Kobe University Repository : Kernel

PDF issue: 2026-08-11

Immunohistochemical study of P53, PCNA and AFP in hepatocellular carcinoma, a comparison between Indonesian and Japanese cases

Marwoto, Wirasmi ; Miskad, Upik Anderiani ; Siregar, Nuryati Chaerani ; Gani, Rino A. ; Boedihusodo, Unggul ; Nurdjanah, Siti ; Suwarso ;…

(Citation)
The Kobe journal of the medical sciences,46(5):217-229

(Issue Date)
2000-10

(Resource Type)
departmental bulletin paper

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/E0001061

Kobe J. Med. Sci. 46, 217/229, October 2000

IMMUNOHISTOCHEMICAL STUDY OF P53, PCNA AND AFP IN HEPATOCELLULAR CARCINOMA, A COMPARISON BETWEEN INDONESIAN AND JAPANESE CASES

Wirasmi MARWOTO[1], Upik Anderiani MISKAD[2], Nuryati Chaerani SIREGAR[1], Rino A. GANI[3], Unggul BOEDIHUSODO[3], Siti NURDJANAH[4], SUWARSO[5], WATADIANTO[6], Poernomo BOEDI[7], H.A. HASAN[7], Nurul AKBAR[3], H.M. Sjaifoellah NOER[3], and Yoshitake HAYASHI[2]

[1]Department of Anatomical Pathology, Faculty of Medicine, Indonesia University, Jakarta, Indonesia

[2]First Division, Department of Pathology, Kobe University School of Medicine

[3]Department of Internal Medicine, Faculty of Medicine, Indonesia University, Jakarta, Indonesia

[4]Department of Internal Medicine, and [5]Department of Clinical Pathology, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia

[6]Department of Anatomical Pathology, and [7]Department of Internal Medicine, Faculty of Medicine, Airlangga University, Surabaya, Indonesia

KEY WORDS

p53; PCNA; AFP; immunostain; hepatocellular carcinoma; Indonesia; Japan

Received for publication: August 21, 2000

ABSTRACT

Mutations of p53 as a tumor suppressor gene in hepatocellular carcinoma (HCC) have been reported to occur with varying frequency in different geographic regions, which might be different etiology for HCC. Overexpressions of p53 (well known for its implications in mutations of the p53 gene), PCNA and α-fetoprotein (AFP) have been reported to be associated with carcinogenesis and/or tumor progression and poor prognosis in various types of cancer. To estimate the geographical difference of the p53 gene, PCNA and AFP in HCC, we examined 14 Japanese HCC cases, 8 Indonesian HCC cases, and 27 Indonesian chronic active hepatitis (CAH) or liver cirrhosis cases, using immunohistochemical approaches. Overexpression of p53 was identified in 37.5% of Japanese HCC, in 62.5% of Indonesian HCC and none in CAH. The mean PCNA Labeling Index of Japanese HCC, Indonesian HCC and CAH was detected in 48.6%, 30.4%, and 43.5%, respectively. AFP was detected in 35.7% of Japanese and 25 % of Indonesian HCC. The rate of p53 overexpression in Indonesian HCC was as high as in HCC of southern part of China, which might share the similar etiology in both regions.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common human malignancies, especially in Asia including Indonesia and Japan. Chronic hepatitis and regenerative changes in the liver, such as can be induced by hepatitis B or C virus infections, aflatoxin, and alcoholic abuse, appear to be the important risk factors for HCC.[18, 20]

There is evidence that carcinogenesis is a multistage process driven by carcinogen induced genetic damage with resulting activation of protooncogenes and/or inactivation of tumor suppressor genes. The p53 tumor suppressor gene has been demonstrated to be the most commonly mutated gene in human neoplasia and the prevalence of p53 mutations is variable, and seems to depend on geographic regions.[17] Mutations of p53 have been reported in 50% of patients with HCC from China and South Africa. These reports suggested an association of p53 mutations with high levels

of aflatoxin in the diet.[11, 3] In Japan exposure to aflatoxin B1 is not considered to be remarkable,[10, 17] but in Indonesia the report of dietary exposure to aflatoxin B1 is prevalent.[5, 23] From these reasons, we assumed that p53 overexpression in Indonesian HCC cases is higher than in Japanese HCC cases. PCNA Labeling Index as a proliferative index has been reported to indicate proliferative activity. This activity has a close correlation with both the histological types and the biological behavior of HCC.[15] The α-fetoprotein (AFP) as a tumor marker of HCC has been also examined in the previous study. But there is no published report on Indonesian cases yet.

We have examined the relationship between histological grades of HCC and their positivity of p53, PCNA and AFP through immunohistochemical staining of HCC and chronic active hepatitis (CAH) and investigated the difference between Japanese and Indonesian cases.

MATERIALS AND METHODS

Samples were obtained from Department of Pathology, Indonesia University, Indonesia (HCC: 8 cases and CAH: 27 cases) and Kobe University, Japan (HCC: 14 cases). The 8 HCC cases consisted of 1 well, 5 moderately and 2 poorly differentiated HCC. Fourteen Japanese cases encompassed 4 well, 5 moderately and 5 poorly differentiated HCC. Those tissues were routinely fixed with 10% buffered formalin solution and embedded in paraffin. Four-μm-thick sections cut from the blocks were stained immunohistochemically for detecting tumor suppressor gene (p53), proliferative activity index (PCNA) and tumor marker for HCC (AFP).

In this study, the histological degree of HCC was defined according to Edmondson's grade as follows: well differentiated, corresponding to grade I or I-II; moderately differentiated, corresponding to grade II or II-III; and poorly differentiated, corresponding to grade III or IV.[7] The histological changes of chronic hepatitis or liver cirrhosis were evaluated according to the modified HAI scoring system by Ishak et al. In staging, architectural changes, fibrosis and cirrhosis were calculated as 0 for no fibrosis; 1 for fibrous expansion of some portal areas with or without short fibrous septa; 2 for fibrous expansion of most portal areas with or without short fibrous septa; 3 for fibrous expansion of most portal areas with occasional portal to portal (P-P)

bridging; 4 for fibrous expansion of portal areas with marked portal bridging (portal to portal (P-P) as well as portal to central (P-C)); 5 for marked bridging (P-P and/or P-C) with occasional nodules (incomplete cirrhosis); and 6 for cirrhosis, probable or definite. In grading, necroinflammatory scores were calculated as the sum of the scores of periportal or periseptal interface hepatitis (piecemeal necrosis, 0-4), confluent necrosis (0-6), focal (spotty) lytic necrosis, apoptosis and focal inflammation (0-4), and portal inflammation (0-4).[13]

Immunohistochemistry was performed using the catalyzed signal amplification system (Dako, Carpinteria, Ca, USA). Paraffin sections were deparaffinized in xylene and rehydrated in a series of graded ethanol. Prior to p53 and PCNA staining, tissue sections were boiled three times for 5 minutes in 10 mM citrate buffer (pH 6.0) in a microwave oven for antigen retrieval. Meanwhile, tissue sections for AFP staining were treated with 0.05 % trypsin solution for 10 minutes at 37°C. After quenching the endogenous peroxidase activity in a solution of 0.1% sodium azide and 3% hydrogen peroxidase in methanol for 15 minutes, non-specific binding was blocked by the treatment with 1.5% rabbit serum for 20 minutes. The primary antibodies (anti-p53 (DO-7, monoclonal antibody, Dako, Denmark) at a 1:50 dilution, anti-AFP (alpha-1-fetoprotein, polyclonal, Immunon, USA) at a 1:50 dilution and anti-PCNA (PC 10, monoclonal antibody, Dako, Denmark) at a 1:50 dilution) were applied to the sections incubated overnight at 4°C in a moist chamber. After washing in 0.05 M Tris HCl buffer (pH 7.4), the sections were then incubated with biotinylated rabbit anti-mouse immunoglobulins (Dako) for 30 minutes at room temperature, followed by a series of additional incubations with streptavidin-biotin complex (Dako) for 20 minutes with washing in Tris buffer between each incubation. Peroxidase activity in each sections was then demonstrated by application color development containing 0.03% diaminobenzidine and 0.01% H_2O_2. Mayer's hematoxylin or methyl green was used as a counterstain. For assessment of the positivity of p53 and PCNA, only nuclear staining was regarded as positive, but the positivity of AFP was evaluated with cytoplasmic staining. The percentage of PCNA Labeling Index in each section exhibiting positive immunostaining was estimated by counting 200 cells. Those HCC and CAH or liver cirrhosis sections that contained more than 10% of cells with positive immunostaing were categorized as being positive. Histological slides were viewed by light microscopy, and

staining intensity was graded on a scale of - to ++.

RESULTS

p53 overexpression was detected in 5 of 14 Japanese HCC cases (37.5%), 5 of 8 Indonesian HCC cases (62.5%), and none positive in CAH or liver cirrhosis cases. Percentage of p53 overexpression in the Indonesian cases was higher than that in the Japanese cases, but the difference was not significant. The distribution of the p53-positive results in Japanese cases was as follows: Edmondson I (4 HCC), none positive; Edmondson II (5 HCC), 1 positive cases (20%); and Edmondson III (5 HCC), 4 positive cases (80%). This study showed the proportion of HCC with overexpression of p53 protein in Japanese cases to be significantly higher in cases with histologically poorly than in the well or moderately differentiated HCC. The distribution of the results in Indonesian HCC was follows: Edmondson I (1 HCC), none positive; Edmondson II (5 HCC), 3 positive cases (60%); and Edmondson III (2 HCC), 2 positive cases (100%).

PCNA Labeling Index was determined without regard for staining intensity, and therefore detected almost all proliferating cells. PCNA positivity was found in the nuclei of all cases of Japanese HCC, Indonesian HCC and Indonesian CAH or liver cirrhosis. The mean PCNA Labeling Index of Japanese HCC cases was 48.6% (range, 15-82 %) in 14 cases. The mean PCNA Labeling Index of Indonesian HCC cases was 30.4% (range, 3-92.5%) in 8 positive cases, and 43.51% (range, 6-83%) in 27 CAH or liver cirrhosis cases. There was no significant difference in the index between tumor and non-tumor cases. AFP was detected in cytoplasm in 5 of the 14 Japanese HCC cases (35.7%), 2 of the Indonesian HCC cases (25%), and none in CAH or liver cirrhosis.

We also examined histological grading and staging in chronic active hepatitis by using modified HAI staging and modified grading scores. From the 27 cases of CAH or liver cirrhosis, all cases were stained positively for PCNA and the mean of PCNA Labeling Index was 43.5%. In this case, p53 and AFP were positive in cancer cells, but negative in non-cancer hepatocytes.

Table I. Immunoreactivity for p53 and AFP in 14 cases of Japanese HCC and 8 cases of Indonesian HCC.

Regions	HCC Edmondson grade	No.of cases	No.of p53-positive cases	% of p53-positive cases	No. of AFP-positive cases	% of AFP-positive cases
Japan	grade I	4	0	0	0	0
	grade II	5	1	20	0	0
	grade III	5	4	80	5	100
	Total	14	5	37.5	5	37.5
Indonesia	grade I	1	0	0	0	0
	grade II	5	3	60	1	20
	grade III	2	2	100	1	50
	Total	8	5	62.5	2	25

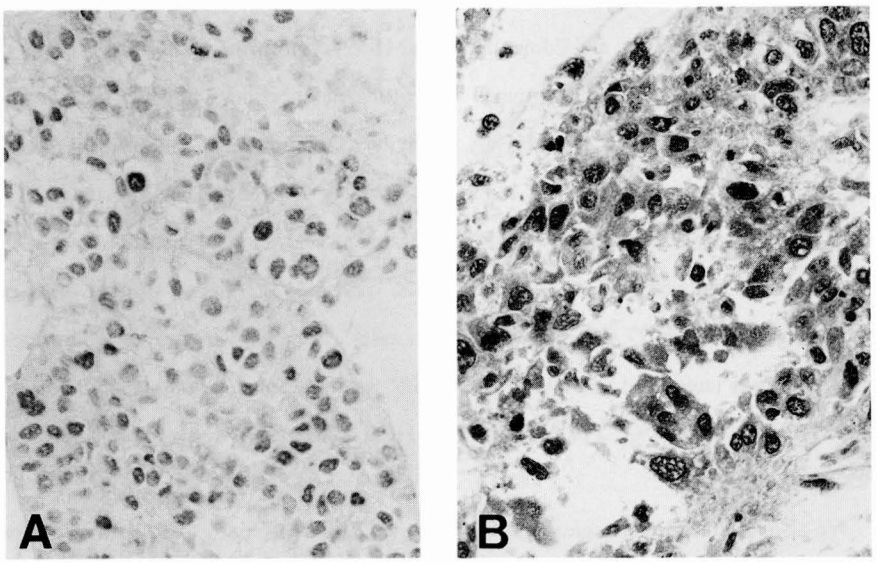

Figure 1. Immunohistochemistry of p53 protein and AFP protein in the poorly differentiated HCC. (A) Positive immunostaining for overexpressed p53 protein was seen in the nuclei of the tumor cells (magnification x40 without counterstain). (B) Positive immunostaining for AFP was seen in the cytoplasm of the tumor cells (magnification x40 counterstained with hematoxylin).

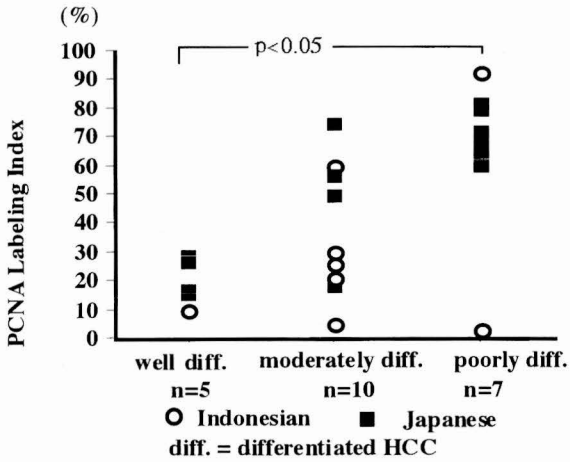

Figure 2. The relationship between PCNA Labeling Index and the histological type of HCC. The labeling index of well differentiated HCC was significantly lower ($p<0.05$) than that of poorly differentiated HCC.

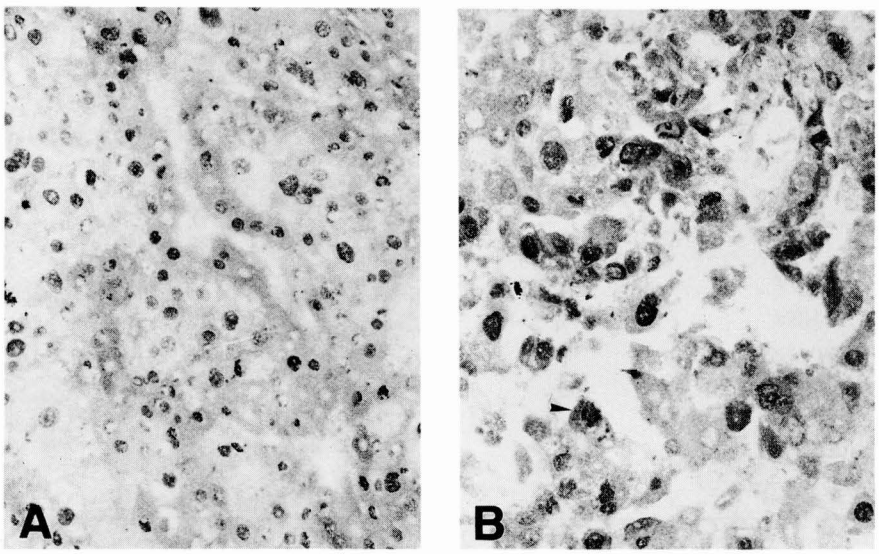

Figure 3. Immunohistochemistry for PCNA as a proliferative index. (A) Well differentiated HCC showed positive for PCNA with the nuclear staining. (B) Poorly differentiated HCC showed positive for PCNA with nuclear and cytoplasmic staining (arrowhead). Magnification (A-B) x40 without counterstain.

Table II. Immunoreactivity for p53, AFP and PCNA Labeling Index in 27 cases of chronic active hepatitis or liver cirrhosis (CAH-LC).

no	Viral infection	Grading	Staging	P53	PCNA	PCNA labeling index	AFP
1	HBV	13	3	-	+	33.5%	-
2	HCV	14	4	-	+	54%	-
3	HCV	12	3	-	+	65%	-
4	-	9	2	-	++	80%	-
5	-	5	1	-	+	33.5%	-
6	HBV	3	1	-	+	29%	-
7	HCV	11	3	-	+	80%	-
8	HBV	8	3	-	+	48.5%	-
9	HBV	6	1	-	+	11.5%	-
10	HCV	13	4	-	+	72%	-
11	-	3	1	-	+	83%	-
12	-	13	4	-	+	47%	-
13	-	7	3	-	+	7.5%	-
14	-	7	1	-	+	10%	-
15	HBV	8	2	-	+	6%	-
16	-	4	1	-	+	68.5%	-
17	-	10	4	-	+	20%	-
18	HBV	6	1	-	+	30%	-
19	HBV	3	1	-	+	33.5%	-
20	HCV	9	3	-	+	44%	-
21	HBV	6	1	-	+	17%	-
22	HBV	7	3	-	+	65%	-
23	HBV,HCV	1	0	-	+	72%	-
24	-	5	1	-	+	46.5%	-
25	HBV	5	0	-	+	13.5%	-
26	-	10	3	-	+	69%	-
27	-	5	0	-	+	35.5%	-
				0%	100%	Mean:43.5%	0%

Modified HAI grading. Activity: Minimal (0-3), Mild (4-8), Moderate(9-12), Severe(13-18). Modified staging. Fibrosis: Absent (0), Mild(1-2), Moderate(3), Severe(4-5), Cirrhosis(6).

DISCUSSION

Hepatocellular carcinoma is one of the most frequently reported malignancies. Depending on the difference in risk factors, the prevalence of HCC shows variations of geographical regions.[21] Many investigators consider that hepatitis B or C viral infection and aflatoxin B1 play an important role in hepatocarcinogenesis,[19] but the precise mechanism is still unclear. The most common cancer related genetic change in tumors is the mutation of p53.[19] The rate of p53 mutation in HCC is variable, and depending on geographic and environmental conditions. Studies from Japan, England, Italy, Germany, and United States similarly demonstrated low percentages of HCC with p53 mutations (37%, 20%, 15%, 13%, and 11% respectively),[17, 18, 4, 16, 9] and showed that the mutation site was not codon 249, whereas the occurrence of p53 mutations in codon 249 was found in 50% of HCC patients from China and sub-Saharan Africa where aflatoxin dietary contamination and HBV infection are very prevalent.[11, 13] The highest frequency of mutations at codon 249 of p53 gene in the literature is 67% in an HCC study in Senegal.[6] Aflatoxin has been regarded as the main factor involved in this mutation, since experimental work has shown that it induces specific mutations (G to T transversions) at the third base in codon 249.[11, 22]

In this study we have shown the overexpression of p53 protein in 5 out of 14 Japanese HCC cases (35.7%), consistent with previous reports. In Japan exposure to aflatoxin B1 seems less remarkable, and the mutation of codon 249 in p53 gene, linked with aflatoxin B1, is not specific in Japanese HCC cases.[10, 17] In contrast, the expression of p53 protein was detected in 5 out of 8 Indonesian HCC cases (62.5%), although not yet proven, suggesting that this is related to high contamination of aflatoxin in Indonesian foods.[5, 23] The rate of p53 overexpression in Indonesian HCC is as high as in that of HCC in southern-China, which might share the similar etiology in both regions.

Although not significant, the percentages of p53 positive cases in Indonesian HCC is higher than in Japanese HCC cases. It has been reported that Indonesia including a high incidence area for HCC, where exposure to HBV, HCV and aflatoxin B1 is prevalent.[2, 5, 23] This result supports the concept of geographical variations in the cause and pathogenesis of HCC. Furthermore, this study showed the rate of p53 overexpression was significantly higher in poorly than in well or moderately

differentiated HCC cases. These results and the absence of detectable p53 protein in well differentiated HCC and non cancer cases (CAH) suggest that p53 mutation might occur after the initial stage of hepatocellular carcinogenesis, and as a relatively late event and might play an important role in the progression of HCC.

PCNA Labeling Index was examined to assess the proliferative activity which will provide useful information about clinical features and prognosis. In this study the PCNA Labeling Index of well differentiated HCC was significantly lower than that of moderately or poorly differentiated HCC, indicating high proliferative activity which correlates with histologic feature of aggressiveness. PCNA positivity in the cytoplasm was found in the 4 cases with poorly differentiated HCC, predicting poor prognosis as previously reported.[1)]

Kawakita et al. examined the proliferating activity in various liver diseases using a monoclonal antibody against PCNA. They described that hepatocytes in acute hepatitis had a higher proliferating activity than hepatoma cells.[14)] In this study, all hepatocytes in non-cancer cases (CAH) proliferated, but there was no difference in the proliferating index between cancers and non-cancer cases.

AFP expression in cases of HCC was dependent on the histological grade. In Japanese cases, the ratio of AFP expression was significantly higher in poorly compared to well or moderately differentiated HCC.

An immunohistochemical study was performed in the non-cancer cases to assess overexpression of p53, PCNA Labeling Index and AFP at premalignant status. The correlation between mutant p53 protein and HbxAg has been reported and suggested that the p53 gene mutation involved in the mechanism of hepatocarcinogenesis caused by HBV.[8)] In this decade, hepatitis C viral infection is considered to play an important role in hepatocarcinogenesis, and the core protein of HCV is thought to repress the promoters of p53. Moreover, chronic viral infection might influence the release of active oxygene from inflammatory leukocytes, which might induce p53 mutation in codon 249.[12)] We failed to find an association between HBV and HCV in premalignant status with p53 overexpression. These results suggest that p53 mutations probably occur after the initial stage of HCC.

In conclusion, overexpressed p53 protein was found to correlate with histological grade, and

with AFP expression and PCNA Labeling Index. The percentage of p53 overexpression in Indonesia was higher than in Japan, and in accordance with previous epidemiological studies and the report of dietary exposure to aflatoxin B1, overexpression of p53 in Indonesia might be caused by aflatoxin as a similar etiology with southern part of China. To support this hypothesis, a further study will be required on the effect of aflatoxin B1 on codon 249 of p53 gene in HCC cases in Indonesia.

REFERENCES

1. Adachi, E., Hashimoto, H., and Tsuneyoshi, M.: Cancer. 1993. 72. 2902-2909. Proliferating cell nuclear antigen in hepatocellular carcinoma and small cell liver dysplasia.
2. Armstrong, B. and Joske, R.A.: Natl. Cancer Inst. Monogr. 1979. 53. 127-132. Incidence and etiology of primary liver cancer in Pacific Basin.
3. Bressac, B., Michael, K., Wands, J., and Ozturk, M.: Nature. 1991. 350. 429-431. Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa.
4. Caruso, M.L. and Valentini, A.M.: Anticancer Res. 1999. 19. 3853-3856. Overexpression of p53 in a large series of patients with hepatocellular carcinoma: a clinicopathological correlation.
5. Chao, T.C., Lo, D.S., Bloodworth, B.C., Gunaseragam, R., Koh, T.H., and Ng, H.S.: Med. Sci. Law. 1994. 34. 289-298. Aflatoxin exposure in Singapore: blood aflatoxin levels in normal subjects, hepatitis B virus carriers and primary hepatocellular carcinoma patients.
6. Coursaget, P., Depril, N., Chabaud, M., Nandi, R., Mayelo, V., Leeann, P., and Yvonnet, B.: Br. J. Cancer. 1993. 67. 1395-1397. High prevalence of mutations at codon 249 of p53 gene in hepatocellular carcinomas from Senegal.
7. Edmondson, H.A. and Steiner, P.E.: Cancer. 1954. 7. 462-503. Primary carcinoma of the liver: a study of 100 cases among 48900 necropsies.
8. Feitelson, M.A.: J. Hepatobiliary. Pancreat. Surgery. 1998. 5(4). 367-374. Hepatitis Bx antigen and p53 in the development of hepatocellular carcinoma.

9. Goldblum, J.R., Bartos, R.E., Carr, K.A., and Frank, T.S.: Am. J. Surg. Pathol. 1993. 17. 1244-51. Hepatitis B and alterations of p53 tumor suppressor gene in hepatocellular carcinoma.
10. Hayashi, H., Sugio, K., Matsumata, T., Adachi, E., Urata, K., Tanaka, S., and Sugimachi, K.: Liver. 1993. 13. 279-281. The mutation of codon 249 in the p53 gene is not specific in Japanese hepatocellular carcinoma.
11. Hsu, I.C., Metcalf, R.A., Sun, T., Welsh, J.A., Wang, N.J., and Harris, C.C.: Nature. 1991. 350. 427-428. Mutational hotspot in the p53 gene in human hepatocellular carcinoma.
12. Hussain, S.P., Anguilar, F., Amstab, P., and Cerruti, P.: Oncogene. 1994. 9. 2277-2281. Oxy-radical induced mutagenesis of hotspot codons 248 and 249 of the human p53 gene.
13. Ishak, K., Baptista, A., Bianchi, L., Callea, F., Groote, J.D., et al.: J. Hepatol. 1995. 22. 696-699. Histological grading and staging of chronic hepatitis.
14. Kawakita, N., Seki, S., Sakaguchi, H., Kuroki, T., Mizoguchi, Y., Kobayashi, K., et al.: AM. J. Pathol. 1992. 140. 513-520. Analysis of proliferating hepatocytes using a monoclonal antibody against proliferating cell nuclear antigen/cyclin in embedded tissue from various liver diseases fixed in formaldehyde.
15. Kitamoto, M., Nakanishi, T., Kira, S., et al.: Cancer. 1993; 72: 1859-1865. The assessment of proliferating cell nuclear antigen immunohistochemical staining in small hepatocellular carcinoma and its relationship to histologic characteristics and prognosis.
16. Kress, S., Jahan, U.R., Buchmann, A., et al.: Cancer Res. 1992. 52. 3220-3223. p53 mutations in human hepatocellular carcinomas from Germany.
17. Murakami, Y., Hayashi, K., Hirohashi, S., and Sekiya, T.: Cancer Res. 1991. 51. 5520-5525. Aberrations of tumor suppressor p53 and retinoblastoma genes in human hepatocellular carcinoma.
18. Ojanguren, I., Castella, E., Llatjos, M., Ariza, A., and Palacios, J.N.: Acta Cytologica. 1996. 40. 1148-1153. p53 Immunoreaction in hepatocellular carcinoma and its relationship to etiologic factors.
19. Sherman, M.: Gastroenterologist. 1995. 3. 55-66. Hepatocellular carcinoma.

20. Sheu, J.C., Huang, G.T., Lee, P.H., et al.: Cancer Res. 1992. 52. 6098-6100. Mutation of p53 gene in hepatocellular carcinoma in Taiwan.

21. Simonetti, R.G., Camma, C., Fiorello, F., Politi, F., D'amico, G., and Pagliaro, L.: Dig. Dis. Sci. 1991. 36. 962-972. Hepatocellular carcinoma: a worldwide problem and the major risk factors.

22. Sohn, S., Jaitovitch, G., Benlimame, N., Galipeaue, J., Batist, G., and Alaoui, J.: Mutation Research DNA Repair. 2000. 460(1). 17-28. Retroviral expression of the hepatitis B virus x gene promotes liver cell susceptibility to carcinogen-induced site specific mutagenesis.

23. Widyastuti, R., Maryam, R., Blaney, B.J., Salfina, and Stoltz, D.R.: Mycopathologia. 1988. 102(1). 45-9. Corn as a source mycotoxins in Indonesian poultry feeds and the effectiveness of visual examination method for detecting contamination.