Pergamon

European Journal of Cancer Vol. 31A, No. 11, pp. 1847–1850, 1995

0959-8049/95 $9.50+0.00

0959-8049(95)00380-0

Original Paper

p53 in Malignant and Benign Liver Lesions

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p53 expression was studied by immunohistochemical methods in benign and malignant human epithelial liver lesions in 46 patients from Hungary. Positive immunostaining for p53 protein, indicating the overexpression or prolonged half-life of p53 protein, was detected in the nuclei of tumour cells of seven of the 16 hepatocellular carcinomas (HCC) (44%), including three HCC patients with hepatitis B virus infection. Immunostaining of p53 was seen in one of the seven hepatoblastomas, none of the 17 focal nodular hyperplasias, and none of the six hepatocellular adenomas. The detection of p53 in a relatively high percentage of the HCC cases in Hungary, a country in which aflatoxin contamination of the diet is rare, suggests that factors other than aflatoxin led to the accumulation or overexpression of p53 in these patients.

Key words: p53, hepatocellular carcinoma, hepatoblastoma, benign liver lesions, immunohistochemistry
Eur J Cancer, Vol. 31A, No. 11, pp. 1847–1850, 1995

INTRODUCTION

THE p53 NUCLEAR phosphoprotein functions in the regulation of the cell cycle and as a tumour suppressor. *TP53* mutations have been detected in human cancers of the lung [1, 2], colorectal tract [3, 4], breast [5], bladder [6] and liver [7–13] and several other tumours. Because of the low intracellular concentration of p53 and its short half-life, the normal or "wildtype" p53 protein usually cannot be detected by immunohistochemical methods [7, 14–16]. Mutations in the *TP53* gene, however, can usually be demonstrated by immunohistochemical staining of p53 protein, perhaps due to metabolic stabilisation and prolongation of the half-life of the mutant protein in the nucleus [14, 15]. However, immunohistochemistry can detect the accumulation of the wild-type p53 protein, representing the overexpression of p53, and it has been demonstrated in different tumours including astrocytomas [17], colorectal adenomas [18] and bone and soft tissue sarcomas [19]. Overexpression of p53 is usually, but not always [20, 21] correlated with *TP53* gene mutation; in all cases, however, its detection suggests that p53 is abnormally regulated.

TP53 gene mutations have been found in a large percentage of hepatocellular carcinomas (HCCs) in association with certain possible aetiologic factors in hepatocarcinogenesis. An association between *TP53* gene mutation in HCC and infection with the hepatitis B virus (HBV) has been suggested [7]. In HCC cases from China and South Africa, *TP53* mutations at codon 249 have been thought to be due to aflatoxin contamination of the diet [9, 10]. However, *TP53* gene mutations have also been described in HCCs from geographical regions where aflatoxin exposure is very low [22–24], although not usually at codon 249 [22–28]. *TP53* mutations have been reported in a few patients with hepatoblastoma (HB) [23, 29–31]; one of these was shown to be at codon 249 [31].

In the present study, the expression of the *TP53* gene at the protein level was examined in HCC, HB and benign liver lesions including focal nodular hyperplasia (FNH) and hepatocellular adenoma (HCA) obtained from patients in Hungary. This is a geographical area where the prevalence of HBV infection is low [32] and the level of dietary contamination with aflatoxin is very low [33]. Thus, it may be possible to evaluate further whether *TP53* mutations or overexpression in HCC and other diseases of the liver can occur in the absence of exposure to large amounts of aflatoxin or to HBV infection.

It has been suggested that certain benign liver lesions may be precursors of HCC; the prevalence of *TP53* mutations in these lesions has not been reported. Cases of HCA and FNH were evaluated in the present study to determine whether p53 can be detected by immunohistochemistry in benign liver lesions, and to see whether its prevalence is consistent with the suggestion that these benign liver lesions can lead to HCC.

PATIENTS AND METHODS

Patients

Resected tumours and adjacent non-tumorous liver tissues were studied from 16 patients with HCC, 7 with HB, 17 with FNH and 6 with HCA. All patients were lifelong residents of Hungary and the lesions were resected at the Semmelweis Medical University and other hospitals throughout Hungary between 1975 and 1993. All resected samples were sent to the Semmelweis Medical University for diagnosis. The male:female

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Revised 4 Apr. 1995; accepted 10 Apr. 1995.

ratio was 8:8 for HCC, 2:5 for HB, 1:16 for FNH and 0:6 for HCA. The mean age was 50 years for HCC (range: 15–70), 2 years for HB (range: <1–11), 39 years for FNH (range: 26–77), and 38 years for HCA (range: 31–48) (Table 1).

Heavy alcohol consumption was documented for 4 HCC patients, but was not known for the remaining patients in this study. 7 of the 17 women with FNH and 3 of 6 women with HCA had been taking oral contraceptives for longer than 2 years, but information was not available on the exact duration of administration and the oestrogen content of the pills.

Hepatitis B surface antigen (HBsAg) and antibody to the hepatitis B core antigen (anti-HBc) were positive in the sera of 3 patients with HCC (detected by radioimmunoassays, Ausria II and Corab, respectively; Abbott Laboratories, North Chicago, Illinois, U.S.A.). Sera from the remaining 14 HCC patients, from all HB patients and from all patients with benign liver lesions were negative.

Cirrhosis was present in the non-tumorous livers of 6 HCC patients (micronodular in all). Of the 6 patients with cirrhosis, alcoholism was known in 3 cases; HBsAg was detected in the serum of 3 patients.

The diagnosis of an intrahepatic tumour and the indication for surgical resection were based on clinical signs and symptoms (including right abdominal mass and pain), abdominal ultrasound, computed tomography, and clinical laboratory abnormalities. The average time between the diagnosis and the surgical resection was 1.5 months (range: 2 weeks–6 months).

Histology and immunohistochemistry

Tissues were fixed in 10% neutral, buffered formalin and embedded in paraffin. 3–5 μm paraffin sections were cut and stained with haematoxylin and eosin (H&E), periodic acid Schiff (PAS) with and without diastase digestion, connective tissue stain according to Mallory, and Prussian blue for iron detection.

For immunohistochemical studies, 3–5 μm paraffin sections were applied to slides coated with poly-L-lysine (Sigma Inc., St Louis, Missouri, U.S.A.), and digested with proteinase K (1 μg/ml in phosphate buffered saline (PBS), pH 7.4) for 10 min at room temperature. For detection of p53 protein, a rabbit polyclonal antibody to recombinant human p53 protein (NCL-p53-CM1, also called CM1, Novocastra Laboratories, Newcastle, U.K.), with reactivity to both wildtype and most mutant forms, was used in a 1:250 dilution. CM1 has been shown to react with p53 on paraffin sections [7, 14]. The immunostaining for p53 was repeated with a monoclonal antibody (Ab-6, derived from clone DO-1, Oncogene Science, Uniondale, New York, U.S.A.), at a 1 μg/ml concentration. Treatment with an antigen retrieval system (HK090-5K, BioGenex, San Ramon, California, U.S.A.) was applied in a microwave oven at 100°C twice for 5 min each before immunostaining with monoclonal antibody, Ab-6. A monoclonal antibody to the hepatitis B surface antigen (anti-HBs) (H-297-05; Medix Biotech Inc., Foster City, California, U.S.A.) was applied for localisation of the hepatitis B surface antigen (HBsAg). Incubation with the primary antibodies was carried out overnight at 4°C. An avidin–biotin–peroxidase technique was used to detect the antigens (Vectastain Elite ABC Kit, Vector Laboratories Inc., Burlingame, California, U.S.A.) with 3'3-diaminobenzidine (DAB) (Polysciences Inc., Warrington, Pennsylvania, U.S.A.) as chromogen, according to the manufacturer's instructions. Immunohistochemical staining of p53 was evaluated by counting the number of nuclei with positive staining among 100 cells in each of ten randomly selected fields (×40 objective).

Controls

Two normal liver samples served as negative controls. These were obtained from 2 female patients, aged 1 and 51 years, who had died of causes unrelated to liver disease (kindly provided by the Liver Tissue Procurement and Distribution System (LTPADS), under a service contract of the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland, U.S.A.).

A human HCC cell line containing multiple copies of the HBV, PLC/PRF/5 (American Type Culture Collection, Rockville, Maryland, U.S.A.) was used as a positive control. This cell line has been shown to have a *TP53* mutation at codon 249 [8].

Statistical evaluation

Fisher's exact test was used to analyse the data. A probability of less than 5% was considered statistically significant.

Table 1. TP53 *overexpression in human liver tumours and benign liver lesions detected by immunohistochemical staining*

Diagnosis	Age (years) Mean (range)	M:F	HBV	Number with p53 staining
Hepatocellular carcinoma	50(15–70)	8:8		
Tumour	(n = 16)		3*	7/(44%)[a]
Non-tumour	(n = 13)		3	0[b]
Hepatoblastoma	2(<1–11)	2:5		
Tumour	(n = 7)		0	1
Non-tumour	(n = 5)			0
Focal nodular hyperplasia	39(26–77)	1:16		
Lesion	(n = 17)		0	0[c]
Non-lesion	(n = 12)			0
Hepatocellular adenoma	38(31–48)	0: 6		
Lesion	(n = 6)		0	0
Non-lesion	(n = 2)			0

M:F, male:female ratio; Fisher's exact test: highly significant (*a* versus *b*: P <0.01, *a* versus *c*: P <0.01). *HBV was detected in the serum and in tissue sections.

RESULTS

p53 protein was detected in the nuclei of tumour cells of 7 of the 16 HCC patients (44%) (Table 1, Figure 1). However, in

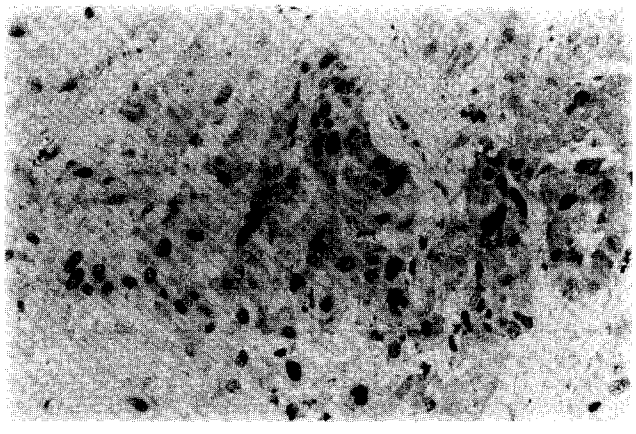

Figure 1. Hepatocellular carcinoma, in which a strong nuclear staining for p53 can be seen in >50% of the tumour cells. (No counterstain; magnification × 250.)

one case, cytoplasmic staining was also observed in scattered cells, with or without nuclear staining in the same cells. The percentage of positive cells per section varied; it was greater than 50% in 2 cases, 25–50% in 2 cases and less than 25% in 3 cases. The intensity of the immunostaining using the monoclonal Ab-6 antibody combined with the antigen retrieval system was much stronger than the polyclonal CM1 antibody without the retrieval system. However, the percentage of positive cells detected in positive cases was the same using either antibody and method.

Strong cytoplasmic staining for HBsAg was observed in hepatocytes in the surrounding liver and in a few tumour cells from the 3 HCC patients with HBsAg in their serum. HCC tissue from all 3 cases that stained positive for HBsAg also stained positive for the p53 protein. HB tissue from 1 of the 7 HB cases stained positive for p53. No staining of p53 protein was seen in any of the 17 FNH or 6 HCA cases.

In the adjacent non-tumorous liver tissue of all cases studied, hepatocytes did not stain positive for p53 protein (Figure 2). No immunohistochemical reactions for p53 protein or for HBsAg were found in the normal control human liver tissues. Strong nuclear staining for p53 protein was noted in most cells of the PLC/PRF/5 cell line. When the primary antibody to p53 protein was replaced with rabbit immunoglobulin on consecutive sections, no staining was noted in the PLC/PRF/5 cells or the positive HCC cases.

DISCUSSION

An association between *TP53* gene mutation at codon 249 in HCC and aflatoxin exposure has been suggested [8–10] and questioned [34]. The data presented here show that the p53 protein can be detected in HCCs from patients in Hungary, a country where contamination of food with aflatoxin is low [33]. Similar results have been reported from other low aflatoxin regions, including the U.S.A. [7] and other European countries [11, 21, 23, 26, 27]. In most studies in Europe, the percentage of HCCs with mutant p53 was relatively low, for instance in France and Italy [11, 23], Great Britain [26] and in Germany [27]. Volkmann and associates [21] demonstrated increased p53 levels due to point mutations in 10 of 22 HCCs from Germany by immunoblotting and immunohistochemistry. The percentage of HCC cases from Hungary with detectable p53 in the present study was 44%, which was closer to the findings in HCC patients from Germany [21] and from two United States populations (30–60%) [7]. In another study in a United States population, 32% of Oriental patients with HCC were positive for p53 by immunohistochemistry compared with 7% among non-Orientals [29].

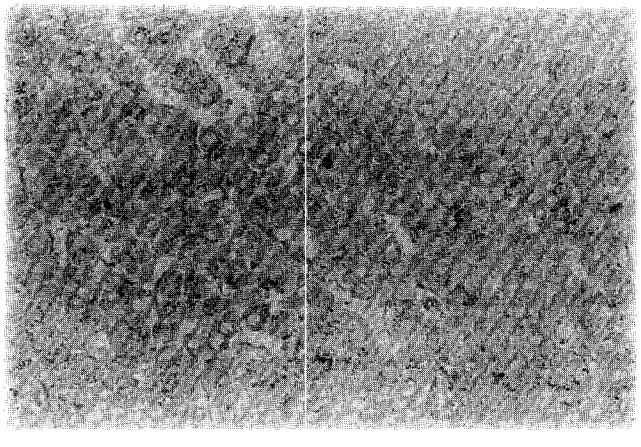

Figure 2. Non-tumorous liver tissue adjacent to hepatocellular carcinoma, in which no immunostaining of p53 is seen. (No counterstain; magnification × 250.)

A possible association has been suggested between *TP53* mutation in HCC and HBV infection [7, 34]. 3 of the 7 HCC cases with detectable p53 in the present study had HBV infections, which would be consistent with an association, although the small number of patients does not permit a conclusion. Others have reported finding no significant difference in p53 immunostaining between HCC cases with and without HBV infection [21, 29].

Immunohistochemical detection of p53 protein is often associated with the presence of a mutation in the *TP53* gene [7, 14]. In most studies comparing immunohistochemistry and direct sequencing, detection of p53 in the nucleus by immunohistochemistry was associated with a *TP53* gene mutation in all [1, 5] or most cases [2]. However, lack of correlation with gene mutation has been reported in some circumstances [18, 19, 20, 35].

The percentage of cells with detectable p53 protein varied in the HCC cases in the present study; it also varied among different areas of the same tumour, as in other studies of HCC [12, 20]. Heterogeneity of p53 staining within the same tumour has been reported in other types of human tumours, such as colon carcinoma [4], glioma [36] and bladder carcinoma [6] and bone and soft tissue sarcomas [19]. The heterogeneity of the p53 immunostaining could be explained by the cell cycle-dependent accumulation of p53 in different cells and/or by its interaction with other cellular proteins, abnormal growth factors, upregulated translational activation, or a post-translational modification, which might cause a difference in the half-life of either mutant or wildtype p53.

It has been shown in glioblastomas that a subcellular compartmentalisation of p53, detected as nuclear or cytoplasmic immunostaining of p53, was associated with different profiles of *TP53* mutation and allele losses on chromosome 17p [36]. It has been suggested that, if the p53 protein is in the cytoplasm, it could be associated with inactivation of normal function [18], or that the cytoplasmic localisation of p53 might represent a conformation change of mutant p53 protein, maintained by binding to the proteins of the heat shock protein family [1, 29]. However, others regard the cytoplasmic staining of p53 as non-specific [12].

Nuclei of one of the 7 HB cases stained positive for presumed mutant p53 protein. For HB cases in the present study, the tumours were relatively large at the time of resection; therefore, even a late mutation of *TP53* would already have been detectable, since the tumours were advanced. In other studies, 1/7 (14%) HB cases from the U.S.A. [29], 1/3 (33%) HB cases from the U.S.A. [31], 1/7 (14%) HB cases from Italy [23], and 4/12 (33%) HB cases from Germany [30] were positive for p53 as detected by immunohistochemistry. The few cases reported by these investigators and the cases reported here indicate that *TP53* mutation is a rare event in HB, and that it probably does not contribute to the development of most cases of HB.

p53 protein was not detected by immunohistochemistry in the 17 FNH and 6 HCA lesions. Premalignant lesions of other tissues have been found to have *TP53* mutations and elevated content of immunohistochemically defined wildtype p53 protein, for instance, in adenomas of the colon [4, 18]. The absence of p53 in the benign liver lesions in the present study and other studies [37] could lead to the conclusion that HCA and FNH are not precursors of HCC. However, *TP53* mutations or

overexpression are believed to occur as late events during carcinogenesis in many human tumours, including HCC [24], although it is possible that *TP53* mutations are involved at an early stage during malignant transformation in a few tumour types, for instance, Barrett's oesophagus [38]. Thus, the absence of mutant p53 protein or undetectable, normal levels by immunohistochemistry, does not rule out the possibility that FNH and HCA are premalignant lesions.

The detection of p53 in a relatively high percentage of HCC cases (44%) in a geographical area with low levels of dietary aflatoxin and a low prevalence of HBV infection suggests that factors other than aflatoxin or HBV led to the abnormal expression of p53 protein in these patients. p53 immunoreactivity is frequently a result of *TP53* gene mutations [1, 2, 5, 7, 14]; even when it is not, its detection by immunohistochemistry has to be considered abnormal. The accumulation of p53 in the nuclei could be a characteristic of changes in cell growth regulation in HCC, regardless of the aetiology of the tumour.

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Acknowledgement—Supported in part by the Hungarian Research Foundation grant T016077 and The Ministry of Welfare T-01 347/93.