p53 Mutations in Human Hepatocellular Carcinomas from Germany

Stefan Kress, Uli-Rüdiger Jahn, Albrecht Buchmann, Peter Bannasch, and Michael Schwarz

German Cancer Research Center, Project Group Tumor Promotion in Liver [S. K., M. S.], and Division of Cytospathology [U.-R. J., P. B.], Immunheinerner Feld 280, 6900 Heidelberg, and Institute of Toxicology, University of Tübingen, Tübingen [A. B.], Germany

Abstract

Mutations in the p53 gene are frequent genetic alterations in human hepatocellular carcinomas. We have examined 13 cases of human hepatocellular carcinomas from Germany for the presence of p53 aberrations. We have examined 13 cases of human hepatocellular carcinomas from Germany for the presence of p53 aberrations in exons 4 to 8 of the gene by single-strand conformation polymorphism and restriction fragment-length polymorphism analyses and by sequencing of polymerase chain reaction products. Single base substitutions occurred in two human hepatocellular carcinomas: a C:G → T:A transition at a CpG site in codon 257, and a T:A → A:T transversion at codon 273. One of these point-mutated tumors and two additional tumors without point mutations demonstrated a loss of one p53 allele. None of the tumors was mutated in codons 12 or 61 of the c-Ha-ras gene.

Introduction

Primary hepatocellular carcinoma is among the ten most important human cancers worldwide (1). Its frequency distribution, however, varies considerably between different geographical areas in the world. While the incidence of HCC is comparatively low in most industrialized countries, there are certain areas of high risk in southern Africa and southern China, where both hepatitis B virus and aflatoxins are suspected etiological risk factors (2). Mutations of the p53 tumor suppressor gene are frequently observed in HCC (3–6). The predominant types of p53 mutations in HCC cases from patients living in high HCC risk areas are G:C → T:A single base substitutions, which tend to cluster at codon 249 of the gene (3–5). This type of mutation is known to be induced by aflatoxin B1 (7). In a very recent study based on HCC samples from patients of 14 different countries including Germany, Ozturk et al. (5) demonstrated a correlation between the presence of mutations at codon 249 of the p53 gene and a high risk of exposure to aflatoxins and hepatitis B virus. The possible occurrence of p53 base substitutions at gene loci other than codon 249, however, was not investigated in their study. In the present investigation we have now screened 13 HCCs from German patients with well-defined disease histories for the presence of aberrations in the evolutionary highly conserved regions (exons 4 to 8) of the p53 gene, where the vast majority of mutations have been localized (8). Aberrations of the p53 gene were examined by sequencing of PCR products, by SSCP analysis, and by RFLP analysis. Our data demonstrate the presence of point mutations in two and hemizygosity at the p53 gene locus in three of the HCCs.

Materials and Methods

Thirteen human hepatocellular carcinomas were examined. Samples were taken from resected tumors or explanted tumor-bearing livers. From each tissue specimen serial cryostat sections were prepared. The first and last sections of each series were stained for glycogen by the periodic acid-Schiff reaction. An additional serial section was stained for hematoxylin and eosin to allow the identification of tumor and normal tissues. The remaining sections were mounted on dialysis membranes, and tumor and normal tissues were dissected with a scalpel.

For PCR amplification of DNA from the evolutionary conserved exons 4 to 8 of the human p53 gene we used the following primers, which were deduced from the published human p53 sequence (9):

- **exon 4**: HD1: 5'-AAG CAA TGG ATT TGA TGC TG-3'
  HE11: 5'-GCC CCA GCT GCT CAC CAT-3'
- **exon 5**: HA1: 5'-CTT CCT CCA GTA GCA TCC CGC-3'
  HC11: 5'-AAC ATG AGC GCT GCT CAG-3'
- **exon 6**: HE11: 5'-ACC ATG AGC GCT GCT CAG CTA ATG-3'
  HE11: 5'-AGT GTC AAA CCA GAC CTC AGG CGG-3'
- **exon 7**: HB1: 5'-GTT GGC TCT GCT TAT ACC ATC-3'
  HB11: 5'-GCT CCT GAC CTT GAG TCT-3'
- **exon 8**: HA2: 5'-CTA TCC TGC GCT GTA ATC TA-3'
  HA11: 5'-GCT TTT CAA CCT CGC TTA GTG-3'

Dissected tissue samples were incubated in a solution containing 10 µl × Taq polymerase buffer (Stehelin, Basel, Switzerland) and proteinase K (0.1 mg/ml) for 3–15 h at 37°C. Subsequently, the samples were overlayed with mineral oil; incubated at 94°C for 20 min; supplemented with primers (final concentration, 0.5 µM each), exonuclease triphosphate (each 200 µM), and 0.1 units Tag DNA polymerase (Stehelin) to a final volume of 50 µl; and amplified for 35 cycles.

Amplification of DNA sequences around codons 12 and 61 of the c-Ha-ras gene and mutation analyses at these gene loci via allele-specific oligonucleotide hybridization were performed essentially as recently reported (10) using primer sets and oligonucleotide probes as described by Verlaan-de Vries et al. (11).

PCR products from exons 5, 7, and 8 of the p53 gene from tumors and surrounding tissues were sequenced following extraction of the PCR reaction mixtures with chloroform-isooamyl alcohol and ultrafiltration using Ultrafree-MC units (Millipore, Bedford, MA). The sequencing primer for exon 5 was 5'-GCC CCA GCT GCT CAC CAT-3', for exon 7, HB1, and for exon 8, 5'-TGG GGC TCT GAC CTT GGT GTC-3'. Sequencing for both directions, additionally using the 5' amplification primers as sequencing primers. Sequencing was performed with 5' 32P-labeled primers using the Sequenase II kit (US Biochemicals, Cleveland, OH).

For SSCP analysis (12), aliquots of the first p53 amplification reactions were reamplified for 15 rounds in a "hot mix" containing unlabeled and 5' 32P-labeled primers in a 9:1 ratio. Exons 4 to 8 were reamplified with the primer pairs mentioned above; for detection of a polymorphic site within intron 7 (13), the primer pair HBI/HAI1 was used, both for the initial amplification and for the hot mix, yielding a 601-base pair fragment. DNA fragments were separated by electrophoresis in a 0.02-cm-thick nondenaturing polyacrylamide gel (3.5% for the 601-base pair fragment, 6% for all other fragments) prepared with 0.5% Tris-borate/EDTA buffer (either with or without 5% glycerol) at 30 W for 2 to 4 h at room temperature under cooling with a fan.

For detection of a p53 polymorphism at codon 72, the purified PCR products of exon 4 were incubated overnight with PnuDII (AGS,
In our study we analyzed HCCs from 13 German patients for the presence of p53 aberrations and mutations at codons 12 and 61 of the c-Ha-ras gene. The histological type of HCC, underlying liver disease, age, and sex of the patients are summarized in Table 1. Most of the HCCs showed trabecular or solid growth patterns with varying degrees of cellular differentiation ranging from highly to poorly differentiated forms. One HCC was of the fibrolamellar type. High cellular differentiation was frequently associated with excessive storage of glycogen, whereas poorly and undifferentiated tumor cells were poor in, or free of, glycogen. However, nuclear atypia was often found in both glycogen-rich and glycogen-poor tumor components. In 4 of the 13 HCCs, pronounced variations in glycogen content was observed in large, well-demarcated subpopulations as exemplified in Fig. 1A. In all of these cases each subpopulation was selectively dissected from serial cryostat sections and studied separately for p53 and c-Ha-ras mutations.

Sequence analysis of the evolutionary conserved exons 5, 6, and 8 of the p53 gene revealed the presence of single base substitutions in two of the HCCs (see Fig. 1 and Table 1). The types of mutations were a T:A —> A:T transversion at the second position of codon 257 (exon 7) in tumor 230/88, which changes the leucine to a glutamine codon, and a C:G —> T:A transition at the first position of codon 273 (exon 8) in tumor 128/89, which results in an arginine-to-cysteine amino acid change. In both cases, the same types of base substitution were present in tissue samples taken from different subpopulations of tumor cells (see Fig. 1). With DNA from tumor 230/88, only the signal representative of the mutated base was detectable at codon 257, suggesting a loss of the wild-type p53 allele in this tumor. In contrast, tumor 128/89 showed signals for both the mutated and the wild-type bases at codon 273 at an approximately 1:1 ratio. Due to the procedure used for the isolation of tumor material, significant contamination by stromal or normal surrounding cells, which would yield wild-type signals, can be excluded.

In additional experiments we examined all HCCs for p53 aberrations and loss of heterozygosity by means of PCR-SSCP and RFLP analyses. PCR-SSCP detects polymorphic sites in exons 6 (15) and intron 7 (13) of human p53, while an RFLP exists at codon 72 of exon 4 which can be analyzed by digestion with FnuDII (14). In total, six tumor cases were informative when combining data from all three polymorphic sites. The SSCP and the restriction banding patterns of p53 PCR products generated from five of these tumors and their corresponding normal tissues are shown in Fig. 2. Comparison between normal and tumor tissue demonstrated the loss of one of the polymorphic SSCP bands in tumors 311/88 and 125/90. RFLP analysis provided additional information about these two HCCs. In accordance with the result obtained by SSCP analysis, tumor 311/88 showed a tumor-specific loss of polymorphism at the FnuDII restriction site at codon 72 of p53, while no such change was detectable in case 125/90. Therefore, this latter HCC is characterized by a loss of only part of one p53 allele around codon 72, while a second part spanning the polymorphic site in intron 7 is undeleted. Tumors 129/89 and 243/90 showed informative exon 6 SSCP banding patterns but no loss of heterozygosity (data not shown).

Since mutations in one of the three ras oncogenes have been found to be a frequent genetic alteration in various human tumors and since mutations in the c-Ha-ras gene are frequently observed in liver tumors from certain strains of mice (10), we additionally examined all HCCs of the present study for all possible single base substitutions at codons 12 and 61 of this latter gene. In accordance with previous findings on the absence of ras mutations in human hepatocellular carcinomas (16), we were not able to detect c-Ha-ras base substitutions in any of the HCCs of this study.

Discussion

HCC is one of the most frequent cancers in certain areas of the world, where both hepatitis B virus and aflatoxins are assumed to represent major etiological risk factors. Analysis of genetic changes in tumors from patients that lived in high HCC incidence areas demonstrated the occurrence of p53 single base substitutions and allele losses in more than 50% of all cases (3-5). The types of base substitutions observed were primarily G:C —> T:A transversions and the mutations were clustered at codon

Table 1 Some characteristics of HCC cases analyzed

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>HCC histology</th>
<th>Surrounding tissue</th>
<th>Base substitution</th>
<th>Allele loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>230/88</td>
<td>72</td>
<td>M</td>
<td>S/T-PD</td>
<td>N</td>
<td>T:A —&gt; A:T (257)</td>
<td>Yes</td>
</tr>
<tr>
<td>281/88</td>
<td>23</td>
<td>F</td>
<td>T-HD/PD</td>
<td>N</td>
<td>N</td>
<td>NI</td>
</tr>
<tr>
<td>311/88</td>
<td>42</td>
<td>M</td>
<td>S-UD</td>
<td>N</td>
<td>N</td>
<td>Yes</td>
</tr>
<tr>
<td>32/89</td>
<td>50</td>
<td>M</td>
<td>S/T-PD/UD</td>
<td>AC</td>
<td>N</td>
<td>NI</td>
</tr>
<tr>
<td>39/89</td>
<td>45</td>
<td>F</td>
<td>T-PD</td>
<td>CC</td>
<td>N</td>
<td>NI</td>
</tr>
<tr>
<td>128/89</td>
<td>64</td>
<td>M</td>
<td>S/T-PD/UD</td>
<td>CC</td>
<td>C:G —&gt; T:A (273)</td>
<td>No</td>
</tr>
<tr>
<td>129/89</td>
<td>54</td>
<td>M</td>
<td>S/T-HD</td>
<td>AC</td>
<td>N</td>
<td>PC</td>
</tr>
<tr>
<td>91/90</td>
<td>50</td>
<td>M</td>
<td>S-PD</td>
<td>PC</td>
<td>PC</td>
<td>No</td>
</tr>
<tr>
<td>125/90</td>
<td>58</td>
<td>M</td>
<td>S/T-HD</td>
<td>PC</td>
<td>N</td>
<td>Yes</td>
</tr>
<tr>
<td>199/90</td>
<td>31</td>
<td>M</td>
<td>S-UD</td>
<td>PC</td>
<td>PC</td>
<td>Yes</td>
</tr>
<tr>
<td>335/90</td>
<td>31</td>
<td>F</td>
<td>FL-PD</td>
<td>N</td>
<td>N</td>
<td>No</td>
</tr>
<tr>
<td>336/90</td>
<td>1</td>
<td>M</td>
<td>M-T-HD</td>
<td>PC</td>
<td>PC</td>
<td>No</td>
</tr>
<tr>
<td>342/90</td>
<td>51</td>
<td>M</td>
<td>S-PD</td>
<td>AC</td>
<td>N</td>
<td>No</td>
</tr>
</tbody>
</table>

* Histology of tumors. S, solid; T, trabecular; HD, highly differentiated; PD, poorly differentiated; UD, undifferentiated; FL, fibrolamellar.

* Histology of surrounding tissue. N, normal; AC, alcoholic cirrhosis; PC, posthepatic cirrhosis resulting from hepatitis B virus infection as demonstrated by serological examination (in two cases HBsAG could also be demonstrated in some hepatocytes by Shikata's orcein stain); CC, cryptogenic cirrhosis.

* p53 aberrations were examined by SSCP, RFLP, and sequence analyses; additional analyses for all possible mutations at codons 12 and 61 of c-Ha-ras by allele-specific oligonucleotide hybridization did not give any positive results.

* Numbers in parentheses indicate the p53 codon affected.

* NI, noninformative.
Fig. 1. Demonstration of p53 base substitutions in HCCs. A, case 230/88. Inset, photomicrograph of a tissue section of the tumor stained for glycogen with periodic acid-Schiff stain. × 2.5; arrow, detail shown at 140-fold magnification. The section demonstrates the presence of three different subpopulations of tumor cells, a glycogen-free (1), a glycogen-rich (2), and an intermediate (3) population. From each of these cell populations, samples were taken for PCR amplification of p53 sequences and subsequent mutation analysis. A, bottom, results of the sequence analysis of p53 exon 7 demonstrating a T:A → A:T transversion at codon 257 with concomitant loss of the wild-type allele in all three subpopulations of cells. NL, normal surrounding liver tissue. B, case 128/89. The sequence analysis of two samples taken from different parts of this tumor demonstrates a C:G → T:A transition at codon 273 of p53 exon 8. Note that this tumor shows both the mutated and the wild-type p53 sequences.

249 of the gene (3–5). There are several lines of evidence suggesting that these particular mutations are causally related to aflatoxin B1; G:C → T:A transversions are the predominant base substitutions in bacteria exposed to activated aflatoxin B1 (7); codon 249 is a mutational target in DNA exposed to the activated carcinogen in vitro (17); and base substitutions at codon 249 of the p53 gene are not observed in HCCs from patients living in areas with lower aflatoxin exposure risk (5, 6).

In Germany, HCC is a comparatively infrequent cancer, with annual incidence rates below 2 per 100,000 inhabitants (18). HCC patients often show concomitant cirrhosis of the liver (in this study, 9 of the 13 cases analyzed), which is often caused by virus infection or chronic alcohol abuse. Aflatoxin B1 is presumably not an important risk factor for HCC in Germany (2), an assumption which is corroborated by our present data regarding the types of p53 base substitutions observed in two of the HCCs. Tumor case 128/89 showed a base substitution at the first position of codon 273. At this particular gene locus, point mutations have frequently been detected in other human tumors including brain, lung, and colon cancers; leukemias; and lymphomas (for a review, see Ref. 8). The type of mutation (C:G → T:A transition), along with its location at a CpG sequence, suggests that this base substitution occurred spontaneously by deamination of 5-methylcytosine and may not be related to any carcinogen exposure (19). The second point mutation detected in tumor 230/88 occurred at codon 257, which is not known as a p53 mutational hotspot. For reasons described above, the T:A → A:T transversion observed in this tumor also contradicts the idea that this mutation was caused by aflatoxins.

In human cancers, p53 base substitutions are often accompanied by a loss of the second wild-type allele. As is best demonstrated for colon cancer, both changes are likely to occur sequentially during the later stages of tumorigenesis (20). Of the two HCCs of this study showing p53 point mutations, only one demonstrated an additional loss of the wild-type allele. In
B. SSCP analysis. A 601-base pair PCR fragment including p53 intron 7 was enzyme detects a polymorphic site at codon 72. The heterozygous restriction clear size. Since the identical p53 base substitution was detected (arrow) which is lost in tumor tissue from patients 311/88 and 125/90, suggesting patients listed under I. Three informative cases show an additional SSCP band studied. Adjacent lanes represent banding patterns from nontumor and tumor of loss of one p53 allele in this tumor. P. nonrestricted PCR product: hp. base pairs. informative cases disappeared in the tumor from patient 311/88. suggesting a pattern (199, 103, and 96 base pairs) present in normal tissues from the five tumor (7") from the same patient are shown in adjacent lanes. The restriction observed in carcinogenicity experiments in rodent liver (21).

Acknowledgments

We express our thanks to Prof. Dr. G. Otto, Surgical Department of the University of Heidelberg and to Prof. Dr. R. Pichlmayr, Surgical Department of the University of Hanover for providing us with surgical specimens. We also thank E. Becker for preparation of the photomicrographic pictures and Dr. H. Glauert for helpful discussion.

References

p53 Mutations in Human Hepatocellular Carcinomas from Germany


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/52/11/3220

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.