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Overexpression of p53 Protein in Hepatocellular Carcinoma: Its Definition and Correlation with Viral Infection

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Summary. **BACKGROUND: No objective definition of p53 protein overexpression is found for hepatocellular carcinoma (HCC) in previous literature. Therefore, data on p53 status differ among investigators. The aim of this paper is to form an objective definition of p53 overexpression in HCC and assess the relationship between p53 overexpression and clinicopathological factors.**
METHODS: Two p53-specific monoclonal antibodies, PAb1801 and DO7, were used to compare p53 protein expression in 91 HCCs and its adjacent non-neoplastic liver tissues (91 patients), six adenomatous hyperplasias (2 patients), and 45 non-neoplastic liver tissues without tumor (45 patients). The p53 labeling index (LI) of each lesion was expressed as the number of p53 positive cells for more than 1,000 cells (range, 1006-1102 cells) in areas which counted the greatest number of positive cells in a homogeneous histological pattern.
RESULTS: Of the 136 patients, 6 patients with cirrhosis and 4 patients with chronic active hepatitis showed p53-positive cells in non-neoplastic hepatocytes with DO7, but no PAb1801 positive cells were found in non-neoplastic hepatocytes in any of the cases. DO7-p53 LI of non-neoplastic hepatocytes in areas with p53-positive cells was less than 1 percent (mean±SD, 0.4±0.2%). Twenty-nine (32%) of 91 HCCs showed more than 1% p53 LI (p53 LI>1%) with either PAb1801 or DO7. The p53 LI>1% was observed in 5% (1/20) of well differentiated HCC, 33% (20/60) of moderately differentiated HCC, and 72.7% (8/11) of poorly and undifferentiated HCC. The ratio of p53 LI>1% was more frequent in patients with serological hepatitis B and/or C viral markers than in patients without these markers ($P=0.003$), but did not correlate with other clinicopathological factors including tumor size, portal invasion or intrahepatic metastasis. The p53 LI>1% in moderately differentiated HCCs showed a positive correlation with Ki-67 labeling index ($P<0.03$).
CONCLUSIONS: p53 overexpression can be defined as greater than 1 percent of p53 LI, and is strongly related to viral chronic infection disease (HBV and/or HCV) and to cell proliferative activity of moderately differentiated HCCs.

Key words—p53 protein overexpression, p53 gene mutation, hepatocellular carcinoma.

INTRODUCTION

The p53 gene, located on the short arm of chromosome 17p[1], encodes as a 53 kDa nuclear phosphoprotein involved in the control of cell proliferation.[2] The exact function of the p53 protein is not fully understood, but it may play a role in DNA replication and regulation of transcription.[3,4] Abnormalities of this gene have been reported in a wide variety of malignant diseases, and mutations of p53 are at present the most commonly recognized genetic change in human cancer.[5-9]

Functional inactivation of the p53 gene is related to some different mechanisms. Most transforming viruses, such as simian virus 40,[10] human adenovirus,[11] human papilloma virus[12] and hepatitis B virus,[13] block its normal function in arresting the cell cycle through the formation of stable nonfunctional complexes between viral proteins.[3] Wild-type-p53 protein can be functionally inactivated by binding to the cellular proteins such as murine double minus-2 pro-

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tein (MDM-2).[14] In carcinoma cells, p53-dependent growth control is often impaired by missense mutations in one allele and the loss of the other.[15] Mutation leads to altered protein conformation and an increase in the p53 protein half-life from 15–20 min to a few hours, resulting in detectability by immunohistochemistry.[16] However, a few scattered p53-positive cells detectable by immunohistochemistry have been found in non-neoplastic conditions and tumors without p53 mutations.[17,18] Therefore, the presence of p53-positive cells itself does not mean an abnormal p53 status, i. e., p53-protein overexpression.

Several immunohistochemical studies have demonstrated a 0%-67% detection rate of p53 protein overexpression in non-neoplastic hepatocytes in which p53 positive cells were few and scattered.[19–23] A review of the literature indicated that the rate of p53 overexpression in moderately differentiated HCC was significantly higher in Japan (34.6%, 9/26) than (13.4%, 27/201) in Western countries ($P=0.005$).[18–20,24–26] Curiously, the criteria of p53 overexpression differed in these papers, i. e., from a small number of positive cells to 50% or more.[19–22,26,28,29] Most of the above data on p53 overexpression were semiquantitative, and did not show the number of tumor cells examined (a numerator, a denominator) or areas examined.

Hepatitis B virus (HBV), hepatitis C virus (HCV), cirrhosis, and aflatoxin B1 are known as the most important risk factors for the development of HCC. In half of the cases of HCC from endemic areas for HBV and aflatoxin B1, a hot spot point mutation at codon 249 has been detected, as previously reported.[30–32] On the other hand, in some European countries where dietary aflatoxin and HBV are not prevalent, p53 gene mutation is a relatively rare event, and few or none of the mutations occur at codon 249.[33–35]

In Japanese HCC, for which HBV and HCV infections are relatively common but aflatoxin exposure is low, Hayashi et al.[36] found no significant correlation between the presence of HBV or HCV infection and p53 mutations. However, Teramoto et al.[37] indicated a higher incidence of p53 abnormalities (45%) in patients who had been infected with either HBV or HCV than in those infected by neither (13%). The detailed relationship between p53 status and viral infection remains to be clarified.

In the current study, we attempted to establish objective criteria for p53 overexpression and investigate whether p53 overexpression in HCC has a correlation with clinicopathological factors and HBV/HCV infection.

MATERIALS AND METHODS

Materials

Surgically resected specimens from 91 primary HCCs were obtained from the archives of the First Department of Pathology at Niigata University from December 1989 to July 1997. The age of the patients ranged from 16 to 79 years old (mean, 61.6 years). The male to female ratio was 64: 27. Serological tests for serum hepatitis B surface antigen (HBsAg) in all 91 patients were performed; 21 patients were positive (23.1%), whereas serum anti-HCV antibody was present in 53 of 91 patients (58.2%). Two patients were positive for both HBsAg and anti-HCV antibody. HBsAg and anti-HCV antibody in the serum were checked by radioimmunoassay and second-generation enzyme-linked immunosorbent assay, respectively.

Surrounding non-neoplastic liver tissues were also sampled in 91 patients. The non-neoplastic liver was normal in 5 cases, hepatitis with/without fibrosis in 42 cases, and cirrhosis in 44 cases. Primary biliary cirrhosis (PBC), Budd-Chiari syndrome, and schistosomiasis japonica associated with HCC were each included in this series. The etiology of chronic liver disease in the rest of patients was alcohol-related (n=9) or unknown (n=7). The patients had received no previous chemotherapy or radiation within 1 month before surgery.

Furthermore, six adenomatous hyperplasias (AHs) (2 patients) and 25 needle or wedge biopsy specimens from chronic liver diseases without tumor (7 cases of cirrhosis: one case of alcoholic liver disease, on case of PBC and five cases of congenital biliary atrasia (CBA); 18 noncirrhotic cases: 8 cases of HCV infection and 10 of CBA) were also examined.

All resected specimens were fixed in 10% formalin. Size and gross appearance of tumors were macroscopically described according to the Eggel and Kanai classification.[38,39] Microscopically, all slides were reviewed to determine the size of small HCC and spread of the tumor by careful examination of 3 μm hematoxylin-eosin (HE) sections from consecutive, serially paraffin-embedded 30×20 mm blocks of the entire resected specimens. A range of two to twenty-four slides of the tumor and/or nontumorous liver (mean±SD of 7.3±4.8) was assessed in each case. The histological grades of tumor differentiation were classified into well, moderately, poorly differentiated, and undifferentiated subtypes according to the General Rules for the Clinical and Pathological Study of Primary Liver Cancer of Japan,[40] which approximately correspond to Edmondson-Steiner's grades I,

II, III, and IV,[41] respectively.

All resected specimens were analyzed for formation of a capsule, infiltration to the capsule, portal invasion, venous invasion, biliary invasion, intrahepatic metastasis, and bile production by tumor cells. These histopathological findings were mapped on color prints. Positive control sections were included in each experiment and consisted of tissue from a colonic adenocarcinoma with p53 positivity and a p53-mutation detected by PCR-SSCP (polymerase chain reaction single strand conformation) and direct sequencing. Specimens from twenty cases of normal liver were used in each experiment as negative controls.

Immunohistochemistry

Four serial 3 μm-thick sections were made from representative blocks of each tumor and/or non-neoplastic liver tissue. The first section was stained with HE. The second and third sections were immunostained for p53 using PAb 1801 and DO7, respectivelly. The fourth section was immunostained for Ki-67. Immunohistochemical staining was performed by a streptavidin-biotin immunoperoxidase method. For p53 and Ki-67 immunostaining, 3 μm-thick paraffinized tissue sections were placed onto poly L-lysine-coated glass slides and air-dried at room temperature. The sections were deparaffinized and rehydrated, then heated in a microwave oven (500W; Hitachi, MR-M220, Tokyo, Japan) for seven cycles of 3 min in a citrate-buffer to retrieve antigenic activity. The sections then were allowed to cool for 60 min at room temperature.[42] Endogeneous peroxidase activity was inhibited by incubation with 0.3% hydrogen peroxidase in methanol for 20 min at room temperature. After the blocking of non-specific reactions with 10% normal rabbit serum, the sections were first incubated with p53 antibody for 1 h at a dilution of 1: 200 or the Ki-67 antibody for 2 h at a dilution of 1: 100 at room temperature. Staining for p53 protein was carried out using the following antibodies: mouse monoclonal antibody PAb1801 (Ab-2, Oncogene Science Inc., Manhasset, NY, U.S.A.), which reacts with a denaturation-resistant epitope in human p53 located between amino acids 32 and 79[43]; and mouse monoclonal antibody DO7 (DO7, Novocastra Laboratories Ltd., Newcastle, UK), which reacts with a denaturation-resistant epitope at the N terminus of p53 between amino acids 20 and 25.[44] These two antibodies are capable of detecting both wild type and mutant type p53 proteins. Cell proliferative activity was determined using mouse monoclonal antibody MIB1 (MIB1; Immunotech, Marseille, France), which reacts with native Ki-67 antigen and recombinant fragments of the Ki-67 molecule.[45-47]

The sections were then incubated with biotinylated rabbit anti-mouse immunogloblin for 30 min and then with the streptavidin-peroxidase complex (Histofine SAB-PO Kit; Nichirei, Tokyo, Japan) for 15 min. Careful rinses were performed with several changes of phosphate-buffered saline between each stage of the procedure. The color of the immunoreactivity was developed with diaminobenzidine for 6 min in both PAb1801 and DO7 staining.[48] The sections were lightly counterstained with hematoxylin and then mounted.

Immunohistochemical analysis

Cells positive for p53 were defined as those with a brown stained nucleus, regardless of staining intensity. Tumors were scored as positive when at least one positive nucleus was visible within the lesion. The staining pattern of p53 immunostaining was classified into four types: negative, no positive cells; scattered, scattered positive cells; nested, focally aggregated positive cells; and diffuse, diffuse positive cells.

The p53 immunostaining was evaluated for LI and staining intensity in the same lesion. The p53 LI was expressed as the number of p53 positive tumor cells for more than 1,000 cells (range, 1006-1102 cells) in the area where the greatest number of positive cells was present in a histologically homogeneous pattern. Staining intensity and p53 LI were compared for corresponding areas of PAb1801 and DO7 stained sections. Nuclear staining intensity was divided into three categories: weak, intermediate, and strong. The scoring patterns of p53 LI were categorized into four groups as follows: $LI \leqq 1\%$, $1\% < LI < 10\%$, $10\% \leqq LI < 50\%$, $50\% \leqq LI$. Ki-67 positive cells were defined as cells with a brown staining of the nucleus, regardless of staining intensity. In suitably fixed praffin sections of liver tissues, Ki-67 positive cells were visualized with a strong nuclear pattern in the germinal center, a site of active lymphocyte proliferation. Mitotic figures were stained strongly in HCC cells. The Ki-67 LI of HCCs ranged from 0.6% to 52.9%. The Ki-67 LI was determined in areas corresponding to those where the p53 LI was measured (Fig. 2A, B, C, and D). In p53-negative cases, the Ki-67 LI was determined in the area counting the greatest number of positive cells in a histologically homogeneous pattern.

Statistical analysis

The Chi-squared and Fischer's exact tests were used for analysis of the categorical data according to the presence or absence of p53 overexpression, whereas Student's t test, and the Mann-Whitney U test were used for continuous data. Probability values of less than 0.05 were considered to be statistically significant.

RESULTS

Comparison of p53 expression in non-neoplastic hepatocytes between PAb1801 and DO7 staining

The p53 immunoreactivity was observed in the nuclei of hepatocytes in hepatitis and cirrhosis but not in the normal liver. The p53 expression of non-neoplastic hepatocytes with each PAb1801 and DO7 staining is presented in Table 1. Of the 136 non-neoplastic samples, p53 expression was detected in only DO7-stained sections, but not in PAb1801 sections. The p53 positive cells were found in hepatocytes in only cirrhosis and chronic active hepatitis at a rate of 6-14%.

Findings of DO7-p53 expression in non-neoplastic hepatocytes

The p53-staining pattern was a scattered pattern in ten p53 positive cases (Fig. 1). All of the staining intensities were intermediate in these 10 cases. The positive hepatocytes showed no histological atypia and were mainly located at the peripheral zone of hepatic lobules close to HCC (range of distance from HCCs: 0.6-9 mm). p53 LI of the non-neoplastic hepatocytes was less than 1 percent (mean±SD, 0.4±0.2%; range, 0.2-0.8%), and p53 LI was lower than Ki-67 LI in the corresponding areas, except for one case with PBC. There was no significant difference in Ki-67 LI at the peripheral zone between p53 positive areas and negative areas (Table 2).

Table 1. Comparison of p53 expression in non-neoplastic hepatocytes between PAb1801 and DO7 staining

Histology	No.of samples	p53 expression	
		PAb1801 positive	DO7 positive
Normal liver without tumor	20	0 (0%)	0 (0%)
Chronic active hepatitis without tumor	18	0 (0%)	1 (6%)
Cirrhosis without tumor	7	0 (0%)	1(14%)
Normal liver with HCC	5	0 (0%)	0 (0%)
Chronic active hepatitis with HCC	42	0 (0%)	3 (7%)
Cirrhosis with HCC	44	0 (0%)	5(11%)
Adenomatous hyperplasia	6	0 (0%)	0 (0%)

HCC, hepatocellular carcinoma.

Table 2. Findings of p53 expression by DO7 staining in non-neoplastic hepatocytes

NG-NO	Histology	Etiology	Staining pattern	Staining intensity	Location of p53-positive cells	Distance from the tumor (mm)	Cell atypia	p53LI	Ki-67 LI in p53-positive area	Ki-67 LI in p53-negative at the peripheral zone
94-2020	Hepatitis	HCV	scattered	Int	Periph	-	(−)	0.6%(6/1012)	1.1%(11/1010)	1.0%(10/1018)
NG8407	Cirrhosis†	PBC	scattered	Int	Periph	-	(−)	0.8%(8/1009)	0.1%(1/1005)	0.2%(2/1014)
NG9516	Hepatitis	HBV	scattered	Int	Periph	0.6-8.5	(−)	0.4%(4/1012)	1.5%(15/1017)	1.6%(16/1011)
NG10062	Hepatitis	HCV	scattered	Int	Periph	0.8-5.5	(−)	0.3%(3/1013)	1.6%(16/1019)	1.8%(18/1015)
NG10361	Hepatitis	HCV	scattered	Int	Periph	6-9	(−)	0.3%(3/1002)	2.4%(26/1065)	2.2%(23/1054)
NG5489	Cirrhosis	HCV	scattered	Int	Periph	1.5-2	(−)	0.2%(2/1006)	1.9%(19/1003)	1.6%(16/1015)
NG9077	Cirrhosis	HCV	scattered	Int	Periph	0.7	(−)	0.3%(3/1017)	1.2%(12/1022)	1.4%(15/1041)
NG9696	Cirrhosis	Unkown	scattered	Int	Periph	1	(−)	0.3%(3/1016)	2.4%(24/1031)	1.9%(20/1038)
NG9830	Cirrhosis	Alcoholic	scattered	Int	Periph	3-8	(−)	0.3%(3/1011)	1.6%(16/1013)	0.8%(8/1016)
NG10253	Cirrhosis	HCV	scattered	Int	Periph	2-5	(−)	0.7%(7/1034)	3.1%(32/1019)	3.4%(36/1062)
								0.4±0.2%*	1.7±0.8%*a	1.6±0.9%*

HCV, hepatitis C virus; PBC, primary biliary cirrhosis; HBV, hepatitis B virus; Periph, peripheral zone.

*LI, labeling index (mean±SD).

†Cirrhosis with atypical adenomotous hyperplasia (high-grade dysplastic nodule).

[a]No significant difference from that without p53 expression by Student's t-test ($P=0.73$).

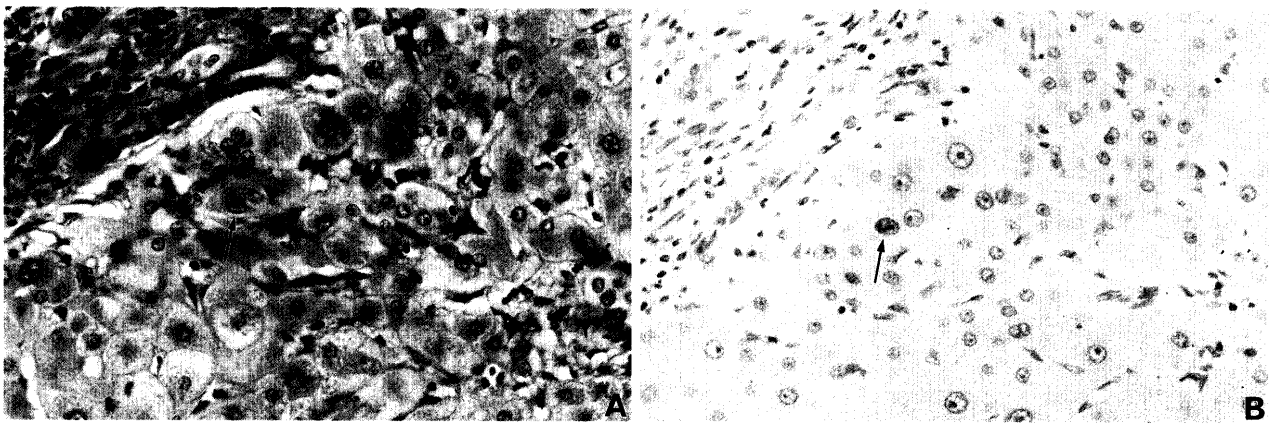

Fig. 1. A scattered p53 staining pattern by DO7 stain in non-neoplastic regenerative hehatocytes. **A.** A case of liver cirrhosis with HCC. **B.** A p53 positive cell (*arrow*) is found. (**A.** HE, ×100; **B.** DO7 staining, ×100)

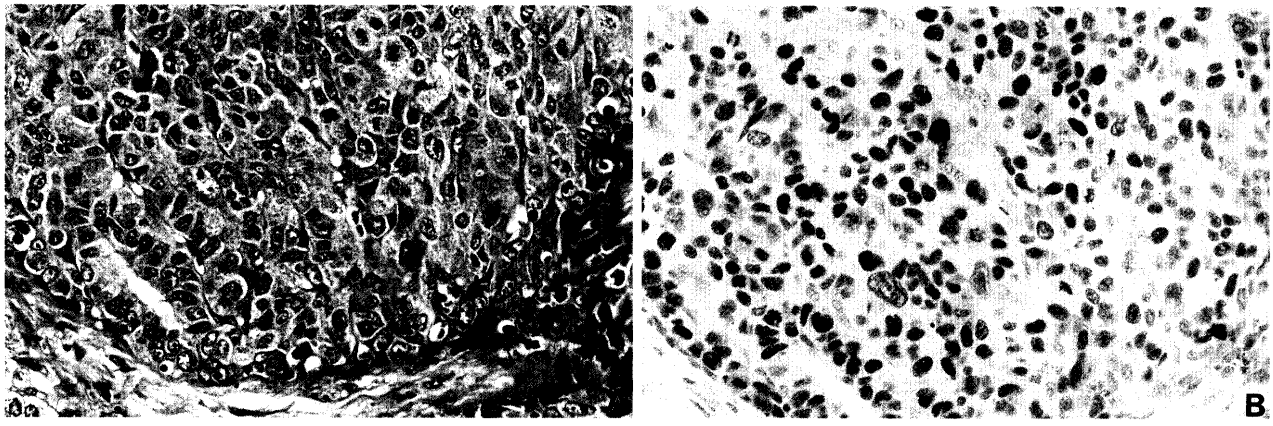

Fig. 2A Fig. 2B

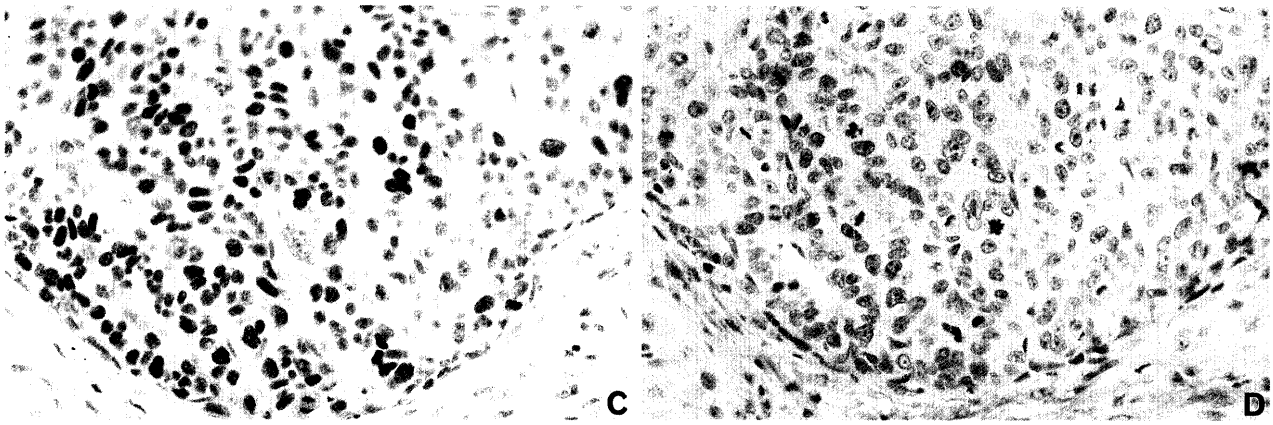

Fig. 2. A diffuse p53 staining pattern of p53 and Ki-67. **A.** A case of moderately differentiated HCC. **B.** PAb1801 and **C.** DO7 show a similar staining pattern. **D.** Ki-67 positive cells show a diffuse pattern in corresponding areas with p53 staining. (**A.** HE, ×100; **B.** PAb1801 staining, ×100; **C:** DO7 staining, ×100; **D:** Ki-67 staining, ×100)

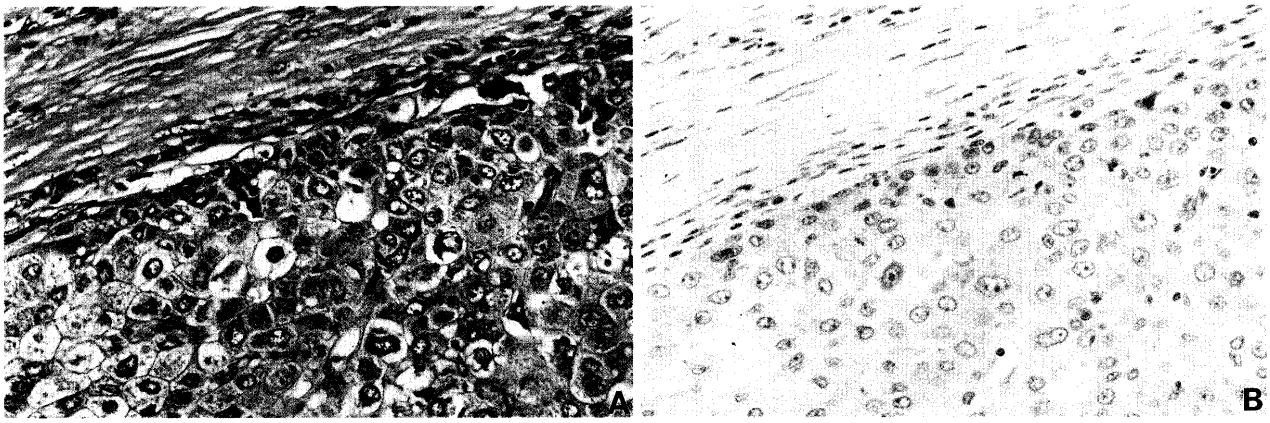

Fig. 3. A nested p53 staining pattern of moderately differentiated HCC. **A.** HE, ×100 **B.** DO7, ×100

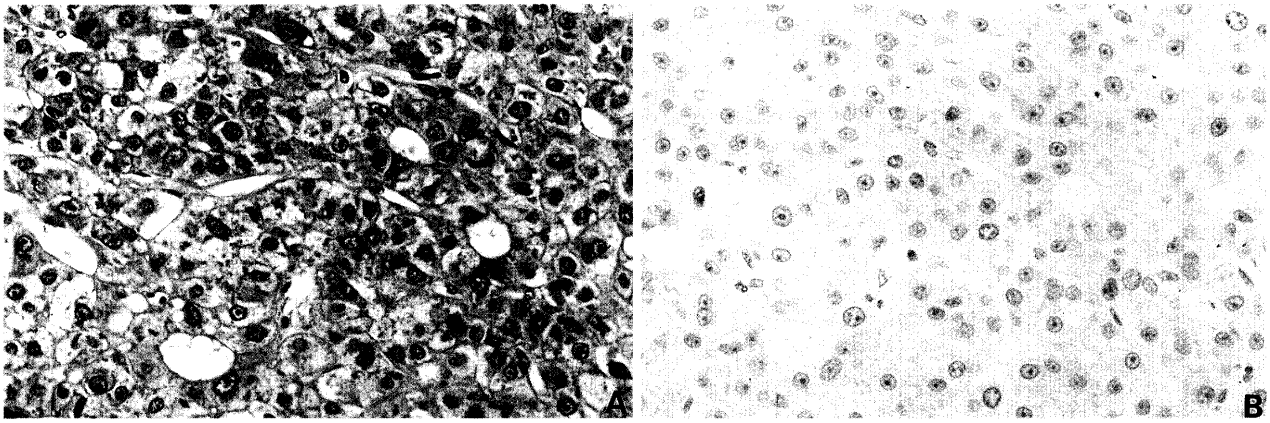

Fig. 4. A scattered p53 staining pattern of moderately differentiated HCC. **A.** He, ×100 **B.** DO7, ×100

Ki-67 LI of non-neoplastic hepatocytes at central and peripheral zones

Ki-67 LI was significantly higher at the peripheral zone than at the central zone of hepatic lobules of the normal liver, in hepatitis and cirrhosis. ($P<0.0001$) (Table 3).

Comparison of p53 immunoreactivity of HCC between PAb1801 and DO7 staining

Staining pattern, intensity, scoring pattern, and the rate of p53 LI>1% of HCCs assessed by the two anti-p53 antibodies are summarized in Table 4. The staining pattern was very similar between the two anti-p53 antibodies used (Fig. 2A, B and C). There was no significant difference in staining pattern, staining intensity, scoring pattern, and the p53 LI between PAb1801 and DO7 for a 6-min reaction time. Two cases were negative for PAb1801, but showed small aggregations of cells by DO7 staining with 2.1 and 3.5% p53 LI (Table 4). The concordance of scoring patterns between PAb1801 and DO7 was 94.5%. The staining intensity was generally strong in the diffuse pattern, but weak or intermediate in the nested and scattered patterns.

p53 LI>1% was detected in 27 (30%) and 29 (32%) of total of 91 HCCs by PAb1801 and DO7, respectively. The incidence of p53 LI>1% was higher in HCCs with a lower histological differentiation: 72.7% (8/11) in poorly and undifferentiated HCCs, 33% (20/60) in moderately differentiated HCCs, and 5% (1/20) in well differentiated HCCs. A statistically significant difference in p53 LI>1% was observed between the former two groups and the latter group ($P<0.02$)

Comparison of clinicopathological factors in p53 expression status in HCC

There was no significant difference between p53 LI>

1% and clinicopathological factors including age, gender, tumor size, bile production, formation of a capsule, infiltration to the capsule, portal invasion, venous invasion, biliary invasion, intrahepatic metastasis and cirrhosis (Table 5).

Comparison of p53 expression status and Ki-67 labeling index in HCC between viral infection and non-infection

Correlation of p53 LI>1% with viral infection was analyzed (Table 6). The frequency of p53 LI>1% was 33% (7/21) in HCCs with HBV infection, 42% (22/53) in HCCs with HCV infection, and 5% (1/19) in HCCs without HBV and HCV infection (NBNC) (HBV vs HCV: $P>0.05$, HBV vs NBNC: $P=0.003$, HCV vs NBNC: $P=0.002$, HBV and/or HCV vs NBNC: $P=0.003$). No correlation was observed between histological grades of HCC and the rate of viral infection. Ki-67 LI was not correlated with the presence of absence of a viral infection, but showed a positive correlation with the histological grade of HCCs alone.

Correlation between p53 expression status and Ki-67 LI of HCC

HCCs with p53 LI>1% showed a significantly higher Ki-67 LI in moderately differentiated HCCs ($P<0.03$. Table 7) than those without, but not in others.

DISCUSSION

Many investigators have reported on a p53 immunohistochemical analysis of HCCs. However, criteria on p53 overexpression have been different and semiquantitative, i.e., a small number of cells,[23] 1%,[22] 2%,[19] 5%,[21] 10%,[28], 25%,[20] 40%[29], and 50%.[26] In addition, there was no description of the number of tumor cells examined or areas selected. Therefore, their definition on p53 overexpression seems to be not reproducible and objective.

The p53 staining pattern is one of a marker to express p53 overexpression. A scattered pattern has been observed elsewhere in non-neoplastic livers and HCCs[19-23] as well as in our study. However, neither a nested nor a diffuse pattern was ever found in non-neoplastic livers. Therefore, a nested or diffuse pattern may be defined as p53 overexpression. Each p53 staining pattern corresponded well to p53 LI, i. e., the scattered pattern (Fig. 4), less than 1% p53 LI; nested pattern (Fig. 3). 1.5-24.9% p53 LI; and diffuse pattern (Fig. 2), 31-84.2% p53 LI. A few p53 positive cells developed in immature regenerative cells of active cholecystitis and esophagitis[6,7] with a lower p53 LI than a Ki-67 LI in the corresponding areas. This held true for the non-neoplastic liver except for a case with PBC. However, p53 staining pattern of the PBC was a scattered one.

p53 positive non-neoplastic hepatocytes did not show histological atypia (Fig. 1) and were mainly located at the peripheral zone close to the tumor (range, 0.6-9 mm), where Ki-67 LI was significantly higher than that of the central zone. From these data, a scattered pattern or p53 LI≦1% may indicate a wild-type p53 gene response to cellular stress or cell proliferation of hepatocytes. To confirm this, it is necessary to study p53 gene mutation by a microdissection method. The data on p53 reactivity of non-neoplastic liver suggest p53-protein overexpression is defined as p53 LI>1%.

According to the criteria mentioned above, p53-protein overexpression was found in 27 (32%) of a total of 91 HCCs, in 5% (1/20) of well differentiated HCCs, 33% (20/60) of moderately differentiated HCCs, and 72.7% (8/11) of poorly and undifferentiated HCC. Alterations of the p53 gene may not always lead to an accumulation of the p53

Table 3. Ki-67 labeling index of non-neoplastic hepatocytes at central and peripheral zones

Histology	Ki-67 labeling index (mean±SD)	
	Central zone	Peripheral zone
Normal liver (n=25)	0.25±0.14%	0.93±0.32%
Hepatitis (n=60)	0.47±0.28%	1.22±0.47%
Cirrhosis (n=51)	1.08±0.56%	2.11±0.80%
		*

SD, standard deviation.

* Mann-Whitney's U test ($P<0.0001$).

Table 4. Comparison of p53 immunoreactivity of HCC between PAb1801 and DO7 staining

Histological grade	Staining pattern			Staining intensity			p53 scoring pattern					No. (%)of p53
	Scattered	Nested	Diffuse	W	I	S	0%	0<LI<1%	1≦LI<10%	10≦LI<50%	50%≦LI	LI>1%
Well differentiated (n=20)												
PAb1801	4	0	0	4	0	0	16	4	0	0	0	0(0%)
DO7	4	1	0	4	1	0	15	4	1	0	0	1(5%)
Moderately differentiated (n=60)												
PAb1801	8	11	8	6	14	7	33	8	10	5	4	19(32%)
DO7	11	12	8	9	14	8	29	11	11	5	4	20(33%)
Poorly differentiated (n=9)												
PAb1801	0	2	5	2	2	3	2	0	2	2	3	7(78%)
DO7	0	2	5	0	2	5	2	0	2	2	3	7(78%)
Undifferentiated (n=2)												
PAb1801	0	0	1	0	1	0	1	0	0	1	0	1(50%)
DO7	0	0	1	0	1	0	1	0	0	1	0	1(50%)
Total (n=91)												
PAb1801	12	13	14	12	17	10	52	12	12	8	7	27(30%)
DO7	15	15	14	13	18	13	47	15	14	8	7	29(32%)

HCC, hepatocellular carcinoma; W, weak; I, intermediate; S strong; LI, labeling index.

* No significant difference between PAb1801 and DO7(Mann-Whitney's U test).

Table 5. Comparision of clinicopathological factors in p53 expression status of HCC

	p53 expression		*P*
	p53 LI>1% (n=29)	p53 LI≦1% (n=62)	
Age (yr)[a]	60.8±13.5	61.9±9.2	NS*
Gender (male/female)	19/10	45/17	NS†
Size (mm)[a]	47.5±28.6	51.1±35.5	NS*
Bile production (%)	14/29(48.3)	35/62(56.5)	NS†
Formation of capsule (%)	27/29(93.1)	54/62(87.1)	NS‡
Infiltration to capsule (%)	17/27(63.0)	38/54(70.4)	NS†
Portal invasion			
Well differentiated (%)	0/1(0)	1/19(5.3)	NS‡
Moderately differentiated (%)	8/20(40.0)	10/40(25.0)	NS†
Poorly differentiated (%)	4/7(57.1)	2/2(100)	NS‡
Undifferentiated (%)	0/1(0)	0/1(0)	NS‡
Venous invasion (%)	3/29(10.3)	6/62(9.7)	NS‡
Biliay invasion (%)	1/29(3.4)	3/62(4.8)	NS‡
Intrahepatic metastasis (%)	16/29(55.2)	26/62(41.9)	NS†
Cirrhosis (%)	16/29(55.2)	28/62(45.2)	NS†

HCC, hepatocellular carcinoma; LI, labeling index; NS, not significant.
[a]Mean±SD. *Mann-Whitney's U test. †Chi-squared test. ‡Fischer's exact probability test.

Table 6. Comparison of p53 expression status and Ki-67 labeling index in HCC between viral infection and non-infection (n=91)

	p53 expression (p53 LI>1%)cases		Ki-67 labeling index (mean±SD)		
			Well	Mod	Poor/undiff
Viral infection					
Positive cases (B and/or C)	28/72(39%)		3.4±2.4%	15.3±11.9%	32.1±12.1%
HBV infection					
Positive cases (B)	7/21(33%)	NS	2.2±2.4%	12.5±10.1%	26.0%
HCV infection					
Positive cases (C)	22/53(42%)	‡	3.7±2.4%	16.3±12.5%	32.9±12.7%
Viral infection		†			
Negative cases (nonBnonC)	1/19(5%)		2.3±1.0%	11.9±8.6%	43.2±4.0%
Alcoholic (nonBnonC)	1/9(11%)		1.9±0.8%	12.6±9.3%	40.3%
Unkown (nonBnonC)	0/7(0%)		3.1±1.1%	11.4±8.6%	-

HCC, hepatocellular carcinoma; LI, labeling index; Well, well differentiated; Mod, moderately differentiated; Poor, poorly differentiated; Undiff, undifferentiated; B, hepatitis B viral infection; C, hepatitis C viral infection; NS, not significant.
*One case each of primary biliary cirrhosis, Budd-Chiari syndrome and schistosomiasis japonica is included.
†$P=0.002$; ‡$P=0.003$; §$P=0.003$.

Table 7. Correlation between p53 expression status and Ki-67 labeling index of HCC (n=91)

Histological grade	Ki-67 labeling index (mean±SD)		*P*
	p53 LI>1% (n=29)	p53 LI≦1% (n=62)	
Well differentiated (n=20)	0.6%(1)	3.2±2.1%(19)	
Moderately differentiated (n=60)	19.1±13.2%(20)	12.5±9.8%(40)	0.03*
Poorly differentiated (n=9)	33.1±9.9%(7)	22.5±5.2%(2)	NS
Undifferentiated (n=2)	52.9%(1)	46.0%(1)	
Total (n=91)	23.0±15.0%	10.5±10.4%	<0.0002†

HCC, hepatocellular carcinoma; SD, standard deviation; LI, labeling index.
* Student's t-test. †Mann-Whitney's U test. ( ), number of HCC.

protein because of nonsense mutation.[27] Nevertheless, this frequency of Japanese HCCs was consistent with the results of others,[36,49] where the rate of p53 mutations was in the range of 27.8 and 36.4% of total HCCs, and was similar even in histological grades of HCCs.

It is controversial whether or not p53 overexpression is correlated with clinicopathological factors. Laurent-Puig et al.[26] reported that p53 overexpression was related to invasion of the portal branches. However, other investigators[28,36] did not find this correlation, including us.

Hayashi et al.[36] and Nagao et al.[22] reported that there was no relationship between p53 abnormalities and the presence of HBV or HCV infection of HCCs. However, their HBV negative and HCV negative cases contained HCV positive and HBV positive cases, respectively. In our study, the patients of HCCs with HBV and/or HCV showed a higher incidence of p53 overexpression (39%) than those infected by neither ($P=0.003$). This result was consistent with the results of others.[26,37,50] This correlation may be explained by the fact that the formation of stable nonfunctional complexes between viral proteins and p53 protein contributes to the functional inactivation of the p53 gene and p53 protein stabilization.[3,27] Moreover, Wang et al.[51] suggested that the inhibition of p53-mediated apoptosis by HBx may contribute to the development of human HCC. However, the detailed mechanisms of cellular transformation induced by HCV remain unknown and require further investigation.

No significant difference was observed between p53 LI>1% and clinicopathological factors. However, cell proliferation and histological grades of HCC are important for the management of patients. A p53 overexpression group of moderately differentiated HCCs showed a significantly higher Ki-67 LI than the p53 non-overexpression group ($P<0.03$. Table 3). In other histological grades of HCC, the correlation of p53 overexpression and Ki-67 LI was not significant because of the small number of cases. This correlation should be studied in the future by increasing the number of cases.

From these data, we can conclude that p53 overexpression of HCCs is defined as p53 LI>1%, and that the overexpression is strongly related to viral infection and the cell proliferative activity of moderately differentiated HCCs but not to tumor invasiveness.

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REFERENCES

1) Isobe M, Emanuel BS, Givol D, Oren M, Croce CM: Localization of gene for human p53 tumour antigen to band 17p13. *Nature* **320:** 84-85, 1986.
2) Baker SJ, Fearon ER, Nigro JM, Hamilton SR, Preisinger AC, Jessup JM, Vantuinen P, Ledbetter DH, Barker DF, Nakamura Y, White R, Vogelstein B: Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science* **244:** 217-221, 1989.
3) Levine AJ, Momand J, Finlay CA: The p53 tumour suppressor gene. *Nature* **351:** 453-456, 1991.
4) Levine AJ: p53, the cellular gate keeper for growth and division. *Cell* **88(3):** 323-331, 1997.
5) Hollstein M, Sidransky D, Vogelstein B, Harris CC: p53 mutations in human cancers. *Science* **253:** 49-53, 1991.

6) Ohashi Y, Watanabe H, Ajioka Y, Hatakeyama K: p53 immunostaining distinguishes malignant from benign lesions of the gall-bladder. *Pathol Int* **45:** 58-65, 1995.
7) Nakagawa S, Watanabe H, Ajioka Y, Nishikura K, Hitomi J, Hatakeyama K: Archival analysis of p53 protein overexpression and genetic mutation in esophageal squamous cell carcinoma. *Acta Med Biol* **44(2)**: 63-69, 1996.
8) Yokoyama N, Hitomi J, Watanabe H, Ajioka Y, Pruyas M, Serra I, Shirai Y, Hatakeyama K: p53 mutations in gallbladder carcinomas in high incidence areas of Japan and Chile. *Cancer Epidemiology Biomarkers & Prevention* **7:** 297-301, 1998.
9) Kuwabara A, Ajioka Y, Watanabe H, Yasuda K, Saito H, Matsuda K, Kijima H, Hatakeyama K: Alteration of p53 clonality accompanied with colorectal cancer progression. *Jpn J Cancer Res* **89:** 40-46, 1998.
10) Lane DP, Crawford LV: T antigen is bound to a host protein in SV40 transformed cells. *Nature* **278:** 261-263, 1979.
11) Yew PR, Berk AJ: Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. *Nature* **357:** 82-84, 1992.
12) Werness BA, Levine AJ, Howley PM: Association of human papilloma virus types 16 and 18 E6 proteins with p53. *Science* **248:** 76-79, 1990.
13) Feitelson MA, Zhu M, Duan LX, London WT: Hepatitis B x antigen and p53 are associated in vitro and in liver tissues from patients with primary hepatocellular carcinoma. *Oncogene* **8:** 1109-1117, 1993.
14) Momand J, Zambetti GP, Olson D, George DL, Levine AJ: The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* **69:** 1237-1245, 1992.
15) Vogelstein B, Kinzler KW: p53 function and dysfunction. *Cell* **70:** 523-526, 1992.
16) Rovinski B, Benchimol S: Immortalization of rat embryo fibroblasts by the cellular p53 oncogene. *Oncogene* **2:** 445-452, 1988.
17) Kang YK, Kim CJ, Kim WH, Kim HO, Kang GH, Kim YI: p53 mutation and overexpression in hepatocellular carcinoma and dysplastic nodules in the liver. *Virchows Arch* **432:** 27-32, 1998.
18) Volkmann M, Hofmann WJ, Müller M, Räth, Otto G, Zentgraf H, Galle PR: p53 overexpression is frequent in European hepatocellular carcinoma and largely independent of the codon 249 hot spot mutation. *Oncogene* **9:** 195-204, 1994.
19) Ojanguren I, Ariza A, Castellà EM, Fernández-Vasalo A, Mate JL, Navas-Palacios JJ: p53 immunoreactivity in hepatocellular adenoma, forcal nodular hyperplasia, cirrhosis and hepatocellular carcinoma. *Histopathology* **26:** 63-68, 1995.
20) Skopelitou A, Hadjiyannakis M, Alexopoulou V, Kamina S, Krikoni O, Agnantis NJ: p53 expression in hepatocellular carcinoma in Greece correlation with epidemiological and histopathological data. *Path Res Pract* **192:** 1100-1106, 1996.
21) Nakopoulou L, Janinis J, Giannoupoulou I, Lazaris AC, Koureas A, Zacharoulis D: Immunohistochemical expression of p53 protein and proliferating cell nuclear antigen in hepatocellular carcinoma. *Path Res Pract* **191:** 1208-1213, 1995.
22) Nagao T, Kondo F, Sato T, Nagato Y, Kondo Y: Immunohistochemical detection of aberrant p53 expression in hepatocellular carcinoma: correlation with cell proliferative activity indices, including mitotic index and MIB-1 immunostaining. *Hum Pathol* **23:** 326-333, 1995.
23) Livni N, Eid P, Ilan Y, Rivkind A, Rosenmann E, Blendis LM, Shouval D, Galun E: p53 expression in patients with cirrhosis with and without hepatocellular carcinoma. *Cancer* **75:** 2420-2426, 1995.
24) D'Errico A, Grigioni WF, Fiorentino M, Baccarini P, Grazi GL, Mancini AM: Overexpression of p53 protein and Ki67 proliferative index in hepatocellular carcinoma: An immunohistochemical study on 109 Italian patients. *Pathol Int* **44:** 682-687, 1994.
25) Choi SW, Hytiroglou P, Geller SA, Kim SM, Chung KW, Park DH, Theise ND, Thung SN: The expression of p53 antigen in primary malignant epithelial tumors of the liver: an immunohistochemical study. *Liver* **13:** 172-176, 1993.
26) Laurent-Puig P, Flejou JF, Fabre M, Bedossa P, Belghiti J, Gayral F, Franco D: Overxepression of p53: a rare event in a large series of white patients with hepatocellular carcinoma. *Hepatology* **16:** 1171-1175, 1992.
27) Bourdon JC, D'errico A, Paterlini P, Walter G, May E, Debuire B: p53 protein accomulation in European hepatocellular carcinoma is not always dependent on p53 gene mutation. *Gastroentrology* **108:** 1176-1182, 1995.
28) Ng IO, Lai EC, Chan AS, So MK: Overexpression of p53 in hepatocellular carcinomas: a clinicopathological and prognostic correlation. *J Gastroenterol Hepatol* **10:** 250-255, 1995.
29) Hsu HC, Tseng HJ, Lai PL, Lee PH, Peng SY: Expression of p53 gene in 184 unifocal hepatocellular carcinoma: Association with tumor growth and invasiveness. *Cancer Res* **53:** 4691-4694, 1993.
30) Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC: Mutational hotspot in the p53 gene in human hepatocellular carcinomas. *Nature* **350:** 427-428, 1991.
31) Bressac B, Kew M, Wands J, Ozturk M: Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. *Nature* **350:** 429-431, 1991.
32) Lunn RM, Zhang YJ, Wang LY, Chen CJ, Lee PH, Lee CS, Tsai WY, Santella RM: p53 mutations, chronic hepatitis B virus infection, and aflatoxin exposure in hepatocellular carcinoma in Taiwan. *Cancer Res* **57:** 3471-3477, 1997.

33) Challen C, Lunec J, Warren W, Collier J, Bassendine MF: Analysis of p53 tumor-suppressor gene in hepatocellular carcinomas from Britain. *Hepatology* **16:** 1362-1366, 1992.
34) Kress S, Jahn UR, Buchmann A, Bannasch P, Schwarz M: p53 mutations in human hepatocellular carcinomas from Germany. *Cancer Res* **52:** 3220-3223, 1992.
35) Debuire B, Paterlini P, Pontisso P, Basso G, May E: Analysis of the p53 gene in European hepatocellular carcinomas and hepatoblastomas. *Oncogene* **8:** 2303-2306, 1993.
36) Hayashi H, Sugio K, Matsumata T, Adachi E, Takenaka K, Sugimachi K: The clinical significance of p53 gene mutation in hepatocellular carcinomas from Japan. *Hepatology* **22(6)**: 1702-1707, 1995.
37) Teramoto T, Satonaka K, Kitazawa S, Fujimori T, Hayashi K, Maeda S: p53 gene abnormalities are closely related to hepatoviral infections and occur at a late stage of hepatocarcinogenesis. *Cancer Res* **54:** 231-235, 1994.
38) Eggel H: Ueber das primäre Carcinoma der Leber. *Beitr z Path Anat u z allgem Pathol* **30:** 506-604, 1901.
39) Kanai T, Hirohashi S, Upton MP, Noguchi M, Kishi K, Makuuchi M, Yamasaki S, Hasegawa H, Takayasu K, Moriyama N, Shimosato Y: Pathology of small hepatocellular carcinoma. *Cancer* **60:** 810-819, 1987.
40) Liver Cancer Study of Japan: The General Rules for the Clinical and Pathological Study of Primary Liver Cancer (ed 3). Kanehara Publications, Tokyo, Japan, 1992.
41) Edmondson HA, Steiner PE: Primary carcinoma of the liver: A study of 100 cases among 48,900 necropsies. *Cancer* **7:** 462-503, 1954.
42) Shi SR, Key ME, Kalra KL: Antigen retrieval in formaline-fixed, paraffin-embedded tissue: An enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J Histochem Cytochem* **39:** 741-748, 1991.
43) Banks L, Matiashewski C, Crawford L: Isolation of human p53 specific monoclonal antibodies and their use in human p53 expression. *Eur J Biochem* **159:** 529-534, 1986.
44) Stephen CW, Heiminen P, Lane DP: Characterization of epitopes on human p53 using phage displayed peptide libraries: Insights into antibody peptide interactions. *J Mol Biol* **248:** 58-78, 1995.
45) Schwarting R: Little missed markers and Ki-67. *Lab Invest* **68:** 597-599, 1993.
46) Cattoretti G, Becker MHG, Key G, Duchrow M, Schlüter C, Galle J, Gerdes J: Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. *J Pathol* **168:** 357-363, 1992.
47) Key G, Becker MHG, Baron B, Duchrow M, Schlüter C, Flad HD, Gerdes J: New Ki-67-equivalent murine monoclonal antibodies (MIB 1-3) generated against bacterially expressed parts of the Ki-67 cDNA containing three 62 base pair repetitive elements encoding for the Ki-67 epitope. *Lab Invest* **68:** 629-636, 1993.
48) Itoi T, Watanabe H, Yoshida M, Kasuya K, Yokota Y: p53 immunohistchemistry of gallbladder carcinoma: A comparison of PAb1801 and DO7 antibodies. *Acta Med Biol* **43:** 53-57, 1995.
49) Murakami Y, Hayashi K, Hirohashi K, Sekiya T: Aberrations of the tumor supressor p53 and retinoblastoma genes in human hepatocellular carcinomas. *Cancer Res* **51:** 5520-5525, 1991.
50) Honda K, Sbisà E, Tullo A, Papeo PA, Saccone C, Poole S, Pignatelli M, Mitry RR, Ding S, Isla A, Davies A, Habib NA: p53 mutation is a poor prognostic indicator for survival in patients with hepatocellular carcinoma undergoing surgical tumour ablation. *Br J Cancer* **77(5)**: 776-782, 1998.
51) Wang XW, Gibson MK, Vermeulen W, Yeh H, Forrester K, Stürzbecher HW, Hoeijmakers JHJ, Harris CC: Abrogation of p53-induced apoptosis by the hepatitis B virus x gene. *Cancer Res* **55:** 6012-6016, 1995.