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Advances in Brief

p53 Mutations in Human Hepatocellular Carcinomas from Germany[1]

Stefan Kress, Uli-Rüdiger Jahn, Albrecht Buchmann, Peter Bannasch, and Michael Schwarz[2]

German Cancer Research Center, Project Group Tumor Promotion in Liver [S. K., M. S.], and Division of Cytopathology [U-R. J., P. B.], Im Neuenheimer 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 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[3] 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 B_1 (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[4] 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.

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: HCC, hepatocellular carcinoma; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism; RFLP, restriction fragment-length polymorphism.

[4] Three of these HCCs were also investigated by Ozturk *et al.* (5) for *p53* codon 249 mutations.

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 ATG ATT TGA TGC TG-3′
HD11:5′-TGG TAG GTT TTC TGG GAA GGG AC-3′
exon 5 HA1: 5′-CCT CTT CCT GCA GTA CTC CCC TG-3′
HC11:5′-AAG ATG CTG AGG AGG GGC CAG AC-3′
exon 6 HE1: 5′-ACC ATG AGC GCT GCT CAG ATA GC-3′
HE11 5′-AGT TGC AAA CCA GAC CTC AGG CG-3′
exon 7 HB1: 5′-GTT GGC TCT GAC TGT ACC ACC AT-3′
HB11:5′-GCT CCT GAC CTG GAG TCT-3′
exon 8 HA2: 5′-CTA TCC TGA GTA GTG GTA ATC TA-3′
HA11:5′-GCT TGC TTA CCT CGC TTA GTG CT-3′

Dissected tissue samples were incubated in a solution containing 10 × 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), dexoynucleotide triphosphate (each 200 μM), and 0.1 units Taq 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-isoamyl 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 GGG CAG CTC GTG GTG-3′. Samples harboring a mutation were sequenced in both directions, additionally using the 5′ amplification primers as sequencing primers. Sequencing was performed with 5′ ^{32}P-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′ ^{32}P-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 HB1/HA11 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 *FnuD*II (AGS,

Heidelberg, Germany), which cleaves the arginine coding sequence of codon 72. This creates two fragments of 103 and 96 base pairs but does not cleave the polymorphic proline coding sequence (14). The DNA samples were separated in a 10% nondenaturing polyacrylamide gel and stained with ethidium bromide.

Results

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. 1*A*. 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 highly 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 *FnuD*II (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 *FnuD*II 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*

Case	Age	Sex	HCC histology[a]	Surrounding tissue[b]	Type of *p53* aberrations[c]	
					Base substitution[d]	Allele loss[e]
230/88	72	M	S/T-PD	N	T:A → A:T (257)	Yes
281/88	23	F	T-HD/PD	N		NI
311/88	42	M	S-UD	N		Yes
32/89	50	M	S/T-PD/UD	AC		NI
39/89	45	F	T-PD	CC		NI
128/89	64	M	S/T-PD/UD	CC	C:G → T:A (273)	No
129/89	54	M	S/T-HD	AC		No
91/90	50	M	S-PD	PC		No
125/90	58	M	S/T-HD	PC		Yes
199/90	31	M	S-UD	PC		NI
335/90	31	F	FL-PD	N		No
336/90	1	M	T-HD	PC		NI
342/90	51	M	S-PD	AC		No

[a] Histology of tumors. S, solid; T, trabecular; HD, highly differentiated; PD, poorly differentiated; UD, undifferentiated; FL, fibrolamellar.
[b] 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.
[c] *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.
[d] Numbers in parentheses indicate the *p53* codon affected.
[e] NI, noninformative.

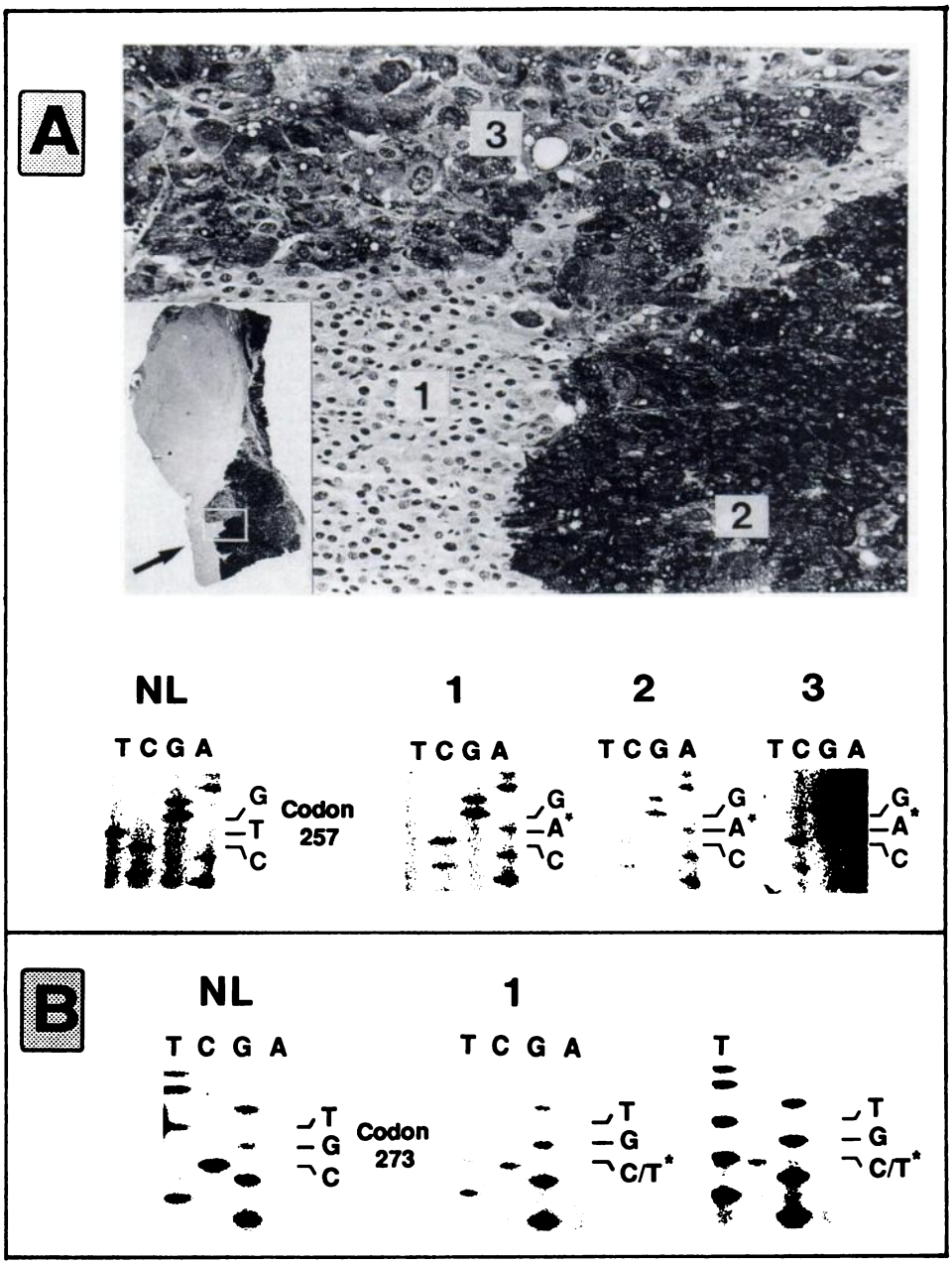

Fig. 1. Demonstration of *p53* base substitutions in HCCs. *A*, case 230/88. *Inset*, photomicrograph of a tissue section of the tumor stained for glycogen with periodic acid-Schiff stain. × 2.5; *arrow*, detail shown at 140-fold magnification. The section demonstrates the presence of three different 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 demonstrate 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 B_1: G:C → T:A transversions are the predominant base substitutions in bacteria exposed to activated aflatoxin B_1 (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 B_1 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

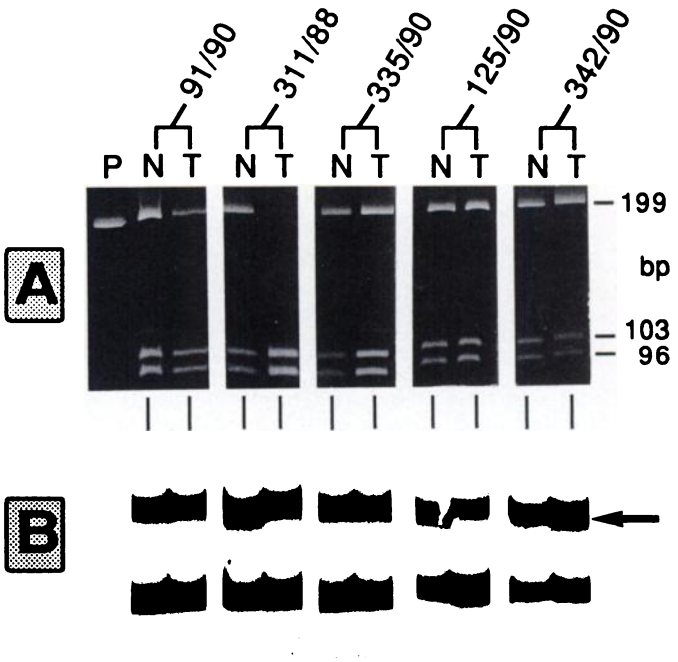

Fig. 2. Demonstration of loss of *p53* heterozygosity in HCCs. *A*, RFLP analysis. *p53* PCR products from exon 4 were digested with *FnuD*II, separated in a polyacrylamide gel, and stained with ethidium bromide. Nontumor (*N*) and tumor (*T*) from the same patient are shown in *adjacent lanes*. The restriction enzyme detects a polymorphic site at codon 72. The heterozygous restriction pattern (199, 103, and 96 base pairs) present in normal tissues from the five informative cases disappeared in the tumor from patient 311/88, suggesting a loss of one *p53* allele in this tumor. *P*, nonrestricted PCR product; *bp*, base pairs. *B*, SSCP analysis. A 601-base pair PCR fragment including *p53* intron 7 was studied. *Adjacent lanes* represent banding patterns from nontumor and tumor of patients listed under *A*. Three informative cases show an additional SSCP band (*arrow*) which is lost in tumor tissue from patients 311/88 and 125/90, suggesting loss of heterozygosity.

total, allele loss was demonstrated in 3 of the 8 cases, where polymorphic sites allowed the identification of loss of heterozygosity by sequencing, SSCP, or RFLP analyses.

An interesting observation is based on the analysis of tumor 230/88. This tumor was composed of well-circumscribed subpopulations of cells which differed considerably with respect to their glycogen content and cellular morphology, including nuclear size. Since the identical *p53* base substitution was detected in all cells, the different subpopulations of this tumor must be clonally derived from only one progenitor cell. This finding favors the assumption of a multistage process leading from glycogen-storing to glycogen-deficient cell populations, as is observed in carcinogenicity experiments in rodent liver (21).

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