Virchows Archiv (1994) 424:613–621

ORIGINAL ARTICLE

Ming Zhao · Nai-Xing Zhang · Jean A. Laissue
Arthur Zimmermann

Immunohistochemical analysis of p53 protein overexpression in liver cell dysplasia and in hepatocellular carcinoma

Received: 11 February 1994 / Accepted: 20 April 1994

Abstract We analysed p53 protein immunoreactivity in hepatocellular carcinomas (HCCs) and in liver cell dysplasia (LCD) of patients from an area in Northern China, using five anti-p53 protein antibodies recognizing different epitopes of the protein. In HCCs, the overall prevalence of p53 protein immunoreactivity was 78.3%. However, prevalence was strongly influenced by the type of antibody used, ranging from 67.5% for antibody PAb-1801 to only 10.8% for antibodies PAb-421 and DO-7. p53 protein immunoreactivity was not related to type or grade of HCC. In contrast to former reports, p53 protein staining was restricted to nuclei only when using the CM-1 antibody, whereas two other antibodies yielded both, nuclear and cytoplasmic or membrane staining, and no nuclear staining was observed with antibodies PAb-421 and DO-7, the latter two, however, demonstrating cytoplasmic and membrane staining. For LCD, three subtypes were morphologically and karyometrically defined. Nuclei of some LCD cells were p53 immunoreactive, but positivity was restricted to the small cell variant of LCD. Positivity was different for cirrhosis with or without associated HCC, amounting to 18.9% in the former and 39.4% in the latter. Interestingly, p53 protein immunoreactivity also occurred in a set of small hepatocytes not showing the typical features of LCD and therefore classified as simple regenerating liver cells.

Key words p53 expression · Hepatocellular carcinoma
Liver cell dysplasia

Introduction

There is now convincing evidence that wild-type (WT) p53 has tumour suppressor activity [45]. Even though not all human tumours have *p53* mutations, this gene is by far the most frequently mutated of all the known suppressor genes and oncogenes [12]. Mutations of the *p53* gene have been observed in human hepatocellular carcinomas (HCCs) [5, 6, 7, 22, 23, 24, 25, 32, 37, 40, 42, 44, 46, 49, 50, 59] and *p53* mutation in HCC appears to occur frequently at the third position of codon 249 (a mutational hotspot with glycine to tyrosine transversion). However, a codon 249 mutation may not represent a general phenomenon because no such mutations were found in 47 North American HCCs [29], in other patients of predominantly Caucasian origin [11, 20, 53] or in 60 HCC patients from Japan [19]. The prevalence of *p53* gene mutations in HCCs varies considerably from one region to another, reported frequencies ranging from 10–15% in Europe [32] to 50–60% in sub-Saharan Africa and the Far East [25] and codons other than 249 can be affected by mutation [40, 59].

In contrast to fully established HCCs, not much is known with respect to p53 expression in HCC precursor lesions. Aflatoxin B1-induced rat hepatic hyperplastic nodules did not exhibit a site-specific mutation within the *p53* gene [26], and it has been surmised that mutation of one *p53* gene allele may be a late event in hepatocarcinogenesis, being preceded by loss of one allele [44].

The aim of the present study was threefold. Firstly, we were interested to test whether heterogeneity of immunohistochemically detectable p53 protein expression would show up in a series of HCCs collected in an area presumed to have a high prevalence of *p53* gene mutations (Northern China). For this purpose, five anti-p53 protein antibodies recognizing different epitopes were used. Secondly, we investigated the relationship between types and grades of HCCs and p53 protein expression patterns. Thirdly, we analysed three types of liver cell dysplasia in cirrhotic livers associated with or without HCC for the presence of immunoreactive p53 protein.

Materials and methods

Biopsies and operation specimens from 37 Chinese patients with liver cirrhosis and HCC, and 66 Chinese patients with cirrhosis but

M. Zhao · J. A. Laissue · A. Zimmermann (✉)
Institute of Pathology, University of Bern, Murtenstrasse 31, CH-3010 Bern, Switzerland

N-X. Zhang
Department of Pathology, Tianjin Medical College, Tianjin, China

without HCC were analysed retrospectively. The selection criterion was the presence of liver cell dysplasia (LCD), identified in a set of 1748 liver tissue samples assessed between 1957 and 1988 in a Northern Chinese centre. The hepatitis B virus (HBV) status of the patients was not known. The material analysed did not contain foci of atypical adenomatous hyperplasia, or borderline lesions defined according to a recent paper [14]. For conventional light microscopy, tissues were fixed in 10% buffered formaldehyde solution, dehydrated and embedded in paraffin. Fixation before embedding was less than 30 h throughout. Sections were stained with haematoxylin and eosin.

Antibodies used in the present study and working dilutions employed are listed in Table 1. For PAb-1801, PAb-240, PAb-421, DO-7 and proliferating cell nuclear antigen (PCNA) antibodies, immunostaining was performed using a modified alkaline phosphatase-anti alkaline phosphatase (APAAP) procedure, whereas the p53 CM-1 antibody was assessed under a modified avidin-biotin peroxidase complex (ABC) technique. In brief, the procedures were as follows: in the modified APAAP method tissue sections were deparaffinized and rehydrated in TRIS-sodium chloride (NaCl) buffer (0.1% TRIS and 1% NaCl, Merck, pH 7.4), followed by exposure of sections to 3% bovine serum albumin (BSA, Sigma), in TRIS-NaCl buffer with 1% normal rabbit serum (Dako) for 1 h at room temperature and incubation with primary antisera for 3 h at the working dilutions indicated. Rabbit anti-mouse immunoglobulin (Dako; 1:30) and APAAP mouse monoclonal antibody (Dako; 1:50) were applied for 45 min each. The AP reaction was run for 20 min in new fuchsin substrate solution, and the reaction was stopped by rinsing sections in cold tap water.

In the modified ABC method preincubation in 3% BSA in Tris-buffered saline (TBS) buffer (0.13 mM NaCl, 7 mM disodium hydrogen phosphate and 3 mM sodium dihydrogen phosphate with 1% normal swine serum (Dako) for 1 h was followed by incubation with CM-1 in TBS-physiogel-NaN_3 (TPA) buffer (10% physiogel and 0.1% sodium in TBS, pH 7.4) for 1 h followed by exposure to biotinylated swine anti-rabbit immunoglobulin (Dako; 1:200) in TPA with 25% normal human serum and AB horseradish peroxidase complex (Dako; 1:20) for 30 min. Phosphate buffered saline buffer was used for triple washing after each incubation step. Diaminobenzidine substrate reaction was developed for 8 min and stopped by rinsing sections in cold tap water.

All incubations were performed in a humidified environment at room temperature. Finally, sections were counter-stained with haematoxylin (Merck) and mounted with Aquadex (Merck). For negative control sections, primary antisera were substituted with normal mouse or rabbit serum (Dako) at a protein concentration of 0.06 mg/ml, and all other steps were carried out with these preparations. As a positive control, three cases of HCC strongly expressing oncoprotein and PCNA were processed in parallel to each working step.

Regenerating hepatocytes in cirrhotic nodules and dysplastic hepatocytes were first classified into four groups on the basis of morphologic features in comparison with normal hepatocytes [64]. Based on haematoxylin as eosin-stained sections, the following working formulation was used: Simple regenerating liver cells/hepatocytes (SRLC) are hepatocytes which are smaller than normal hepatocytes (NLC), however with apparently normal nuclear size. The main difference between SRLC and NLC is the tendency of the former to form small foci or clusters with nuclear crowding within cirrhotic nodules, where they usually occur in peripheral parts.

Large liver cell dysplasia with nuclear hypochromasia (LLCDo) are large hepatocytes with a large nucleus and one or several prominent nucleoli [1], frequently showing an abundant, eosinophilic or clear cytoplasm. LLCDo usually occur in clusters and occupy a part of a cirrhotic nodule, but may form entire nodules as well.

Large liver cell dysplasia with nuclear hyperchromasia (LLCDe) describes cells which have some features in common with LLCDo, but they usually exhibit markedly hyperchromatic or even polymorphous nuclei. Mean nuclear diameter as determined by karyometry (see below) is 13.5 μm in comparison with 14.5 μm for LLCDo. Similar to LLCDo, LLCDe may form clusters of cells of entire nodules.

Finally, small liver cell dysplasia (SLCD; [62]) defines cells which when compared with LLCD cells, have a cell and nuclear size distinctly smaller (mean nuclear diameter: 10.1 μm). In contrast to SRLC, the cytoplasm is basophilic and the nuclei are hyperchromatic and may show atypia. Cells of SLCD in most instances locate in peripheral parts of cirrhotic nodules, where they form clusters or small foci, as previously reported [62], but they may also form entire nodules.

In order to better define these types of LCD, karyometry was performed. Our method is described in detail in a previous report [64]. Three parameters, nuclear area (NA), nucleic acid content (NAC), and nucleic acid density (NAD) were estimated. NA as defined here is the estimated projected area of a nuclear slice represented in a section of 5 μm thickness. NAC is the microphotometrically determined amount of Feulgen-positive material within such a nuclear slice. NAD represents the calculated ratio NAC/NA. A total of 2762 cells (2417 hepatocytes and 345 lymphocytes used as controls) were analysed for the 130 cases using a scanning microspectrophotometer [64].

HCCs were classified according to published criteria [18, 39]. Grading was performed following guidelines previously reported [13].

The PC 10 monoclonal mouse anti-PCNA antibody (Dako; Table 1) was used and has been shown to stain biopsies more reliably than 19A2 [19]. The tissue samples had been fixed in formalin for less than 30 h which is of importance because, besides other factors [10], the duration of fixation may affect the accessibility of PCNA/cyclin [19]. With the APAAP method used, nuclei that had reacted were stained either pink or brightly red. Thus, even though immunoreactivity was sharply defined (nuclear staining only), its intensity was not uniform, as previously reported [31]. Therefore, nucleated hepatocytes and HCC cells were considered positive for PCNA only if a distinctly red staining of the nucleus was identified. PCNA labelling was scored using a × 40 objective and a × 10 eyepiece, and sections were scanned to identify areas that were most evenly labelled, but within these zones areas to be analysed were randomly chosen. The extent of PCNA positivity was evaluated by determining N-pos/1000 nuclei (permille), and this value was used as a PCNA labelling index. (SRLC and LCD only) [30].

For each of the five anti-p53 antibodies used, immunoreactivity was registered as either positive or negative, based on the presence or absence of clearly red or brown reaction product for the APAAP and ABC assays, respectively. In addition to nuclear staining, reaction product was assessed in cytoplasm and at the cell surface.

Values are indicated as mean± SD. For group comparisons, Pearson's Chi-square test (McNemar symmetry and Yates corrected) and Fisher's two-tailed test were employed.

Table 1 Antisera used in this study (*WT* wildtype, *PCNA* proliferating cell nuclear antigen)

Antibody	Dilution	Source	Specificity
CM-1	1:200	Medac Diagnostika	Recombinant WT p53
PAb-1801	1:20	Oncogene Sciences	Amino acids 32–79
PAb-240	1:10	Oncogene Sciences	Amino acids 156–335
PAb-421	1:5	Oncogene Sciences	Amino acids 370–378
DO-7	1:100	Dako	Amino acids 35–45
PCNA (PC 10)	1:50	Dako	36 kDa cyclin

Results

As seen in Table 2, 47.6% of HCCs examined showed nuclear immunoreactivity for PCNA. Nuclear PCNA staining mostly involved numerous tumour cells, but with considerable regional differences, as previously demonstrated [41]. Due to this phenomenon, we did not attempt to determine PCNA labelling indices in HCCs.

Immunoreactivity for p53 protein in HCCs was predominantly nuclear (Fig. 1A, B) but, depending on the antibody used, cytoplasmic or peripheral cell membrane

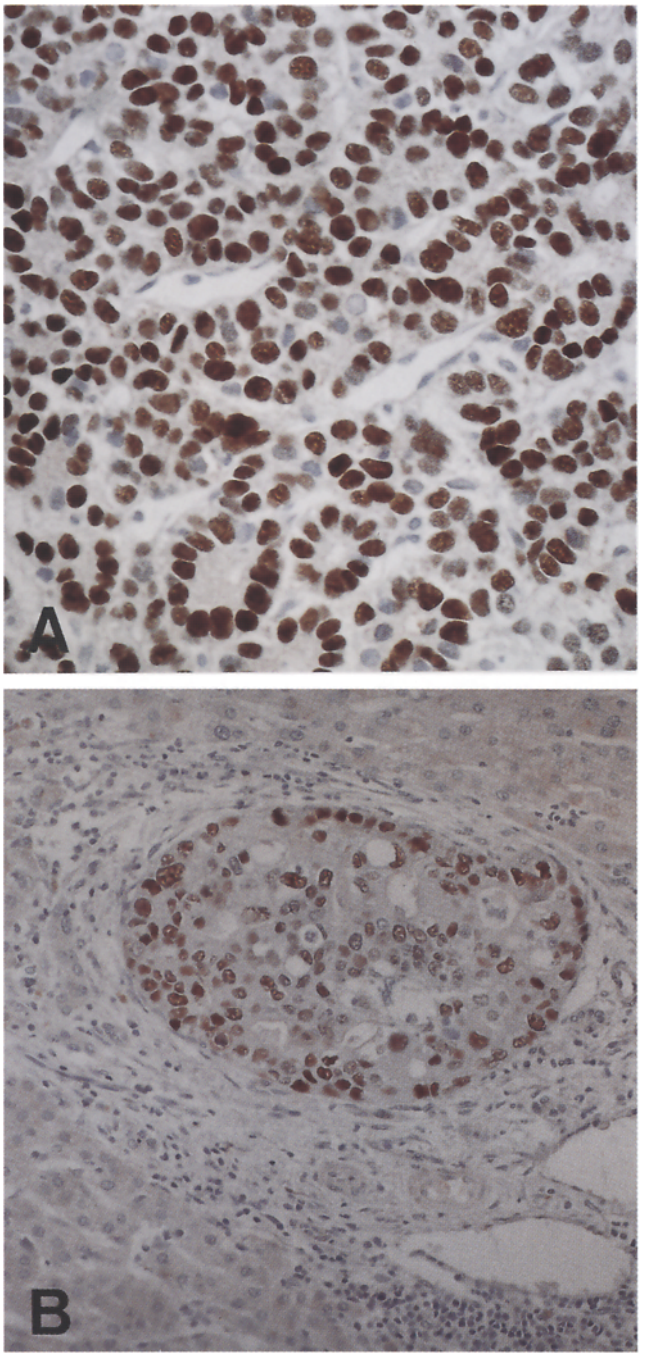


Fig. 1 **A** Hepatocellular carcinoma, mixed trabecular and acinar type, grade 2. p53 protein immunoreactivity is seen as a strong staining of nuclei in most neoplastic cells [CM-1 antibody; avidin-biotin peroxidase complex (ABC) method, × 250]. **B** Hepatocellular carcinoma tissue is localized within a portal vein branch. Whereas most of tumour cell nuclei are p53 protein positive, normal hepatocytes are negative (CM-1 antibody; ABC method, × 150)

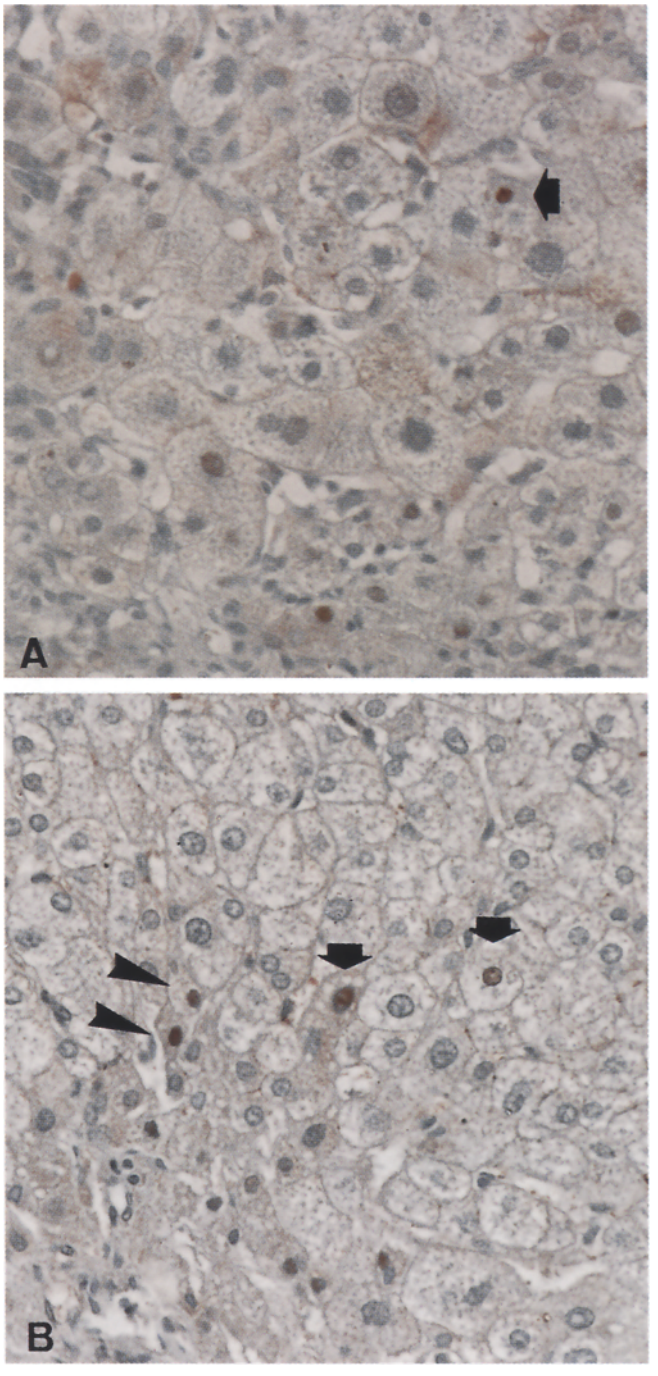


Fig. 2 **A** Part of a cirrhotic nodule showing one small liver cell dysplasia (SLCD cell; *arrow*) exhibiting nuclear p53 protein staining. No nuclear immunostaining is observed in large liver cell dysplasia (LLCD) cells (CM-1 antibody; ABC method, × 250). **B** Cells containing p53 protein-positive nuclei in this area are classified as simple regenerating liver cells (SRLC; *arrows*) or SLCD (*arrowheads*; CM-1 antibody; ABC method, × 250)

Table 2 Histopathology and immunohistochemistry of hepatocellular carcinomas (HCCs; HCC type: *T*, trabecular (sinusoidal) type; *P*, pseudoglandular (acinar) type; *C*, compact (solid) type; *Sc*, scirrhous (sclerosing) type; *F*, fatty HCC. Staining pattern: *N*, nuclear staining; *C*, cytoplasmic staining; *M*, membrane staining)

Case	Histopathology										Immunohistochemistry (+ or –/staining pattern)					
	Type						Grade[a]				PCNA	CM-1	PAb-1801	PAb-240	PAb-421	DO-7
1	T			Sc			1	2			–	–	–	–	–	–
2	P	P						2			+/N	–	–	–	–	–
3					F					4	–	–	–	–	–	–
4	T	P						2	3		+/N	+/N	+/N	–	–	+/N
5	T	P		Sc			1	2			+/N	+/N	+/M	+/CM	+/CM	+/CM
6	T								3	4	+/N	+/N	+/C	–	–	–
7		P	C			Sa			3	4	+/N	+/N	–	–	–	–
8			C					2			+/N	+/N	+/C	–	–	–
9						Sa				4	+/N	+/N	+/C	–	–	–
10	T							2			+/N	+/N	+/C	–	–	–
11	T			Sc			1	2			+/N	+/N	+/C	–	–	–
12		P						2	3		+/N	+/N	+/NC	–	–	–
13		P							3		+/N	–	+/C	–	–	–
14			C					2	3		–	–	–	–	–	–
15	T								3	4	+/N	–	+/C	+/C	–	–
16	T								3		+/N	–	+/C	–	–	–
17	T							2			+/N	+/N	+/N	–	–	–
18	T			Sc					3	4	+/N	+/N	–	–	–	–
19	T									4	+/N	+/N	+/C	–	–	–
20			C					2	3		+/N	+/N	+/N	+/N	–	–
21				Sc	F					4	+/N	–	+/C	–	–	–
22	T								3		+/N	+/N	+/C	–	–	–
23	T		C					2	3		+/N	–	–	–	–	–
24	T							2			+/N	+/N	+/C	–	–	–
25	T							2			+/N	+/N	+/N	–	–	–
26	T							2			+/N	–	–	–	–	–
27	T							2			+/N	+/N	+/C	–	–	–
28	T							2	3		+/N	+/N	–	–	–	–
29		P						2	3		+/N	+/N	+/NC	+/CM	+/C	+/C
30			C			Sa				4	+/N	+/N	+/NC	–	–	–
31	T									4	–	–	–	–	–	–
32		P				Sa				4	+/N	+/N	+/N	–	–	–
33	T								3	4	+/N	–	–	+/M	+/M	–
34	T							2	3		+/N	+/N	+/N	–	–	–
35	T		C					2	3		–	–	–	–	–	–
36	T								3		+/N	–	+/C	–	–	–
37	T							2			+/N	+/N	+/NC	+/C	+/CM	+/C
Total	24	8	7	5	2	4	3	21	18	12	32/37	23	25	6	4	4
%	64.86	21.62	18.92	13.51	5.41	10.81	8.11	56.76	48.65	32.43	86.49[b]	62.16	67.57	16.22	10.81	10.81

[a] Grades 1–4 according to Edmondson and Steiner

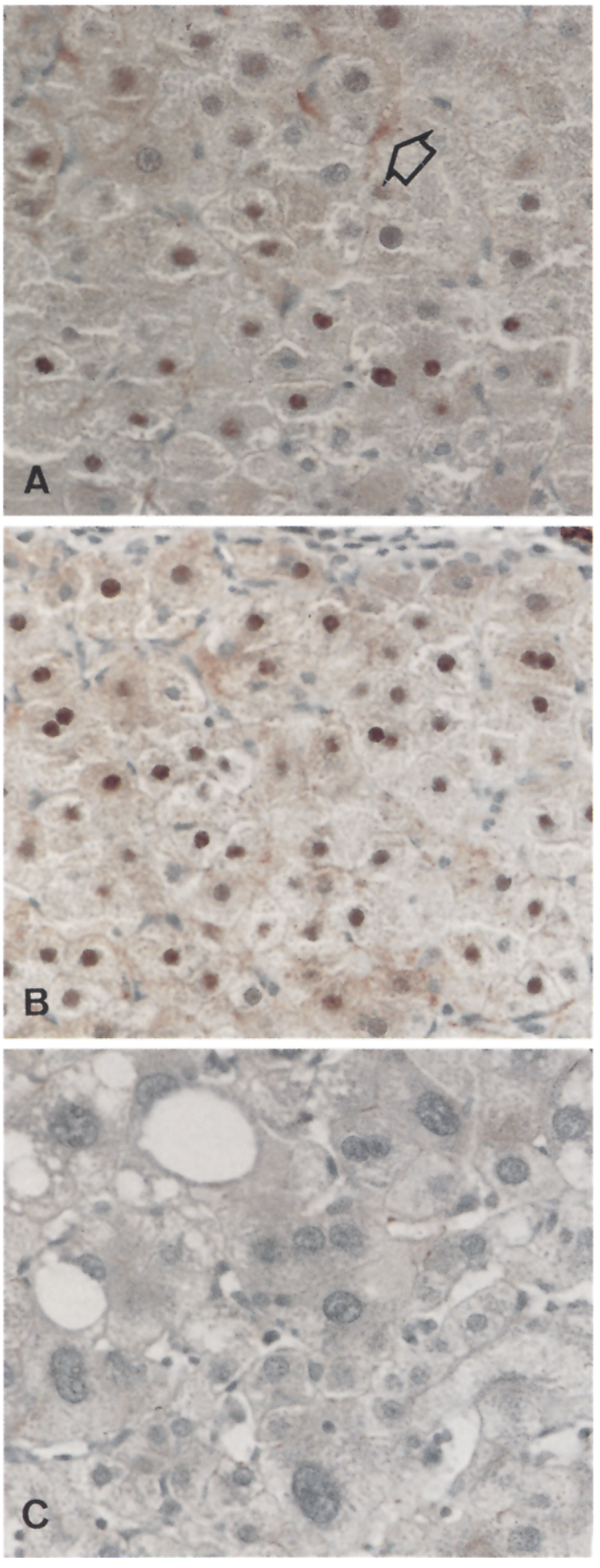
[b] The total PCNA-positivity of tumour cells is 47.6%, and the PCNA-positivity of cases is 86.49

Table 3 Karyometric features of dysplastic vs hyperplastic hepatocytes [*LCD* liver cell dysplasia; *SRLC* simple regenerating liver cells/hepatocytes; *LLCDo* large liver cell dysplasia with nuclear hypochromasia; *LLCDe* large liver cell dysplasia with nuclear hyperchromasia; *SLCD* small liver cell dysplasia; *NA* nuclear area (μm^2); *NAC* nucleic acid content (arbitrary units); NAD nucleic acid density (ratio NAC/NA)]

Cell type (*n*)	NA[a]	NAC[a]	NAD[a]
SRLC (492)	46.79± 10.21	25.49± 6.81	0.54± 0.07
LLCDo (477)	164.73± 58.62	88.48± 34.61	0.53± 0.07
LLCDe (438)	142.07± 45.51	86.88± 27.94	0.61± 0.11
SLCD (1010)	79.25± 36.39	85.18± 39.76	1.07± 0.12
Lymphocytes (345) (control)	20.15± 4.01	15.17± 03.09	0.76± 0.12

[a] Mean± SDM

staining was also observed. Overall positivity for p53 (all five antibodies included) was 78.3%, but there were considerable differences from one antibody to the other (Table 2). The highest yield was obtained with antibody PAb-1801 (67.5%), followed by CM-1 (62.1%), PAb-240 (16.2%), PAb-421 (10.8%), and DO-7 (10.8%). The main localization of reaction product varied among the five antibodies; assays with CM-1 resulted in an exclusively nuclear staining, whereas testing with the other four antibodies disclosed considerable heterogeneity (Table 2). With PAb-1801, 18/25 cases had cytoplasmic, 10/25 both nuclear and cytoplasmic, and 1/25 membrane staining. With PAb-240, only one of six cases showed nuclear staining, and no nuclear staining was noted with both PAb-421 and DO-7, the latter two, however, demonstrating cytoplasmic and membrane staining.



There was no relationship between positive immunoreactivity for p53 protein and WHO types of HCC, and reactivity with one or more antibodies was also detected with HCCs showing a strong development of stroma or exhibiting sarcomatoid features (Table 2). In contrast, one out the two HCCs with important macrovesicular fatty change (fatty HCCs) was p53-negative, the other case reacting with one antibody only (PAb 1801; cytoplasmic staining).

The majority of HCCs contained components corresponding to grades 2 and 3 according to the Edmondson-Steiner grading system (56.7% and 48.6%, respectively), whereas components with grade 4 or grade 1 amounted to 32.4% and 8.1%. No relationship between tumour grade and p53 protein immunoreactivity was observed.

Immunoreactivity for PCNA and p53 protein in NLC, SRLC, and LCD was analysed separately for the two groups with or without associated HCC. Karyometric data for SRLC and LCD are compiled in Table 3.

In the group with HCC, morphologically "normal" NLC had an overall PCNA labelling of 3.0%. As shown in a previous study [64], SRLC exhibited a labelling which was higher than that for NLC and LCD (6.4%, Table 4). For the three types of LCD (Table 3), PCNA varied considerably, the small cells of SLCD showing the highest labelling (4.5%), whereas both types of large cell dysplasia (LLCDo and LLCDe) had definitely lower labelling, that of LLCDo being about eight times lower than that of NLC (Table 4).

For SRLC, 8/37 cases showed p53 protein staining with the CM-1 antibody, and 2/37 with the PAb 1801 antibody, mostly in a nuclear pattern (overall positivity: 27%, Fig. 2). For the group of LCDs, only SLCD disclosed immunoreactivity (5/37 with the CM-1 antibody, 2/37 with the PAb 1801 antibody; overall positivity: 18.9%; Table 4; Fig. 2). No p53 protein immunoreactivity was observed with NLC and LLCD (Fig. 3), and no reactivity was noted when using the PAb 240, PAb 421, and DO-7 antibodies.

The corresponding data for the group of cases not associated with HCC are shown in Table 4. With respect to PCNA immunostaining, a similar overall pattern ensued, however with quantitative differences. Whereas PCNA labelling for NLC was similar to the group with HCC (3.1%), values for SRLC and LCD were somewhat higher, but again LLCD exhibiting the lowest values. In contrast with the group with HCCs, p53 protein staining in the group without HCC yielded higher values for both, SRLC and SLCD. By use of CM-1, 43.9% of SRLC and 33.3% of SLCD nuclei were positive, respectively, and

Fig. 3 **A** Cirrhotic nodule with a mixture of normal liver cells and SRLC. Some nuclei are p53 protein-positive. Note that reaction product is also visualized at the canalicular surface of some cells (*arrow*; CM-1 antibody; ABC method, × 250). **B** Area showing SLCD and SRLC cells. In a few binuclear hepatocytes, both nuclei are p53 protein-positive. (CM-1 antibody; ABC method, × 250). **C** Area with several dysplastic hepatocytes of the LLCD type, showing no p53 protein immunostaining (CM-1 antibody; ABC method, × 250)

Table 4 PCNA and p53 protein staining of normal liver cell (*NLC*), SRLC and LCD in cirrhosis with or without HCC

Cell type	PCNA	CM-1	PAb-1801	PAb-240	PAb-421	DO-7	Total positivity
A) Cirrhosis associated with HCC:							14/37 (37.8%)
NLC	3.00‰	0	0	0	0	0	
SRLC	6.4‰	8/37*	2/37***	0	0	0	
SLCD	4.5‰	5/37**	2/37***	0	0	0	
LLCDo	0.4‰	0	0	0	0	0	
LLCDe	1.6‰	0	0	0	0	0	
B) Cirrhosis not associated with HCC:							42/66 (64.6%)
NLC	3.1‰	0	0	0	0	0	
SRLC	8.3‰	29/66*	3/66***	0	2/66	0	
SLCD	5.5‰	22/66**	4/66***	0	0	0	
LLCDo	1.7‰	0	0	0	0	0	
LLCDe	0.7‰	0	0	0	0	0	

* $P<0.05$ (A vs B) ** $P<0.05$ (A vs B) *** Not significant (A vs B)

for all antibodies used, overall positivities for SRLC and SLCD were 51.5% and 39.4%, respectively. For CM-1, but not for PAb-1801, staining yields in the groups with or without HCC were significantly different for both, SRLC and SLCD (Table 4). In some hepatocytes, p53 protein immunoreactivity was observed at the canalicular surface, but only when using the CM-1 antibody (Fig. 3A).

Discussion

In HCCs collected in a distinct area of Northern China, overall p53 protein staining amounted to 78.4%. When positive cases were, however, broken down with respect to antibodies used, it turned out that the prevalence of immunoreactivity differed significantly among the five antibodies. The highest positive yield was obtained with antibody PAb-1801 (67.5%), which recognizes a denaturation-resistant epitope in human p53 protein located between amino acids 32 and 79 [2]. This finding is in contrast with a previous study where only 15% of HCCs reacted with this antibody tested in a group of white patients of predominantly French origin [39]. A similarly high prevalence (62.1%) was noted when using antibody CM-1 raised against recombinant human WT p53 protein [3, 35]. This value found in Northern Chinese patients is almost the same as that observed in a previously published series of patients from China (61%) [24], but higher than that obtained with HCCs from Britain (1/19 cases) [7] and from North America (20/47 cases) [29]. A higher detection rate of p53 protein in HCCs of Orientals has also been demonstrated by use of another antibody (BP53-12) [8]. These findings illustrate the significance of regional differences in the prevalence of positive immunostaining, and are further underlined by the observation that the yield of positive results by use of CM-1 may even differ for two regions within the United states, being 30% for National Institutes of Health (NIH) patients and 60% for patients from Hawaii [24].

In contrast to both PAb-1801 and CM-1 antibodies, a significantly smaller prevalence of immunoreactivity was noted with the remaining three antibodies used in this study. With PAb-240, which recognizes an epitope located between amino acids 156 and 335 of p53 protein [16], PAb-421 (residues 370 to 378) [2, 60] and DO-7 (residues 35 to 45) [58], positive immunoreactivity amounted to 16.2%, 10.8%, and 10.8%, respectively. Taken together the results suggest that, even for a region with HBV and/or aflatoxin B1 as risk factors and a presumably high mutation rate of the *p53* gene in HCCs [5, 20, 25, 46], positive immunostaining for a mutated gene product may strongly depend on the type of antibody used.

The staining results obtained with the five antibodies did, however, not only differ with respect to the overall yield of immunoreactivity, but also to the localization of reaction product in the neoplastic cells. Even though the *p53* gene product is a nuclear protein [12, 45], in HCCs only assays using CM-1 antibody resulted in an exclusively nuclear localization of reaction product, whereas PAb-1801 and PAb-240 exhibited nuclear and extranuclear staining, and no nuclear staining was obtained with PAb-421 and DO-7. In contrast, testing of CM-1 in non-neoplastic hepatocytes resulted, in some cells, in staining of the canalicular surface. Differential expression of p53 protein in either the nuclei, the cytoplasm, or the cell membrane may well reflect a relevant phenomenon, because the regulation of p53 tumour suppressor activity may be influenced by altering its subcellular location [63]. Previously, elevated levels of p53 were detected in the cytoplasm of inflammatory breast carcinoma cells and of normal lactating breast cells [36]. The mechanism for the exclusion of p53 protein from the nucleus, or alternatively, its retention in cytoplasm or at the cell membrane level, has not yet been determined [63].

In the present series of HCCs, prevalence and cellular localization of p53 protein immunoreactivity were not related to tumour type or grade. It appears that a broad array of morphological patterns occurring in HCCs may be associated with p53 protein immunoreactivity, with the notable exception that the two cases of fatty HCC exhibited the least staining.

LCD was originally detected in the liver of HBsAg-positive Ugandan patients and emphasized as a preneo-

plastic change [1], but several other studies arrived at controversial interpretations with respect to its biological significance [64]. More recent immunohistochemical and morphometrical findings suggest, however, that LCD similar to atypical adenomatous hyperplasia or borderline lesions of the liver [14] may represent a cell population prone to neoplastic evolution [for review, see [64]). Foci of LCD may coexist with HCC in other parts of the liver [15, 38], but its prevalence may differ as a function of the subtype of LCD involved, only SLCD and LLCDo being more frequently observed in cirrhosis associated with HCC [47, 64]. Subtypes of LCD can be distinguished objectively by the use of karyometry, as shown in the present and previous studies [64], and by microspectrophotometry [34, 64]. Using a correlative karyometric analysis we have shown that the highest degree of similarity occurred between LLCDe and HCC of high differentiation followed by the pair, SLCD and HCC of low differentiation, suggesting that poorly differentiated HCCs reflect a karyometric pattern very similar to that of SLCD [64]. We now show that LCD, but only SLCD can express p53 protein. This finding has not been previously reported. Immunoreactivity was only found when using the antibodies, CM-1 and PAb-1801. Positivity yields differed significantly between the cirrhosis group with or without HCC, amounting to 18.9% in the former and to 39.4% in the latter. In both groups, SLCD cells also exhibited the highest PCNA labelling among the three subtypes of LCD, but being lower than that of SRLC. Immunoreactivity for p53 protein has been observed in situations where epithelial dysplasia is still better defined; in bronchial dysplasia [43], in dysplasia of the upper aerodigestive tract [9], in gastric epithelial dysplasia [48], in human oesophageal precancerous lesions [61], in colorectal adenomas [27], and in epithelial dysplasias of the gallbladder [28]. It thus appears that overexpression of p53 occurs in several dyplastic cell populations including SLCD cells of the liver. However, in experimental rat hepatocarcinogenesis, p53 increases predominantly in oval cells, and not in dysplastic hepatocytes [4] but in chimpanzees harboring HCCs, microscopic foci of hepatocytes in the nontumorous part of the liver were found to stain for the p53 protein [51].

The higher PCNA labelling of SLCD cells is of interest in the light of the finding that DNA synthetic potency in noncancerous cirrhotic tissue from patients with liver cirrhosis complicated by HCC was significantly higher than that in cirrhotic livers not associated with HCC, suggesting that HCC may develop in cirrhotic patients with high DNA synthesis rate [52]. Moreover, flow cytometric analyses have shown that DNA anomalies are more frequent in LCD of high grade than in low grade [55]. p53 overexpression in HCC appears to be correlated with proliferative activity [21], and higher PCNA labelling may be linked to expression of mutant p53 protein.

The finding that cells not fulfilling the morphologic and karyometric criteria for LCD (SRLC cells) are immunoreactive for p53 protein is intriguing. Hyperplasia of karyometrically identified small hepatocytes without nuclear atypia in cirrhotic livers with HCC has been demonstrated previously [54]. SRLC cells exhibited the highest PCNA labelling, but still clearly below 5%, and much lower than in HCCs [30]. The yield of p53 immunostaining of SRLC cells was, like that of SLCD, higher in the non-HCC group. Even though we cannot exclude some overlap in the morphological and karyometric assessment of SRLC vs SLCD, we believe that the majority of SRLC cells represents a distinct cell population. The finding of p53 immunoreactivity in regenerating liver cells has also been obtained in experimental liver regeneration in the rat. When growth is stimulated in the normally quiescent rat liver by partial hepatectomy, steady state levels of mRNAs for c-*fos*, c-*myc* and *p53* increase sequentially during the prereplicative phase, suggesting that a transient expression of protooncogenes occurs in liver regeneration [56]. A similar phenomenon may ensue in replicating cells forming cirrhotic nodules in the human liver. The reason why p53 is detectable by use of immunohistochemistry is not clear, because WTp53 protein should not be visualized due to its short life-time. However, even in tumours where the gene is not mutated, altered levels of p53 protein can be detected [59]. One might, therefore, theorize that the population of cells defined as SRLC may in some way be related to dysplastic hepatocytes.

Acknowledgements We thank Erna Müller for her expertise in writing the manuscript. The technical help of Mary Economou and Iris Blaha is gratefully acknowledged.

References

1. Anthony PP (1976) Precursor lesions for liver cancer in humans. Cancer Res 46:2579–2583
2. Banks L, Matlashewski G, Crawford L (1986) Isolation of human-p53-specific monoclonal antibodies and their use in the studies of human p53 expression. Eur J Biochem 159:529–534
3. Bartek J, Bartkova J, Vojtesek B, Staskova Z, Lukas J, Rejthar A, Kovarik J et al. (1991) Aberrant expression of the p53 oncoprotein is a common feature of a wide spectrum of human malignancies. Oncogene 6:1699–1703
4. Braun L, Mikumo R, Fausto N (1986) Production of hepatocellular carcinoma by oval cells: cell cycle expression of c-*myc* and *p53* at different stages of oval cell transformation. Cancer Res 49:1554–1561
5. Bressac B, Kew M, Wands J, Ozturk M (1991) Selective G to T mutations of *p53* gene in hepatocellular carcinoma from Southern Africa. Nature 350:429–431
6. Buetow KH, Sheffield VC, Zhu M, Zhou T, Shen FM, Hino O, Smith M, McMahon BJ, Lanier AP, London WT, Redeker AG, Govindarajan S (1992) Low frequency of *p53* mutations observed in a diverse collection of primary hepatocellular carcinomas. Proc Natl Acad Sci USA 89:9622–9626
7. Challen C, Lunec J, Warren W, Collier J, Bassendine MF (1992) Analysis of the *p53* tumor-suppressor gene in hepatocellular carcinomas from Britain. Hepatology 16:1362–1366
8. Choi SW, Hytiroglou P, Geller SA, Kim SM, Chung KW, Park DH, Theise ND, Thung SN (1993) The expression of p53 antigen in primary malignant epithelial tumors of the liver: an immunohistochemical study. Liver 13:172–176
9. Coltrera MD, Zarbo RJ, Sakr WA, Grown AM (1992) Markers for dysplasia of the upper aerodigestive tract. Suprabasal ex-

pression of PCNA, p53, and CK19 in alcohol-fixed, embedded tissue. Am J Pathol 141:817–825
10. Coltrera MD, Skelly M, Gown AM (1993) Anti-PCNA antibody PC10 yields unreliable proliferation indexes in routinely processed, deparaffinized, formalin-fixed tissue. Appl Immunohistochem 1:193–200
11. Conte D, Neri A, Lodi L, Croce LS, Fraquelli M, Masutti F, Tiribelli C (1993) *p53* and H- and K-*ras* in hepatocellular carcinoma (HCC). Data from an area at intermediate HCC prevalence (abstract). Hepatology 18:182
12. Donehower LA, Bradley A (1993) The tumour suppressor p53. Biochim Biophys Acta 1155:181–205
13. Edmondson HA, Steiner PE (1954) Primary carcinoma of the liver. A study of 100 cases among 48900 necropsies. Cancer 7:462–503
14. Ferrell L, Crawford JM, Dhillon AP, Scheuer PJ, Nakanuma Y (1993) Proposal for standardized criteria for the diagnosis of benign, borderline, and malignant hepatocellular lesions arising in chronic advanced liver disease. Am J Surg Pathol 17:1113–1123
15. Furuya K, Nakamura M, Yamamoto Y, Togei K, Otsuka H (1988) Macroregenerative nodules of the liver: a clinicopathologic study of 345 autopsy cases of chronic liver disease. Cancer 61:99–105
16. Gannon JV, Greaves R, Iggo R, Lane DP (1990) Activating mutations in *p53* produce a common conformational effect. A monoclonal antibody specific for the mutant form. EMBO J 9:1595–1602
17. Gelb AB, Kamel OW, LeBrun DP, Warnke RA (1992) Estimation of tumor growth fractions in archival formalin-fixed, paraffin-embedded tissues using two anti-PCNA/cyclin monoclonal antibodies. Factors affecting reactivity. Am J Pathol 141:1453–1458
18. Gibson JB, Sobin LH (1978) Histological typing of tumours of the liver, biliary tract and pancreas. International histological classification of tumours 20. World Health Organization, Geneva
19. Hayashi H, Sugio K, Matsumata T, Adachi E, Urata K, Tanaka S, Sugimachi K (1993) The mutation of codon 249 in the *p53* gene is not specific in Japanese hepatocellular carcinoma. Liver 13:279–281
20. Hayward NK, Walker GJ, Graham W, Cooksley E (1991) Hepatocellular carcinoma mutation. Nature 352:764
21. Hofmann WJ, Volkmann M, Haag D, Otto G, Galle PR (1993) p53 overexpression in HCC is correlated with the proliferative activity but not with EGF-R expression of the tumors (abstract). Hepatology 18:185
22. Hollstein MC, Wild CP, Bleicher F, Chutimataewin S, Harris CC, Srivatanakul P, Montesano R (1993) *p53* mutations and aflatoxin B1 exposure in hepatocellular carcinoma patients from Thailand. Int J Cancer 53:51–55
23. Hosono S, Lee CS, Chou MJ, Yang CS, Shih CH (1991) Molecular analysis of the *p53* alleles in primary hepatocellular carcinomas and cell lines. Oncogene 6:237–243
24. Hsia CC, Kleiner DE, Axiotis CA, DiBisceglie A, Nomura AMY, Stemmermann GN, Tabor E (1992) Mutations of *p53* gene in hepatocellular carcinoma: roles of hepatitis B virus and aflatoxin contamination in the diet. J Natl Cancer Inst 84:1638–1641
25. Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC (1991) Mutational hotspot in the *p53* gene in human hepatocellular carcinomas. Nature 350:427–428
26. Hulla JE, Chen ZY, Eaton DL (1993) Aflatoxin B1-induced rat hepatic hyperplastic nodules do not exhibit a site-specific mutation within the *p53* gene. Cancer Res 53:9–11
27. Kaklamanis L, Gatter KC, Mortensen N, Baigrie RJ, Heryet A, Lane DP, Harris AL (1993) p53 expression in colorectal adenomas. Am J Pathol 142:87–93
28. Kamel D, Pääkö P, Nuorva K, Vähäkangas K, Soini Y (1993) p53 and c-erbB-2 protein expression in adenocarcinomas and epithelial dysplasias of the gallbladder. J Pathol 170:67–72
29. Kar S, Jaffe R, Carr BI (1993) Mutation at codon 249 of *p53* gene in a human hepatoblastoma. Hepatology 18:566–569
30. Kitamoto M, Nakanishi T, Kira S, Kawaguchi M, Nakashio R, Suemori S, Kajiyama G, Asahara T, Dohi K (1993) The assessment of proliferating cell nuclear antigen immunohistochemical staining in small hepatocellular carcinoma and its relationship to histologic characteristics and prognosis. Cancer 72:1859–1865
31. Koukoulis G, Rayner A, Tan KC, Williams R, Portmann B (1992) Immunolocalization of regenerating cells after submassive liver necrosis using PCNA staining. J Pathol 166:359–368
32. Kress S, Jahn UR, Buchmann A, Bannasch P, Schwarz M (1992) *p53* mutations in human hepatocellular carcinomas from Germany. Cancer Res 52:3220–3223
33. Laurent-Puig P, Flejou JF, Fabre M, Bedossa P, Belghiti J, Gayral F, Franco D (1992) Overexpression of p53: a rare event in a large series of white patients with hepatocellular carcinoma. Hepatology 16:1171–1175
34. Li ZS (1990) Analysis of DNA content of cell from liver cells dysplasia and hepatocellular carcinoma. Chung-hua Ping Li Hsueh Tsa Chih 19:215–217
35. Midgley CA, Fisher CJ, Bartek J, Vojtesek B, Lane D, Barnes DM (1992) Analysis of p53 expression in human tumours: an antibody raised against human p53 expressed in *Escherichia coli*. J Cell Sci 101:67–74
36. Moll UM, Riou G, Levine AJ (1992) Two distinct mechanisms alter *p53* in breast cancer: mutation and nuclear exclusion. Proc Natl Acad Sci USA 89:7262–7266
37. Murakami Y, Hayashi K, Hirohashi S et al. (1991) Aberrations of the tumor suppressor *p53* and retinoblastoma genes in human hepatocellular carcinomas. Cancer Res 51:5520–5525
38. Nakanuma Y, Terada T, Terasaki S, Ueda K, Nonomura A, Kawahara E, Matsui O (1990) "Atypical adenomatous hyperplasia" in liver cirrhosis: low-grade hepatocellular carcinoma or borderline lesion? Histopathology 17:27–35
39. Nakashima T, Kojiro M (1987) Hepatocellular carcinoma. An atlas of its pathology. Springer Verlag, Tokyo, Berlin Heidelberg New York
40. Ng IOL, Chung LP, Tsang S, Lam CL, Lai ECS, Fan ST, Ng MMT (1993) *p53* gene mutation spectrum in hepatocellular carcinoma in Hong Kong Chinese (abstract). Hepatology 18:191
41. Ng IOL, Lai ECS, Fan ST, Ng MMT, Chan ASY, So MKP (1993) Expression of proliferating cell nuclear antigen in hepatocellular carcinoma (abstract). Hepatology 18:191
42. Nishida N, Fukuda Y, Kokuryu H, Toguchida J, Yandell DW, Ikenaga M, Imura H, Ishizaki K (1993) Role and mutational heterogeneity of p53 in hepatocellular carcinoma. Cancer Res 53:368–372
43. Nuorva K, Soini Y, Kamel D, Autio-Harmainen H, Risteli J, Vähäkangas K, Pääkö P (1993) Concurrent p53 expression in bronchial dysplasia and squamous cell lung carcinomas. Am J Pathol 142:725–732
44. Oda T, Tsuda H, Scarpa A, Sakamoto M, Hirohashi S (1992) Mutation pattern of the *p53* gene as a diagnostic marker for multiple hepatocellular carcinoma. Cancer Res 52:3674–3678
45. Oren M (1992) *p53*: the ultimate tumor suppressor gene? FASEB J 6:3169–3176
46. Ozturk M, Bressac B, Kew M, Volkman M, Bozcall S, Kar S, Carr BI (1991) *p53* mutation in hepatocellular carcinoma after aflatoxin exposure. Lancet 338:1356–1359
47. Paterson AC, Kew MC, Dusheiko GM, Isaacson C (1989) Liver cell dysplasia accompanying hepatocellular carcinoma in Southern Africa. Differences between urban and rural populations. J Hepatol 8:241–248
48. Rugge M, Shiao YH, Correa P, Baffa R, DiMario F (1992) Immunohistochemical evidence for p53 overexpression in gastric epithelial dysplasia. Cancer Epidemiol Biomarkers Prev 1:551–554
49. Scorsone KA, Zhou YZ, Butel JS, Slagle BL (1992) *p53* mutations cluster at codon 249 in hepatitis B virus-positive hepatocellular carcinomas from China. Cancer Res 52:1635–1638
50. Sheu JC, Huang GT, Lee PH, Chung JC, Chou HC, Lai MY, Wang JT, Lee HS, Shih LN, Yang PM, Wang TH, Chen DS

(1992) Mutation of *p53* gene in hepatocellular carcinoma in Taiwan. Cancer Res 52:6098–6100
51. Tabor E, Hsia CC, Muchmore E (1993) Hepatocellular carcinoma (HCC) in two chimpanzees; studies of transforming growth factor alpha (TGF alpha) and p53, and detection of oval cells (abstract). Hepatology 18:194
52. Tarao K, Shimizu A, Ohkawa S, Harada M, Ito Y, Tamai S, Kuni Y, Okamoto N, Inoue T, Kanisawa M (1992) Development of hepatocellular carcinoma associated with increases in DNA synthesis in the surrounding cirrhosis. Gastroenterology 103:595–600
53. Terris B, Marcio A, Tiollais P, Dejean A (1993) The *p53* gene in human hepatocellular carcinomas. J Hepatol 17:422
54. Tezuka F, Sawai T (1983) Hyperplasia of small hepatic cells in the precancerous condition of cirrhotic livers. Tohoku J Exp Med 139:171–177
55. Thomas RM, Bermann JJ, Yetter RA, Moore W, Hutchins GM (1992) Liver cell dysplasia: a DNA aneuploid lesion with distinct morphologic features. Hum Pathol 23:496–504
56. Thompson NL, Mead JE, Braun L, Goyette M, Shank PR, Fausto N (1985) Sequential protooncogene expression during rat liver regeneration. Cancer Res 46:3111–3117
57. Vojtesek B, Lane DP (1993) Regulation of p53 protein expression in human breast cancer cell lines. J Cell Sci 105:607–612
58. Vojtesek B, Bartek J, Midgley CA, Lane DP (1992) An immunohistochemical analysis of the human nuclear phosphoprotein p53. New monoclonal antibodies and epitope mapping using recombinant p53. Immunol Methods 151:237–244
59. Volkmann M, Hoffmann WJ, Müller M, Klein R, Willemann B, Zentgraf H, Räth U, Galle PR (1993) *p53* in European hepatocellular carcinoma: genomic alterations associated with increased steady state levels and heterogenous functional characteristics in vitro (abstract). Hepatology 18:196
60. Wade-Evans A, Jenkins AR (1985) Precise epitope mapping of the murine transformation-associated protein, p53. EMBO J 4:699–706
61. Wang LD, Hong JY, Qui SL, Gao H, Yang CS (1993) Accumulation of p53 protein in human esophageal precancerous lesions: a possible early biomarker for carcinogenesis. Cancer Res 53:1783–1787
62. Watanabe S, Okita S, Harada T, Kodama T, Numa Y, Takemoto T, Takahashi T (1983) Morphologic studies of the liver cell dysplasia. Cancer 51:2197–2205
63. Zambetti GP, Levine AJ (1993) A comparison of the biological activities of wild-type and mutant *p53*. FASEB J 7:855–865
64. Zhao M, Zhang NX, Du ZY, Laissue JL, Zimmermann A (1994) Three types of liver cell dysplasia (LCD) in small cirrhotic nodules are distinguishable by karyometry and PCNA labelling, and their features resemble distinct grades of hepatocellular carcinoma. Histol Histopath 9:73–83