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Absence of *p53* gene mutations in hepatocarcinomas from a Mediterranean area of Spain

A study of 129 archival tumour samples

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Abstract The incidence of *p53* gene abnormalities in human hepatocellular carcinoma (HCC) varies in different geographical areas, being higher in regions where hepatitis virus infection and dietary exposure to aflatoxin B1 are the most common aetiological agents. These mutations are less frequently encountered in Europe, although some studies have reported p53 protein overexpression in up to 45% of cases analysed. We have analysed 129 tumour samples of primary malignant hepatic neoplasms recovered from paraffin blocks processed in two pathology laboratories in a Mediterranean area of Spain (Valencia and Gerona). Among 14 cases in which p53 immunohistochemistry expression proved positive, 5 stained in more than 50% of the cell nuclei. By PCR-SSCP analysis we could detect the complete sequence from exon 5 through 8 in 70 cases and part of this region in the remaining cases, but no mutations were found. We found no relationship with the clinical stage, tumour stage or clinical outcome. We conclude that *p53* gene alterations are not a major event in the malignant transformation of hepatic cells in this region of the Mediterranean. The variable incidence of *p53* gene alterations in other geographical areas may reflect a different genetic background for the aetiology of HCC.

Key words *p53* mutation · Hepatocellular carcinoma

Introduction

Abnormalities of the *p53* gene are the most common molecular events in human cancers [14]. The most frequent type is missense mutation in the evolutionary conserved regions located in exons 5–8, coding for the central domain of the protein that mediates DNA binding and transcriptional activation. This results in the inhibition of its growth suppressor function. As a consequence, the stability of the p53 protein increases, and it accumulates in the cell nuclei and can be detected immunohistochemically. Nevertheless, other non-mutational changes, such as interactions with endogenous or exogenous proteins, may also stabilize the p53 protein [12], producing a positive nuclear staining.

Several studies have investigated the spectrum of *p53* gene mutations in human hepatocellular carcinoma (HCC). In high-risk areas where chronic hepatitis virus infections and high dietary exposure to aflatoxin B1 are the most common aetiological agents in the molecular pathogenesis of the HCC, *p53* gene mutations appear in 45–75% of cases and affect mostly codon 249 [4, 8, 11, 16, 27, 28, 32]. In Europe and other developed regions the incidence of *p53* mutations in HCC is much lower, there being no preferential "hotspots" [6, 9, 33]. These variable results in different geographic areas obscure the mechanisms by which *p53* genetic lesions operate in the multistep progression of hepatocarcinoma.

The aim of the present study was to determine, in a group of HCC from a Mediterranean area of Spain (Valencia and Gerona), the extent of involvement of *p53* gene alterations at structural and expression levels, using molecular and immunohistochemical techniques.

Materials and methods

A total of 129 samples recovered between 1972–1996 was studied, being 87 from the Hospital Josep Trueta (Gerona) and 42 from the Hospital Clínico (Valencia). They consisted of small pieces or cylinders of hepatic tumour tissue, fixed routinely in 10% buffered formalin and paraffin embedded. One hundred and three patients were male and 26 female. The mean age was 66.15 years. Fourteen percent of patients had a single HCC nodule less than 5 cm in diameter; 16.3% had a single nodule more than 5 cm or more than one nodule, but in only one lobule; 69.8% had multiple HCC nodules. Further, 44.7% were patients whose daily alcohol intake had been more than 80 g in the last 10 years. In serum, HbsAg and

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ANTI Hc were detected by enzyme-linked immunosorbent assay (Abbot Laboratories); 9.6% were positive to HbsAg and 68.6% to ANTI Hc. Histopathological analysis was performed after conventional staining (HE, PAS, Reticulin, and Goldner stains) and samples with predominantly normal or nontumour tissue were discarded. All samples were hepatocellular carcinomas, except for 2 cholangiocarcinomas. We evaluated the histological differentiation grade as poorly, moderately and well differentiated [1].

High-molecular-weight DNA was isolated from three to five 10-µm sections of paraffin blocks. After treatment with xylol and graded ethanols to extract the paraffin, the tumour tissue was lysed in 400 µl of a buffer containing 10 mM Tris-HCl pH 8.3, 59 mM KCl, 2.5 mM MgCl2, 0.45% Tween-20, and 0.5 mg/ml proteinase K at 55°C for 1 h. The DNA was purified by extraction with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated in ethanol, and dissolved in ultrapure water.

DNA from exons 5 through 8 of the *p53* gene was amplified using the polymerase chain reaction (PCR) performed in 50 µl reaction mixture containing approximately 100 ng genomic DNA, 0.5 µM of each primer, 200 µM of each dNTP and 1 U Taq polymerase (Gibco BRL, Paisley, UK). The following primers were used:

5A: 5'-TTCCTCTTCCTGCAGTAC-3';
5B: 5'-GCCCCAGCTGCTCACCA-3'

6A: 5'-GGGCTGGTTGCCCAGGGT-3';
6B: 5'-TAGTTGGAAACCAGACCTC-3'

7A: 5'-GTGTTGTCTCTTAGGTTG-3';
7B: 5'-TGGCAAGTGGCTCCTGAC-3'

8A: 5'-CCTATCCTGAGTAGTGGT-3';
8B: 5'-GTCCTGCTTGCTTACC-3'.

The fragment sizes ranged between 142 and 214 base pairs.

Thirty-five cycles of denaturation: 94°C, 10 s, annealing: 55°C (60°C for exon 6) 45 s and extension: 72°C, 25 s were performed.

For SSCP analysis we used the same basic method as described by Orita et al. [25] and Spinardi et al. [29]. After 2.5% agarose gel electrophoresis to check the quality of the PCR product, we took 3–5 µl of this product and mixed it with an equal volume of formamide loading dye. The mixture was heated at 95°C for 10 min, chilled on ice and applied to a 10% nondenaturing polyacrylamide gel containing 5% glycerol. Electrophoresis was performed at 20 V/cm in 0.5×Tris–boric acid–EDTA for approximately 5 h. The temperature was maintained at 20°C with constant recirculation of the buffer between the upper and the lower chambers. The gels were stained using the Silver Stain kit (BioRad, Hercules, Calif.).

The PCR-amplified DNAs were purified with Centricon columns (Amicon, Beverly, Mass.) and sequenced on an Applied Biosystem model 373 automated sequencer (Foster City, Calif.) using the dideoxy dye terminator method with the Taq polymerase and the sense and antisense PCR primers. We used as control other tumour samples from our laboratory with well-known *p53* mutations [26].

Five-micrometre sections were deparaffinized and endogenous peroxidase was inhibited with 3% hydrogen peroxide for 30 min, washed in phosphate-buffered saline (PBS), and then incubated with 10 mM buffer citrate and heated in a microwave oven for 10 min (700 W). Sections were blocked with 20% horse serum in PBS and incubated with primary antibody for 1 h. The time of incubation for the secondary antibody and avidin–biotin complexes was 30 min at room temperature. Sections were extensively washed and the immunoreaction developed using DAB (0.05% 3'3'diamino-benzidine in 0.1% hydrogen peroxide). Negative controls included substitution of the primary antibody by mouse ascitis or PBS alone.

Monoclonal antibody Bp 53-11 (Progen Biotechnik, Heidelberg, Germany) that recognizes the wild and mutant forms of the p53 protein was used [2]. This antibody is a mouse immunoglobulin of the IgG2a subclass and was used at a concentration of 10 µg/ml in PBS containing 2% bovine serum albumin (PBS/BSA). As secondary antibody a biotinylated rabbit anti-mouse IgG (heavy and light chain specific) affinity purified antibody (1:200 dilution in PBS) was used, followed by avidin–biotin peroxidase complexes at 1:100 dilution in PBS (Vector Labs, Burlingame, Calif.). The *p53* nuclear immunoreactivities were classified as follows: negative (<10% tumour cells displaying nuclear staining); low (10–20% tumour cells with nuclear reactivity); medium (20–50% tumour cells with nuclear reactivity) and high (>50% tumour cells with intense nuclear staining).

Results

Of the 129 patients in this series, we were able to study exons 6 through 8 of the *p53* gene for only 70. In 10 cases the DNA degradation did not allow amplification of any exon, and in the remaining cases some exons failed to give an amplified product.

Electrophoretic mobility shifts on SSCP analysis were observed in only 2 cases (exon 6). Sequencing detected the same point mutation type in these cases: an A-to-G transition in the third base position of codon 213, result-

Table 1 Results of *p53* gene characterization in IHQ positive tumours

Patient	Age	Sex	Extension	Alcohol	Virus	Survival	Dif.	IHQ	Mutation (exons 5–8)
G013	57	M	3	–	–	16 m	MD	+++	Normal
G043	74	F	3	–	–	1 m	PD	+++	Normal
G074	78	M	3	–	–	3 m	WD	++	Normal
G146	67	M	3	–	–	2 m	PD	++	Normal
795	?	M	2	–	C	4 y	PD	++	213 Neutral mutation
G068	62	M	3	+	–	3 m	PD	+	Normal
G088	60	M	2	+	C	4.5 y	BD	+	Normal
G106	71	F	3	–	C	1 m	WD	+	Normal
G118	62	F	3	–	–	18 m	WD	+	Normal
G144	63	M	3	–	–	4 m	MD	+	Normal
754	65	F	2	–	?	13 m	MD	+	Normal
766	73	M	2	–	–	> 10 y	WD	+	Normal
774	65	F	2	+	B	1 m	PD	+	Normal
775	?	F	?	?	?	?	PD	+	Normal

(*M* male, *F* female, extension: *1* 1 nodule <5 cm, *2* 1 nodule >5 cm or >1 nodule in 1 lobule, *3* multiple nodules, *Alcohol* + daily intake >80 g/day – daily intake <80 g/day, *Virus*: *B* HbsAg + *C* ANTI Hc +, Survival: *m* months, *y* years, *PD* poorly differentiated, *MD* moderately differentiated, *WD* well-differentiated, *IHQ* p53 immunoreactivity)

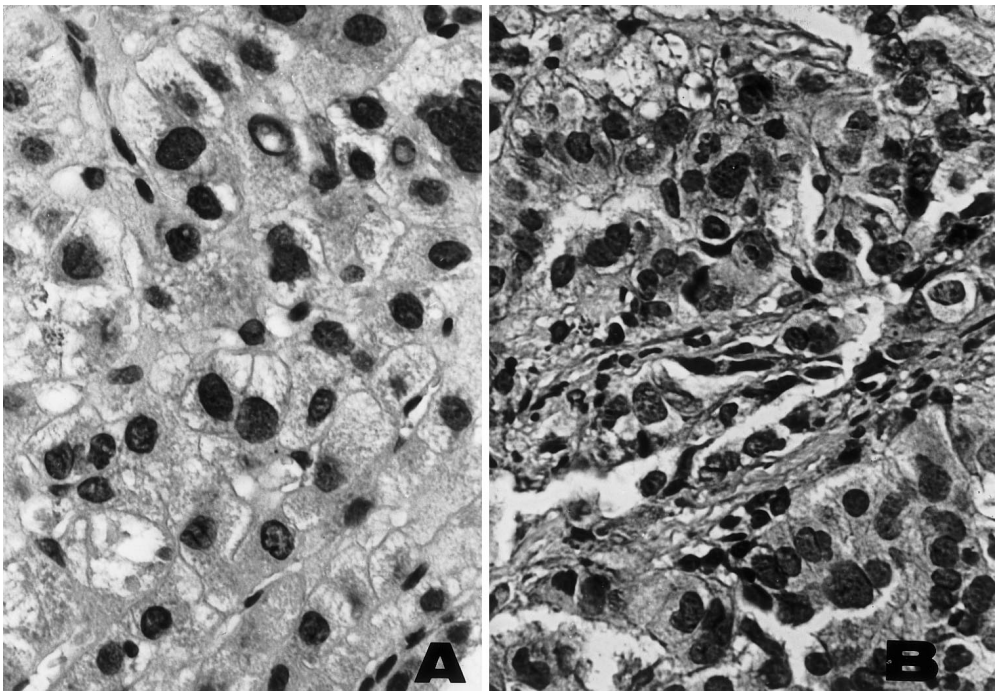

Fig. 1A, B Two hepatocellular carcinomas showing positivity for p53. **A** Case 754 corresponds to a moderately differentiated tumour with presence of p53 nuclear staining. **B** Case 774 displays a poorly differentiated neoplasm with regular positivity for p53. HE counterstaining, ×40

ing in no amino acid substitution, because both sequences coded for arginine. Thus, the mutation has no effect on the amino acidic sequence of the protein (silent or neutral mutation). Case 18 without SSCP alteration was selected at random and sequenced for different exons. In no case we did find abnormalities in the sequence.

Abnormal nuclear expression of p53 protein appeared in nuclei of 14 cases: 2 with >75% positive cells, 3 with 50% immunostained nuclei and 9 with positive staining in under 20% nuclei, either dispersed or focally located (Fig. 1). There was no signal in the remaining 115 tumours, or only isolated (less than 10%) positive nuclei. One case with neutral codon 213 mutation showed immunostaining in 50% of the nuclei, and the other was negative. Neither of the two reviewed cholangiocarcinomas expressed p53. There was no significant relationship between any possible aetiological factor (alcohol intake, viral) and p53 positivity. Moreover, the clinical outcome of the patients was not correlated with histochemical expression of p53 in their tumour cells (Table 1).

Discussion

Primary hepatocellular carcinoma is one of the most frequently reported malignancies in the world. Depending on different risk factors, the prevalence shows a broad spectrum of variation in distinct geographical regions [13]. However, the molecular mechanisms in hepatocarcinogenesis are poorly understood.

Studies on HCCs from high-risk areas detected *p53* gene aberrations in a significant percentage of tumours (45–75%) and a common hotspot for *p53* mutations at codon 249, although other studies have found a lower incidence (25%) [4, 5, 15]. This mutation has been considered to be a direct result of aflatoxin B1 adduction to DNA guanine residues [10], but it has also been found in regions with no aflatoxin B1 exposure [33].

The importance of *p53* mutations in the aetiology of HCC cannot be confirmed in European countries, where no AFB1 exposure occurs. Thus, the frequency of *p53* gene mutations detected at level of changes in DNA sequence is low. They occur at several distinct codons in the core domain of the gene, there being no preferential 249 codon incidence [3, 18, 19]. Nevertheless, parallel analysis of p53 protein by immunohistochemical techniques has revealed overexpression in up to 45% of cases studied [33].It has been shown that, in the absence of gene mutations, the p53 protein can be stabilized by other endogenous cellular proteins, such as mdm2 [24]. Viral proteins can also enlarge the half-life of p53 protein [22, 31]. Consequently, it has been postulated that the X transactivator protein of hepatitis B virus may bind to p53 protein and abrogate its normal cellular function [34].

We made a retrospective analysis of a large number of HCC samples fixed in 10% buffered formalin and embedded paraffin, using PCR-SSCP techniques and sequencing. In 70 cases all the coding regions of exons 5, 6, 7 and 8 were determined, which are the most frequently mutated in human cancers. Only 10 cases resisted our technical approach, owing to the small size of the sample and probable DNA degradation following fixation. These dificulties may also explain the isolated exon amplification failures in the remaining cases.

In two HCCs a previously described neutral mutation was found at codon 213 that seems not to be implicated in carcinogenic processes [20]. There were no further cases with electrophoretic shifts in the SSCP analysis.

Nevertheless, we sequenced 18 samples of different exons in several tumours and found no mutation. Some difficulties may exist in the interpretation of sequencing results, probably due to DNA fragmentation in paraffin-embedded tissue storage, in addition to odd errors introduced by TaqI polymerase in the DNA elongation process. The sequencing method we used overcomes this problem because only those mutations that coincide strictly with sense and antisense primers in the sequencing runs can be regarded as DNA alterations. Similar caution must also be exercised when sequencing is performed by cloning strategies [19]. The detection of two neutral mutations validates our technical approach, even if we cannot exclude the possibility of mutations that have escaped because of inadequate sensitivity of the SSCP technique or are located in isolated exons that were not analysed.

Immunohistochemical analysis demonstrated accumulation of nuclear staining in more than 50% of nuclei in five tumours, most of them poorly differentiated and of greater size [21], but there was no relation with age, sex or presence of hepatitis virus [23, 30] (Table 1). Other isolated positive nuclei were observed in 9 cases of HCC.

The low incidence of *p53* alterations detected in the present analysis indicates that this genetic abnormality is not necessarily involved in the malignant transformation of the hepatic cells in this Mediterranean area. Since the histopathological characteristics do not differ in HCC from various geographic areas, the discrepancy in frequency of *p53* alterations may indicate that these changes do not represent a primary oncogenic event but are a late event in tumour progression [7, 17]. Alternatively, the existence of a different genetic background for the aetiology of HCCs in different human populations could be postulated.

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