and C (23.5%), P < 0.0001 (Table 2).
Table 2  Expressions of p53 protein and mRNA in relation to survival of patients with unincertic HCC

<table>
<thead>
<tr>
<th>Overexpression of p53 protein/p53 mRNA</th>
<th>Survival</th>
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<tr>
<td></td>
<td>1 year</td>
</tr>
<tr>
<td>Group A (+/+,-,-)†</td>
<td>34/52</td>
</tr>
<tr>
<td>(65.4)§</td>
<td>(26.1)‖</td>
</tr>
<tr>
<td>Group B (-/-)</td>
<td>18/27</td>
</tr>
<tr>
<td>(60.7)§</td>
<td>(26.3)‖</td>
</tr>
<tr>
<td>Group C (-/+?)</td>
<td>22/22</td>
</tr>
<tr>
<td>(100.0)§</td>
<td>(62.5)‖</td>
</tr>
</tbody>
</table>

P value: a <0.0008  b <0.0005  c <0.0001  d <0.0001

‡ Detected by PCR-SSCP (see text for detail). Seven of the 10 mutations in groups B and C are not missense mutation: splice junction mutations (4 cases), deletion (2 cases), and insertion (1 case).

§ Abbreviations: +, yes; -,-, no; ? , not examined.

‖ Number of patients/number of patients (%).

See text for explanation of comparisons.

Correlation of p53 Expression with Patient Outcome. p53 protein expression alone did not correlate with patient survival. According to the patterns of p53 protein and mRNA expression, we found that group C HCC had a favorable outcome (Table 2) that was significantly better than group A and group B; the 4-year survival rates for groups A, B, and C were 62.5, 26.3, and 26.1%, respectively (A versus B, P < 0.005; A versus C, P < 0.02).

DISCUSSION

p53 protein expression in HCC has been reported, but little is known of its biological significance because of the rarity (15.5%) of the protein expression (19). Using immunocytochemical study in 184 unifocal primary HCCs in Taiwan, we detected a significantly higher p53 protein expression (31.5%) than in French HCC (19). There are biological implications for p53 gene expression. The p53 protein was detected more frequently in large than in small HCCs (39.0 versus 25.5%, P < 0.03), in poorly differentiated than in well-differentiated HCC (34.8 versus 20%, P < 0.04), and in HCC associated with elevated serum AFP level (37.9 versus 24.7%, P < 0.03), in accordance with our previous observation that AFP gene expression correlated with poorly differentiated HCC (26). More important, the p53 protein was more often expressed in the invasive than in the well-encapsulated, noninvasive HCC (35.1 versus 13.3%, P < 0.01). The p53 protein was expressed in 42–55% of colorectal carcinoma (15, 33) but only rarely (8%) in colonic adenomas (15). Although the noninvasive HCC cannot be regarded as an adenoma, it does portend a significantly more favorable outcome than the invasive HCC (21). These findings suggest that p53 gene overexpression closely correlates with tumor dedifferentiation, cellular oncofetal gene expression, and tumor progression of HCC. Acquisition of p53 expression may be one of the major steps for transformation of the tumor cells into cells with more aggressive biological behavior during the course of tumor growth.

The clinical implication of p53 gene expression in human cancer is controversial (35–37). p53 protein expression has been shown to be associated with poor prognosis in breast carcinoma (36) but not clear in HCC. We found that HCC with p53 protein overexpression had a worse prognosis but not of statistical significance. However, p53 protein accumulation is related to protein stability, p53 mRNA expression and genetic alterations, and cell cycle (39). We found that p53 protein expression was more frequently associated with p53 mRNA overexpression (75 versus 44.4%, P < 0.01), and p53 gene mutation (76.9 versus 19.2%, P < 1 x 10^-7). Therefore, three expression patterns of p53 protein and mRNA were identified: group A, with p53 protein; group B, negative for p53 protein and mRNA overexpression; group C, with no p53 protein but with p53 mRNA overexpression. We found that group A HCC, which had the most frequent p53 mutation, had a very unfavorable outcome (4-year survival, 26.1%). Among HCCs with no p53 protein expression, the outcome was significantly better for group C than group B (4-year survival, 62.5 versus 26.3%, P < 0.02). The reasons for the favorable outcome and failure to detect p53 protein in group C HCC with p53 mRNA overexpression are unclear. The low frequency of p53 mutation suggests that the p53 protein is probably wild-type p53 in most of the cases and, hence, undetectable by immunocytochemistry because of the very short half-life. Our results demonstrate that the pattern of p53 gene expression closely correlates with p53 mutation and patient outcome and may serve as a molecular prognostic factor.

Studies suggest that the detection of p53 protein by immunohistochemistry means mutant p53 (14–16). This study showed that p53 protein expression was indeed often accompanied by p53 mutation (76.9%), but the concordance rate (79.5%) was not very satisfactory. A serious discrepancy in the estimates of mutation based on immunocytochemistry (~60%) and DNA analysis (~40%) has been noticed in breast cancer (38). In group A HCC with p53 protein overexpression, 9 tumors did not show p53 mutation by PCR-SSCP analysis. In a separate study of DNA analysis covering exons 2–11 of p53 gene, we found that the concordance of PCR-SSCP and DNA sequencing was 97%, suggesting that PCR-SSCP is a reliable method for the detection of p53 mutation. The reasons for the increased p53 protein in these tumors are unclear. It is shown that p53 is involved in cell cycle progression and the wild-type p53 is overexpressed in some nontransformed cell lines after serum stimulation (39). The conformation and stability of p53 protein also can be affected by culture temperature (40). Therefore, our results are in accordance with the suggestion that the overexpression of p53 protein does not always mean overexpression of mutant p53 (38). On the other hand, mutation was detected in nearly one-quarter of group B and C HCCs which had no protein expression. We found that 7 of the 10 mutations in groups B and C were deletions, insertions, and splice junction mutations; these mutations would result in abnormal protein products and escape the immunocytochemical detection. Therefore, it is clear that the detection of p53 protein alone may overestimate and underestimate the p53 mutation rates and should be interpreted carefully for the determination of p53 gene mutation rate.

REFERENCES


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