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Mutation of tumor suppressor gene p53 in hepatocellular carcinomas from Korea

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Abbreviations: HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HCV, hepatitis C virus; SSCP, single strand conformation polymorphism

Abstract

Mutation of the p53 gene in hepatocellular carcinoma has been recognized as one of the most important genetic alterations to occur during hepatocarcinogenesis. This study was performed to analyze the frequency and nature of p53 mutations in advanced hepatocellular carcinomas from Korea. Tissue samples were obtained by laparoscopic biopsy from 35 patients; adjacent nontumorous liver tissue was also obtained from 24 of them. Mutations of the p53 gene were identified in 11/35 (31%) of hepatocellular carcinomas. These included 7 missense mutations and 4 deletion mutations. Only one mutation was detected at codon 249, a "hot spot" at which mutations have been found frequently in hepatocellular carcinomas from some geographic areas; however, this was an A-to-T transversion at the first nucleotide, thus differing from commonly reported G-to-T transversion at the third nucleotide of codon 249 in hepatocellular carcinomas. Patients whose serum alkaline phosphatase levels were higher than the mean value were more likely to have p53 mutations, compared to patients whose alkaline phosphatase levels were lower than the mean value [55% (6/11) *vs*. 21% (5/24)] ($p<0.05$). Thus, p53 mutations are found in many hepatocellular carcinomas in Korea. However, mutations commonly thought to be due to aflatoxin B_1 (G-to-T transversion at codon 249) were not found, suggesting that aflatoxin-B_1 does not play an important role in the etiology of hepatocellular carcinoma in Korea.

Keywords: reverse transcription-polymerase chain reaction, polymerase chain reaction-single strand conformation polymorphism, p53, tumor suppressor gene, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma is one of the most common cancers in the world. Chronic infection with hepatitis B virus and hepatitis C virus are important etiologic factors for the development of hepatocellular carcinoma, but it is also possible that other factors such as chemical carcinogens may contribute to carcinogenesis. The tumor suppressor gene p53 has been found to be commonly mutated in various human tumors including hepatocellular carcinoma (Nigro *et al*., 1989; Levine *et al*., 1991; Hollstein *et al*., 1991; Harris and Hollstein, 1993). A mutation at p53 codon 249 with a G-to-T transversion at the third nucleotide has been found frequently in hepatocellular carcinomas from southern Africa and Qidong, China, areas in which dietary aflatoxin B_1 may play an important role in the etiology of hepatocellular carcinoma (Hsu *et al*., 1991; Bressac *et al*., 1991; Ozturk *et al*., 1991). However, hepatocellular carcinomas from geographic areas where aflatoxin is uncommon in the diet do not have such a mutational "hot spot" for p53 (Ozturk *et al*., 1991; Unsal *et al.*, 1994). These observations indicate that the molecular mechanisms of p53 gene damage or dysfunction in hepatocarcinogenesis may be heterogeneous. In the present study, exons 3 to 9 of the p53 gene were investigated to determine the frequency, nature, and significance of mutations in these genes in advanced hepatocellular carcinomas from Korea.

Materials and Methods

Liver tissues

Hepatocellular carcinoma tissues were collected from 35 patients with advanced hepatocellular carcinoma (TNM stage III or IV), who were seen at the Department of Internal Medicine, St. Mary's Hospital, Catholic

University Medical College, Seoul, Korea, between 1990 and 1993. Laparoscopic biopsy was performed on all 35 patients; hepatocellular carcinoma was available from 35 and adjacent nontumorous liver was available from 24.

Patients ages were 18 to 74 years (median, 52 years); 31 patients were male, 4 patients were female. Twenty-six patients (74%) had hepatitis B surface antigen (HBsAg) in serum, including 13 hepatitis B e antigen (HBeAg)-positive and 13 HBeAg-negative patients. Four patients (11%) had antibody to hepatitis C virus (anti-HCV) and no detectable HBsAg. One other patient was positive for both HBsAg and anti-HCV and 4 patients were negative for both. As determined by visual inspection at laparoscopy, 23 patients (66%) had cirrhosis, 8 patients (23%) had chronic active hepatitis and 4 patients (11%) had normal liver surface. As determined by computerized tomography, 6 patients had hepatocellular carcinoma with TNM stage III and 29 patients had TNM stage IVa. Histologic grade based on the Edmondson-Steiner classification was available for 33 tumors; 3 were grade I (9%), 15 were grade II (46%), and 15 were grade III (46%). Tissues obtained at laparoscopy were snap-frozen and stored in liquid nitrogen until use.

Serological markers

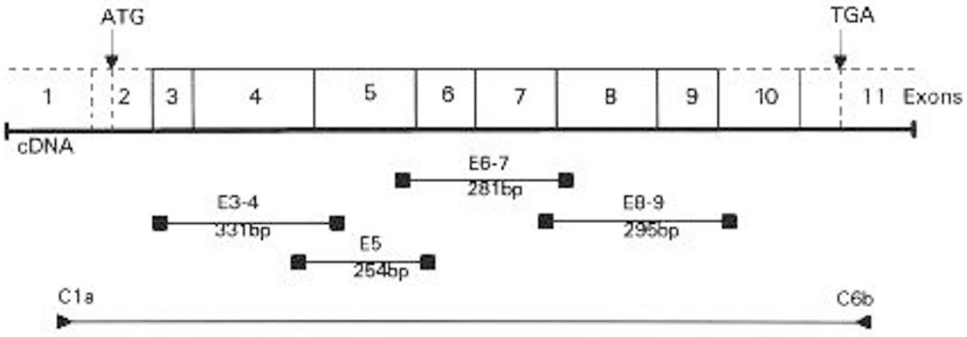
HBsAg, antibody to HBsAg (anti-HBs), HBeAg, antibody to HBeAg (anti-HBe), and total antibody to hepatitis core antigen (anti-HBc) were assayed using commercially available radioimmunoassay kits (Abbott Laboratories, Chicago, IL). Anti-HCV was assayed with a second generation enzyme immunoassay kit (Abbott Laboratories).

Extraction of total RNA and reverse transcription

Total RNA was prepared from hepatocellular carcinoma and liver tissue by the acid guanidinium thiocyanate-phenol-chloroform method (Chomçzinski and Sacchi, 1987). Each RNA sample (2 μg) was reverse-transcribed with 10 pmol of an antisense primer (5'-CTGACGCACACCTATTGCAA) derived from the exon 11 of the p53 gene, and 200 units of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD) at 37°C for 60 min in a total reaction volume of 10 μl.

PCR-single strand conformation polymorphism (PCR- SSCP)

The sense and antisense primers used for amplifying exons 3 to 9 were made with published primer sequences (Nagai *et al*., 1991) (Figure 1). A DNA segment extending from the distal part of exon 1 to the proximal part of exon 11 was amplified in a 50 μl reaction volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM each of the four deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), 1 μM each of outer sense primer (5'-TCCACGACGGTGACACGCTT) and outer antisense



E3-4a	sense	5'-ACTTCCTGAAAACAACGTTC
E3-4b	anti-sense	5'-GCAAAACATCTTGTTGAGGG
E5a	sense	5'-TTGCATTCTGGGACAGCCAA
E5b	anti-sense	5'-CCTTCCACTCGGATAAGATG
E6-7a	sense	5'-ACCATGAGCGCTGCTCAGAT
E6-7b	anti-sense	5'-TCAAAGCTGTTCCGTCCCAG
E8-9a	sense	5'-CACCATCATCACACTGGAAG
E8-9b	anti-sense	5'-GAGTTCCAAGGCCTCATTCA
C1a	sense	5'-TCCACGACGGTGACACGCTT
C6b	anti-sense	5'-CTGACGCACACCTATTGCAA

Figure 1. A strategy of reverse transcription-PCR and single-strand conformation polymorphism analysis. Reverse transcription reaction was performed with the C6b primer. The first stage of PCR was performed with the C1a and C1b primers. The second stage of PCR was performed with the radiolabelled inner primers: the primer sets cover exons 3 and 4 (E3-4), exon 5 (E5), exons 6 and 7 (E6-7), and exons 8 and 9 (E8-9), respectively.

primer (5'-CTGACGCACACCTATTGCAA), 0.5 units of *Taq* polymerase, and 10 μl of cDNA. PCR was processed for 15 cycles (denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, and polymerization at 72°C for 2 min).

For the SSCP, PCR was carried out with the SSCP primer sets (Figure 1), of which the 5'-terminus was labeled with [γ-^{33}P]ATP (Amersham, Arlington Heights, IL) using T4 polynucleotide kinase (Promega, Madison, WI). The reaction volume of the PCR was 5 μl, containing 125 μM each of the dNTPs, 0.1 μM each of radiolabeled sense and antisense SSCP primers, 0.5 units of *Taq* polymerase, and 1 μl of the first PCR products as described above. PCR was performed for 30 cycles (denaturation at 95°C for 30 sec, primer annealing at 55°C for 30 sec, and polymerization at 72°C for 1 min). Each PCR product was diluted 10 fold with distilled water and 1 μl each of the PCR diluents was mixed with 3 μl of gel loading dye containing 95% formamide, 20 mM EDTA, 0.05% xylene cyanol FF, and 0.05% bromophenol blue. The sample heated at 95°C for 3 min, immediately cooled on ice, and loaded on 5% polyacrylamide gel (acrylamide:N,N-methylene-bis-acrylamide = 49:1) with or without 10% glycerol. The electrophoresis buffer contained 45 mM Tris-borate (pH 8.3) and 1 mM EDTA. The gel (40 cm x 40 cm x 0.04 cm in size) was run at 10 watts overnight with the temperature maintained at 23-24°C. In some cases, an additional minigel (10 cm x 10 cm x 0.7 cm in size) with 10% polyacrylamide gel (49:1) was run at 200-300 volts for 3 h with the temperature maintained at 10°C with cold-water circulation. After electrophoresis, the gels were exposed to x-ray film (XOMAT-AR5) with an intensifying screen at -70°C overnight.

DNA Sequencing

Sequencing was performed according to the Sanger dideoxy chain termination method using a direct sequencing kit ("fmol", Promega). Each SSCP primer, which was end-labeled with [γ-^{33}P]ATP (Amersham) as described above, was used as a sequencing primer. The first PCR products of the samples with abnormal SSCP bands were used as sequencing templates. Sequencing products were electrophoresed with 6% polyacrylamide/7 M urea gel, dried at 80°C for 1 h with a gel drier, and autoradiographed at -70°C overnight.

Statistical Analysis

Data was analyzed using unpaired *t*-test, chi-square test, and Mann-Whitney U test.

Results

Abnormalities of the p53 gene were identified in 11 of 35 hepatocellular carcinomas (31%), including 7 missense mutations in exons 5, 6, 7, and 8, 3 short deletions (15-, 18- and 63-bp each) in parts of exons 4 and 8, and 1 large deletion including exons 2 to 6 (Table 1, 2).

Of the 7 missense mutations, 4 were transitions and 3 were transversions. One of the missense mutations was in codon 249 with an A-to-T transversion (AGG to TGG; Arg to Trp). The only G-to-T transversion was in codon 176 (TGG to TTC, Cys to Phe) (Table 1 and Figure 2).

Seven of the 26 hepatocellular carcinomas (27%) from patients with serum HBsAg and no detectable anti-HCV had p53 mutations. Mutations or deletions

Table 1. Mutations of the p53 gene in hepatocellular carcinoma

Case	HBsAg	Anti-HCV	Exon	Codon	Nucleotide Change		Amino Acid change
K6	+	–	5	179	Transversion	CAT → GAT	His → Asp
K22	+	–	6	215	Transition	AGT → GGT	Ser → Gly
K23	+	–	6	220	Transition	TAT → TGT	Tyr → Cys
K26	+	–	7	249	Transversion	AGG → TGG	Arg → Trp
K5	+	–	8	278	Transition	CCT → TCT	Pro → Ser
K16	+	–	8	277-281	15-base deletion without frameshift[a]		
K15	+	–	2-6		Deletion of 5 exons (2 to 6)		
K32	–	+	7	258	Transition	GAA → GGA	Glu → Gly
K35	+	+	4	44-50	18-base deletion without frameshift[b]		
K37[d]	–	–	4	105-126	63-base deletion without frameshift[c]		
K40	–	–	5	176	Transversion	TGC → TTC	Cys → Phe

[a] GCC TGT CCT GGG AGA

[b] G ATG CTG TCC CCG GAC GA

[c] G GGC AGC TAC GGT TTC CGT CTG GGC TTC TTG CAT TCT GGG ACA GCC AAG TCT GTG ACT TGC AC

[d] HBV-DNA-positive by PCR

Table 2. Mutation patterns of the p53 gene and hepatitis virus infection

Mutations	No. of Cases (n=35)	Hepatitis Virus Markers			
		HBsAg (+) / anti-HCV(-) (n=26)	HBsAg(-) / anti-HCV (+) (n=4)	HBsAg (+) / anti-HCV (+) (n=1)	HBsAg(-) / anti-HCV (-) (n=4)
Transition					
A to G	3 (9%)	2	1	0	0
C to T	1 (3%)	1	0	0	0
Transversion					
C to G	1 (3%)	1	0	0	0
A to T	1 (3%)	1	0	0	0
G to T	1 (3%)	0	0	0	1
Deletion					
In one exon	3 (9%)	1	0	1	1[b]
Multiple exons	1 (3%)[a]	1	0	0	0
Total Number	11 (31%)	7 (27%)	1 (25%)	1 (100%)	2 (50%)

[a] Deletion of 5 exons (2 to 6)

[b] HBV-DNA-positive by PCR

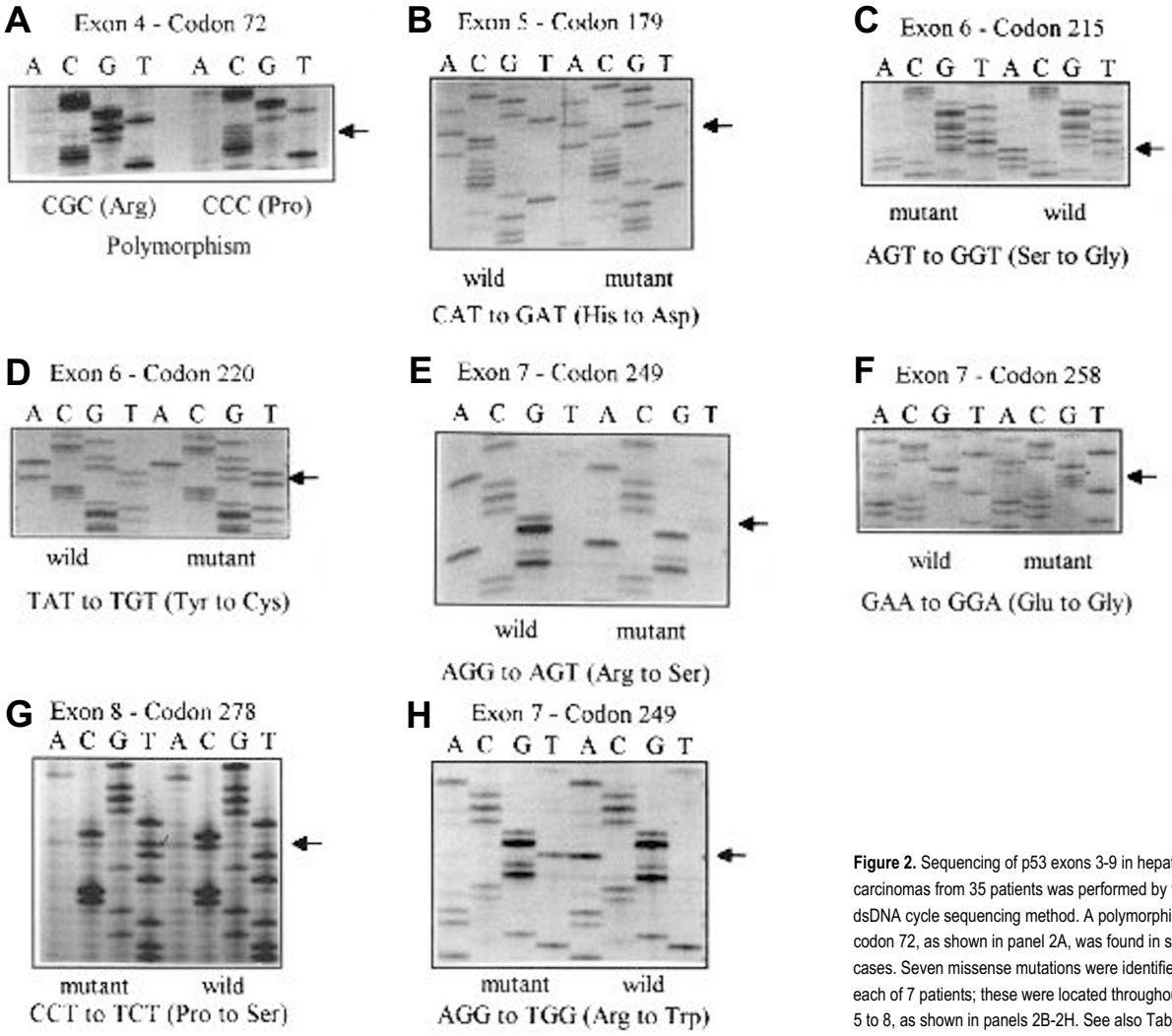


Figure 2. Sequencing of p53 exons 3-9 in hepatocellular carcinomas from 35 patients was performed by the dsDNA cycle sequencing method. A polymorphism at codon 72, as shown in panel 2A, was found in several cases. Seven missense mutations were identified in each of 7 patients; these were located throughout exons 5 to 8, as shown in panels 2B-2H. See also Table 1.

were found in one of 4 hepatocellular carcinomas from patients with anti-HCV and no detectable HBsAg, one of one with both HBsAg and anti-HCV, and two of four negative for both viral markers (Table 2).

None of the 24 samples of non-tumorous liver tissues adjacent to hepatocellular carcinomas had p53 mutations. Tumor size, portal vein thrombosis, and differentiation grade of the hepatocellular carcinomas did not show a significant relationship with p53 mutations. Among the laboratory parameters, only the serum alkaline phosphatase level was correlated with the p53 mutations; p53 mutations were found more often in those patients with serum alkaline phosphatase level higher than the mean value, compared to patients with alkaline phosphatase level lower than the mean value (55% [6/11] vs 21% [5/24], p=0.046) (Table 3, 4 and 5).

Discussion

Mutation in the p53 tumor suppressor gene may be an important event in the transformation of hepatocytes (Bressac *et al*., 1990). p53 mutations are common in hepatocellular carcinomas throughout Asia. In Chinese patients, including those from Hong Kong and Taiwan, where hepatitis B virus is closely associated with hepatocellular carcinoma, p53 mutations have been

Table 3. p53 mutations and characteristics of the hepatitis B virus infection

Characteristics of the HBV infection		No. analyzed	p53 mutations
HBeAg[a]	Positive	13	4 (31%)
	Negative	13	3 (23%)
HBV-DNA[b]	Positive	25	8 (32%)
	Negative	9	3 (33%)
Precore Codon 28[c]	Wild-type	5	1 (20%)
	Mutant	7	1 (14%)
	Mixed	9	5 (56%)

[a] All cases are HBsAg-positive.

[b] HBV-DNA detectable in serum by PCR.

[c] Sequence variations in precore codon 28 of HBV genome: wild-type, TGG (Trp); mutant, TAG (stop) mutation; mixed, coexistance of both wild-type and mutant HBV. For each parameter, differences in prevalence of p53 mutations between the groups were not statistically significant.

Table 4. Characteristics of hepatocellular carcinomas and p53 mutation

HCC Characteristics		Number of Cases	
		Analyzed	p53 Mutations
TNM Stage	III	6	2 (33%)
	IVa	29	9 (31%)
Sizea	<10 cm	12	3 (25%)
	> 9 cm	19	7 (37%)
Portal Vein Thrombosis	Negative	10	2 (20%)
	Positive	20	8 (40%)
Configuration of Tumorb	Massive	7	3 (43%)
	Multiple	19	8 (42%)
Differentiation	Well to Moderately	18	4 (22%)
	Poorly	15	6 (40%)

[a] Size (mean maximum diameter of the tumor) was determined by computerized tomogram.

[b] Configuration of tumor as determined by computerized tomogram: Massive, single large mass (>5cm) with or without visible daughter nodules; multiple, multi-focal masses in single lobe or both lobes. For each parameter, the differences in prevalence of p53 mutations between the groups were not statistically significant.

Table 5. Mutations of the p53 gene and age, laparoscopic findings, alpha-fetoprotein (AFP) and alkaline phosphatase (AP)

		Number of Cases	
		Analyzed	p53 Mutations
Age (Mean)	< 51 years	15	7 (47%)
	> 51 years	20	4 (20%)
Laparoscopic findings	cirrhosis	23	5 (22%)
	hepatitis	8	3 (38%)
	non-specific changes	4	3 (75%)
AFP (ng/ml) (Mean)	< 793	12	3 (25%)
	> 793	19	7 (37%)
AP (IU/L) (Mean)[a]	< 471	24	5 (21%)
	> 471	11	6 (55%)

[a] p=0.046.

reported in 23-33% (Sheu *et al*., 1992; Hsu *et al*., 1993; Hsu *et al*., 1994; Ng *et al*., 1994). In Japan, where hepatitis C virus is the predominant virus associated with hepatocellular carcinoma, p53 mutations were reported in 30-65% (Murakami *et al*., 1991; Oda *et al.*, 1992; Nishida *et al.*, 1993; Nose *et al.*, 1993; Teramoto *et al.*, 1994). In the present study, p53 mutation was identified in 31% (11/35) of advanced hepatocellular carcinomas from Korea, a country where hepatitis B virus is the virus most commonly associated with hepatocellular carcinoma.

In general, compared to other tumor suppressor genes such as the Rb and APC genes, missense mutations are much more common in the p53 gene; usually comprising more than 90% of all the p53 mutations (Harris, 1993), and transversions are most common in hepatocellular carcinomas in large part due to G-to-T transversion at codon 249 (Harris and Hollstein, 1993). Among hepatocellular carcinomas from Taiwan, 71% of the alterations in the p53 gene were due to missense mutations (Hsu *et al.*, 1994). In the present study, 64% were missense mutations, in which transversions were not dominant, and 36% were deletion mutations.

Aflatoxin B_1 is strongly associated with a G-to-T transversion in the third nucleotide position of codon 249 of the p53 gene (Hsu *et al*., 1991). Mutations at this hot spot were found in 58% in hepatocellular carcinoma patients from China (Scorsone *et al*., 1992; Li *et al*., 1993; Fujimoto *et al*., 1994), but in 10% of hepatocellular carcinoma patients from Taiwan (Sheu *et al*., 1992; Hsu *et al*., 1994) and in few (Oda *et al*., 1992) or none of Japanese hepatocellular carcinomas (Murakami *et al*., 1991; Nishida *et al*., 1993; Nose *et al*., 1993; Teramoto *et al*., 1994). In the present study, none of the cases had a G to T transversion at p53 codon 249, indicating that aflatoxin B_1 may not play an important role in hepatocarcinogenesis of Korean hepatocellular carcinomas.

This G-to-T transversion in p53 codon 249 was also found in the nonmalignant liver from some patients with hepatocellular carcinoma from Qidong (Aguilar *et al*., 1994). However, none of the adjacent nontumorous liver tissues of Korean patients with hepatocellular carcinoma had p53 mutations.

In hepatitis virus-associated hepatocellular carcinomas, it has been suggested that the occurrence of the genetic aberrations in the p53 gene may be a late event in hepatocarcinogenesis (Teramoto *et al*., 1994). Additionally, it was also suggested that the p53 mutations in hepatocellular carcinoma may be correlated with the higher serum alpha-fetoprotein level, the presence of thrombosis of the main portal vein, or poorly differentiated hepatocellular carcinoma (Hsu *et al*., 1994). However, the present study did not show statistically significant differences in these factors. These findings were similar to those reported by other researcher (Hong *et al.*, 1995) The only significant factor associated with the occurrence of p53 mutations in hepatocellular carcinomas was a higher serum alkaline phosphatase level.

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