

Original Article

Geographic Characterization of Hepatitis Virus Infections, Genotyping of Hepatitis B Virus, and p53 Mutation in Hepatocellular Carcinoma Analyzed by In Situ Detection of Viral Genomes from Carcinoma Tissues: Comparison among Six Different Countries

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SUMMARY: We investigated the relationship of infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) to p53 mutation in hepatocellular carcinomas (HCC) from six countries, including Japan, China, Korea, Vietnam, Spain, and the United States. For this purpose, we used formalin-fixed, paraffin-embedded liver tissues obtained from 449 patients with HCC to detect the viral and p53 genes by polymerase chain reaction (PCR). HBV was the most prevalent in Korea (69.1%), China (66.1%), Vietnam (60.5%), and Spain (38.6%). In contrast, HCV was the most prevalent in Japan (59.8%) and in the United States (41.5%). Type C of HBV was the most common genotype (78.6%) encountered in HCC in these countries. Importantly, among 125 intrahepatic HBV DNA-positive patients, 44 (35.2%) were serologically negative for HBsAg (occult hepatitis B). Based on PCR, immunohistochemical, serological, and clinical findings, 4.8% of HCC patients were diagnosed with non-B, non-C. A point mutation at exon 7 of p53 was detected in 20 of the 239 HCC samples examined, including those from 9 Chinese, 5 American, 2 Japanese, 2 Korean, and 2 Spanish patients, respectively. Interestingly, a point mutation with an amino acid substitution at codon 251 (Ile → Asn) was detected frequently in 11 of 20 (55%) cases. A specific mutation induced by aflatoxin B₁ at codon 249 was seen in two patients, both Chinese. Our results suggest that genotype C of HBV may play an important role in hepatocarcinogenesis in different geographic regions, and that in situ detection of HBV genomes could be important for clarifying the agent(s) of unknown etiology related to HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. Risk factors such as hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, and aflatoxin ingestion are strongly associated with HCC occurrence, although the precise mechanism underlying the development of HCC is still not clear (1-5). It is known that the prevalence of HBV and HCV infections differs relative to geographical area. For example, HBV is a more predominant agent in liver diseases in Southeast Asia and Africa, whereas HCV plays a more predominant role in Japan and the United States (U.S.). Furthermore, distribution of the genotype of HBV and HCV has also shown geographic characteristics, and an association between genotype-related differences and severity of liver diseases has been discussed (6-13).

The relationship of HCC to HBV and HCV has been

investigated worldwide, though investigations have been based mainly on seroepidemiological studies. To understand the relationship between viral infection and cancer development, it is very important to demonstrate the localization of a virus in the carcinoma tissues directly by in situ detection. We previously reported on a highly sensitive method of detecting and identifying sequences of DNA/RNA genomes in formalin-fixed, paraffin-embedded (FFPE) tissues by polymerase chain reaction (PCR) assay (14). In the present study, using this method to assess the pathological roles of HBV and HCV, respectively, in the origin of HCC, we carried out a retrospective study to determine the detection rate of the HBV and HCV genomes in FFPE specimens from HCC patients from six countries. We also investigated the genotypic distribution of HBV and the p53 gene mutation.

PATIENTS AND METHODS

Patients: We selected sequential liver samples of all HCC tissues encountered at the participating institutions. Four hundred and forty-nine FFPE-liver specimens from HCC patients, including 122 Japanese (88 males and 34 females,

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mean age 63 ± 9 years, collected from 1984 to 1996 at the International Medical Center of Japan, Tokyo), 112 Chinese (98 males and 14 females, mean age 50 ± 12 years, collected from 1954 to 2001 at Harbin Medical University Hospital, Harbin, China), 55 Korean (42 males and 13 females, mean age 53 ± 9 years, collected from 1989 to 1995 at Yonsei University Hospital, Seoul, Korea), 38 Vietnamese (30 males and 8 females, mean age 54 ± 13 years, collected from 1990 to 2000 at the Hospital of University of Medicine and Pharmacy, Ho Chi Minh City, Vietnam), 57 Spanish (45 males and 12 females, mean age 55 ± 10 years, collected from 1984 to 1996 at the Hospital of Bellvitge, Barcelona, Spain), and 65 American (41 males and 24 females, mean age 58 ± 10 years, collected from 1989 to 1995 at the Mount Sinai Medical Center Hospital, New York, USA) patients, were analyzed. In total, the sex ratio (male/female) was 344/105, and the mean age was 56 ± 11 years. All of these patients underwent liver surgery and were diagnosed with HCC by histopathological examination. We used paraffin blocks that included nontumor areas in a number of cases, although these blocks consisted mainly of tumor area.

DNA/RNA extraction from FFPE liver specimens: Extraction of the nucleic acids (DNA and RNA) from FFPE-liver specimens and PCR were performed as described previously (14). In brief, sections were cut and placed in 1.5 ml microtubes, then deparaffinized in xylene at 60°C for 10 min. The sections were incubated for 5 h at 60°C in a lysis buffer containing 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 2% sodium dodecyl sulfate, and 500 $\mu\text{g/ml}$ proteinase K (Merck, Tokyo). The nucleic acids were purified by phenol/chloroform extraction followed by precipitation with isopropanol. The resulting pellet was resuspended in RNase-free water then used as a template to detect HBV DNA (X region), HCV RNA (5'-noncoding region), and p53 DNA (exon 7).

Detection of HBV DNA and HCV RNA by multiplex PCR: The sequences of PCR primers for HBV and HCV were reported previously (6). To obtain simultaneous detection of hepatitis B and C viral genomes, we used a multiplex PCR method as described previously (15). The sensitivity of HBV and HCV PCR allowed detection up to at least 10 copies of HBV DNA and HCV RNA, respectively.

Genotyping of HBV by PCR: Genotyping of HBV was carried out by the method originally reported by Naito et al. (16) with slight modification. We designed new primer combinations for the first PCR in order to obtain higher sensitivity for application to FFPE specimens. Two different primer pairs for first PCR were newly designed. That is, mix A-1, for genotypes A through C, consists of B1 (sense, types A- to C-specific, 5'-GCA GTC AGG AAG RCA GCC TAC T-3', nt 3152-317) and S4R (antisense, universal, 5'-AGA AGA TGA GGC ATA GCA GC-3', nt 417-436), and mix-B-1, for genotypes D through F, consists of P1 (sense, universal, 5'-TCA CCA TAT TCT TGG GAA CAA GA-3', nt 2823-2845) and PS6R (antisense, types D to F-specific, 5'-GCA RTA GTC GGA RCA GGG TT-3', nt 86-105). Product sizes were 506 bases for combination of B1/S4R and 503 bases for P1/PS6R, respectively. Second-round PCR primers (inner primer pairs) were designed on the basis of the conserved nature of nucleotide sequences in regions of pre-S1 through the S gene, irrespective of the six HBV genotypes, as reported previously (16). The second PCR was performed using mix A-2 for genotypes A through C and mix B-2 for genotypes D through F, respectively. B2 (sense, 5'-GCA GTC AGG AAG RCA GCC TAC T-3') was used as the inner primer, in combi-

nations with mix A-2. Mix A-2 consists of antisense primers BA1R (type A-specific, 5'-CTC GCG GAG ATT GAC GAG ATG T-3'), BB1R (type B-specific, 5'-CAG GTT GGT GAG TGA CTG GAG A-3'), and BC1R (type C-specific, 5'-GGT CCT AGG AAT CCT GAT GTT G-3'). B2R (antisense, 5'-GGA GGC GGA TYT GCT GGC AA-3') was used as the inner primer in combinations with mix B-2. Mix B-2 consists of sense primers BD1 (type D-specific, 5'-GCC AAC AAG GTA GGA GCT-3'), BE1 (type E-specific, 5'-CAC CAG AAA TCC AGA TTG GGA CCA-3'), and BF1 (type F-specific, 5'-GYT ACG GTC CAG GGT TCA CA-3'). These primer combinations for the second-PCR reaction were designed on the basis of the differences in size of the genotype-specific bands (i.e., 68 bases for type A, 281 bases for type B, 122 bases for type C, 119 bases for type D, 167 bases for type E, and 97 bases for type F).

Detection of the p53 gene by PCR: In this study, we targeted exon 7, including either the 5' or 3' splice site of intron 6 and intron 7 of the p53 gene. The sequences of PCR primers for p53 were as follows: 53-1 (sense, 5'-TTG CCA CAG GTC TCC CCA AG-3', nt 13942-13961) and 53-2R (antisense, 5'-CAG GCT CCA TCT ACT CCC AA-3', nt 14361-14380) for the outer primer pairs (439 bp), and primers 53-1 and 53-1R (antisense, 5'-AGG TGG GAG GAG AAG CCA CA-3', nt 14301-14320) for the inner primer pairs (379 bp). The nucleotide positions were deduced from the full sequence of a human p53 isolate (GenBank accession No. X54156). The first PCR was done using a buffer containing 50 μl of a reaction buffer prepared as follows: 50 ng of each outer primer, 200 μM of each of the four deoxynucleotides, 1.25 unit of Takara Ex TaqTM DNA polymerase (Takara, Tokyo), and 1X reaction buffer containing 2 mM MgCl_2 . The thermocycler was programmed first to preheat at 94°C for 1 min for denaturing followed by 40 cycles consisting of 94°C for 30 s, 66°C for 30 s, and 72°C for 40 s using a Perkin-Elmer 2700 or 9700 Thermal Cycler (Perkin-Elmer, Norwalk, Conn., USA). For the second reaction, 2 μl (1/25 volume) of the first PCR product were added to a tube containing the second set of each inner primer, deoxynucleotides, Takara Ex TaqTM DNA polymerase, and the PCR buffer used in the first reaction. Amplification was performed for 40 cycles under the same condition as that for the first round PCR. The PCR products were run on 2% agarose gel, stained with ethidium bromide, and evaluated under UV light. The sizes of the PCR products were estimated according to the migration pattern of a 50 bp DNA ladder (Pharmacia Biotech, Piscataway, N.J., USA). To avoid the risk of false-positive results, PCR assays were done with strict precautions against cross-contamination. Furthermore, all PCR assays were performed in duplicate to confirm reproducibility.

Nucleotide sequencing of amplified p53 gene: PCR products were separated by 2% agarose gel electrophoresis and purified using a QIAquick gel extraction kit (Qiagen, Inc., Chatsworth, Calif., USA). Recovered PCR products were subjected to direct sequencing using the ABI PRISM BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Sequences of amplified cDNA were determined using a sequencer (ABI PRISMTM 310 Genetic Analyzer; Applied Biosystems, Foster City, Calif., USA).

Statistical Analysis: The data were analyzed using a standard statistical software package (Stat View; Brain Power Inc., Calabasas, Calif., USA). Statistical differences were evaluated using the Student's *t* test and Fisher's exact probability test where appropriate. Analysis of variance was

used for multiple sets of data.

RESULTS

HBV was the most prevalent in Korea (69.1%), China (66.1%), Vietnam (60.5%), and Spain (38.6%) (Table 1). HCV was the most prevalent in Japan (59.8%) and the U.S. (41.5%), but its frequency was very low in Korea (5.5%), China (2.7%), and Vietnam (2.6%). Based on PCR genotyping of HBV

using type-specific primers, type C of HBV was found to be the most common genotype encountered in HCC in these countries (Table 2). Neither genotype E nor F was detected in these countries. Importantly, among 125 intrahepatic HBV DNA-positive patients, 44 cases (35.2%) were serologically negative for HBsAg (occult HBV infection). Furthermore, 15.5% of HCC patients (9/58) who were negative for all HBV markers had intrahepatic HBV DNA. Based on PCR, immunohistochemical, serological, and clinical findings, 4.1% of

Table 1. Detection rate of HBV DNA and HCV RNA in liver tissues from 449 HCC patients from different geographic regions

Country	n	Age (yrs)	Gender (M/F)	HBV DNA	HCV RNA	HBV+HCV	Non-B, non-C ¹⁾
Japan	122	63±9	88/34	34 (27.9)	73 (59.8)	11 (9.0)	5 (4.1)
China	112	50±12	98/14	74 (66.1)	3 (2.7)	2 (1.8)	unknown ²⁾
Korea	55	53±9	42/13	38 (69.1)	3 (5.5)	2 (3.6)	0
Vietnam	38	54±13	30/8	23 (60.5)	1 (2.6)	0	0
Spain	57	55±10	45/12	22 (38.6)	7 (12.3)	2 (3.5)	7 (12.3)
U.S.	65	58±10	41/24	10 (15.4)	27 (41.5)	2 (3.1)	4 (6.2)
Total	449	56±11	344/105	201 (44.8)	114 (25.4)	19 (4.2)	16 (4.8) ³⁾

¹⁾Based on PCR, immunohistochemical, serological and clinical findings.

²⁾Due to no serologic data.

³⁾Not included China.

Numbers in parentheses indicate percentage.

Table 2. Distribution of the HBV genotype among HCC patients from different geographic regions

Country	n	HBV genotype (%)						
		A	B	C	D	E	F	UC ¹⁾
Japan	34	2 (5.9)	1 (2.9)	23 (67.6)	0	0	0	8 (23.5)
China	74	2 (2.7)	0	70 (94.6)	0	0	0	2 (2.7)
Korea	38	0	0	33 (86.8)	1 (2.6)	0	0	4 (10.5)
Vietnam	23	3 (13.0)	3 (13.0)	15 (65.2)	0	0	0	2 (8.7)
Spain	22	4 (18.2)	0	8 (36.4)	3 (13.6)	0	0	7 (31.8)
U.S.	10	0	0	9 (90.0)	0	0	0	1 (10.0)
Total	201	11 (5.5)	4 (2.0)	158 (78.6)	4 (2.0)	0	0	24 (11.9)

¹⁾UC=unclassified.

Numbers in parentheses indicate percentage.

Table 3. Pathological features of non-B, non-C HCC

Case	Country	Age/sex	Non-cancerous region	
			Cirrhosis	Chronic inflammatory cell infiltration
1	Japan	53/M	Yes	Yes
2	Japan	67/M	Yes	Yes
3	Japan	59/M	Chronic hepatitis	Yes
4	Japan	68/M	Chronic hepatitis	Yes
5	Japan	59/M	Yes	Yes
6	U.S.	60/F	Yes	No
7	U.S.	55/M	Yes	No
8	U.S.	69/M	Yes	No
9	U.S.	51/M	Yes	No
10	Spain	61/M	Yes	No
11	Spain	54/M	Yes	Yes
12	Spain	51/F	Yes	Yes
13	Spain	49/M	Yes	Yes
14	Spain	67/M	Yes	Yes
15	Spain	52/F	No	No
16	Spain	62/F	Yes	Yes

Table 4. p53 mutation detected in HCC tissue samples from patients in different countries

Country	n	Exon 7 mutation detected	
		whole region	codon 249 ¹⁾
Japan	17	2 (11.8%)	0
China	80	9 (11.3%)	2 (2.5%)
Korea	42	2 (4.8%)	0
Vietnam	24	0	0
Spain	19	2 (10.5%)	0
U.S.	57	5 (8.8%)	0
Total	239	20 (8.4%)	2 (0.8%)

¹⁾Aflatoxin B₁-induced specific mutation.

Table 5. Point mutation at exon 7 of the p53 gene detected in HCC tissue samples

Nucleotide change (nt position)	Amino acid change (codon number)	Number of cases (country)
ATC → AAC (14079)	Ile → Asn (251)	11 (China 4, U.S. 4, Japan 1, Korea 1, Spain 1)
AGG → AGT (14074)	Arg → Ser (249) ¹⁾	2 (China)
AGT → GGT (14045)	Ser → Gly (240)	1 (Japan)
TCC → TGC (14049)	Ser → Cys (241)	1 (Spain)
GGC → GTC (14058)	Gly → Val (244)	1 (China)
AAC → AGC (14067)	Asn → Ser (247)	1 (U.S.)
CGG → TGG (14069)	Arg → Trp (248)	1 (Korea)
ACC → ACT (14017)	No change (230)	1 (Japan)
AAC → AAT (14068)	No change (247)	1 (China)
CTC → CTA (14083)	No change (252)	1 (China)

¹⁾Aflatoxin B₁-specific mutation.

Japanese, 6.2% of American, and 12.3% of Spanish patients with HCC were diagnosed with non-B, non-C (Table 1). Histological findings of non-B, non-C HCC showed that 81% of cases were accompanied by liver cirrhosis and 62.5% had chronic inflammatory cell infiltration in the portal tracts of nontumorous regions, which findings suggested persistent infection by unknown virus(es) (Table 3).

A point mutation at exon 7 of p53 was detected in 20 of 239 (8.4%) HCC tissue samples examined, including 9 from 80 Chinese, 5 from 57 American, 2 from 17 Japanese, 2 from 42 Koreans, and 2 from 19 Spanish patients; and in none of 24 Vietnamese patients (Table 4). Interestingly, a point mutation with an amino acid substitution at codon 251 (Ile → Asn) was detected in 11 of 20 (55%) cases, including 4 Chinese, 4 American, and one each of the Japanese, Korean, and Spanish patients (Table 5). An aflatoxin-specific mutation at codon 249 was seen in two Chinese patients. Among 20 cases with the p53 mutation, 9 were positive for HBV and 2 were positive for HCV, and the remaining 9 cases were non-B, non-C. No splicing mutation at either the 5' or 3' splice site of introns 6 and 7 was seen. No significant differences in the pathological features of HCC based on histological grade were observed among HBV, HCV, and the p53 mutation. Reproducibility of the PCR results was confirmed by duplicated examination.

DISCUSSION

HBV and HCV are the major causes of post-transfusion hepatitis and are also involved in the etiology of sporadic cases of hepatitis. Most importantly, chronic infection with these hepatitis viruses is linked to the development of HCC. Since the discovery of the HCV genome in 1989 (17), HCV

infection is becoming recognized in many parts of the world as a more important etiologic factor than HBV infection in the pathogenesis of HCC. The relationship of HCC to HBV and HCV has been investigated worldwide, mainly via sero-epidemiological studies. The prevalence of HCV antibody-positive patients with HCC has been found to be 70-90% in Japan, 75% in Spain, 65% in Italy, 29% in South Africa, and 29% in the U.S. (18-22). Thus, this virus has become the most common cause of HCC occurrence among patients in many countries. However, HBV infection remains one of the main causes underlying the occurrence of HCC. For example, the positive rate of HBsAg was found to be 26% in Japanese patients with HCC who had previously undergone hepatic resection (23). Therefore, both HBV and HCV infections are considered to be endemic in the occurrence of HCC. It has been suggested that HBV genotypes may correlate with clinical outcomes including that of HCC (24,25). Other studies have reported that genotype C of HBV is associated with the presence of more serious liver conditions (26,27). Our data, obtained by direct detection of the HBV genome, support their findings. Genotype C of HBV is the most predominant genotype in many Asian countries and may be one of the main factors underlying the presence of HCC in many Asian patients. Clarification of the relationship between the HBV genotype and its pathogenicity in chronic liver diseases including HCC is awaited with great interest.

The high prevalence of HCV infection in patients with HCC in Japan and the U.S. investigated in this study strongly indicates that HCV infection plays a more overwhelming role than HBV infection in HCC. The most likely explanation for the HCC association with HCV being higher than that with HBV is that the prevalence of HBV infection in these countries

in recent years has been on the decline. These results also indicate that the replication of HCV is still active when the tumor is developing, which is probably important in the contribution of HCV to the development of HCC. Interestingly, our results showed that nearly 35% of HCC patients who were sero-negative for HBsAg (occult HBV infection) were found to have HBV DNA in HCC tissues. Furthermore, 15.5% of our HCC patients who were negative for all markers of HBV-related antigen-antibody had intrahepatic HBV DNA. These results suggest that seronegativity for all viral markers or seropositivity for anti-HBs does not exclude the existence of a low copy number of HBV DNA in liver tissues. In certain cases, serology must be complemented with PCR analysis to obtain a correct viral diagnosis. Our results also indicate that in situ detection of viral genomes by a highly sensitive method is as important as serology.

Furthermore, in our results, the etiology of HCC was unknown in 4.1% of Japanese, 6.2% of American, and 12.3% of Spanish patients. This result suggests that there is still an unknown agent(s) that induces chronic inflammation, considering that most of the cryptogenic HCC patients had cirrhosis associated with chronic inflammatory cell infiltrations in the portal tracts of the noncancerous region.

Hot spot mutations at codons 175, 248, 249, and 273 of tumor suppressor p53 were found frequently in a wide variety of human malignant tumors (28,29). Aflatoxin exposure associated with a point mutation at codon 249 is commonly found among HCC patients, and is especially frequent in regions characterized by dietary aflatoxin exposure and a high prevalence of HBV infection, such as South Asia and Sub-Saharan Africa, and so on (3,30,31), which suggests that HBV acts as a confounder or synergistic partner in the development of the 249 mutation or any type of p53 mutation (32,33). Our results in the present study showed a point mutation at exon 7 of p53 in 8.4% of HCC patients. Interestingly, a point mutation with an amino acid substitution at codon 251 (Ile→Asn) was the most frequent (55%) to appear among patients with a p53 mutation. To the best of our knowledge, there have been no reports regarding the mutation of p53 at codon 251 in HCC samples. Further studies are needed to clarify the significance of this specific mutation in codon 251 as it relates to the occurrence of HCC. A specific base change in codon 249 (AGG to AGT) at exon 7 of the p53 gene is known as a hot-spot mutation closely related to exposure to foods highly contaminated with aflatoxin B₁. The incidence of this hot spot mutation varies relative to geographic region. Codon 249 mutation has been demonstrated at very high rate in some parts of China and southern Africa (30,34-36). However, HCCs from Taiwan, Thailand, and Mexico show a mutation at codon 249 at a lesser frequency, and this type of mutation in India, Japan, Korea, Germany, Spain, and the U.S. is very rare (37-42). In this study, we found a codon 249 mutation only in HCC tissues from Harbin, China. Harbin is a city in Heilongjiang province situated in the northeast part of China, where HCC is a common cancer. However, contrary to expectation, the detection rate of p53 codon 249 mutation was low. The average annual temperature in Harbin is 4°C. This may be one of the main reasons why aflatoxin contamination in food is low in this area, although aflatoxin-related HCC is endemic in China. Lai et al. reported that point mutations at the splice site of introns of p53 could play an important role in the development of human cancers (43). However, our results showed that no mutations were found at the splice site of introns 6 and 7.

In conclusion, using routinely-processed FFPE specimens, we showed that HBV, particularly type C, may play an important role in hepatocarcinogenesis under a variety of geographical conditions. Approximately 5% of the HCC patients examined have been diagnosed as non-B, non-C. Attention should be paid to the fact that nearly 40% of HCC patients sero-negative for HBsAg were found by PCR to have HBV DNA in HCC tissue samples. A p53 mutation at exon 7 was detected in 8.4% of HCC patients, and in 55% of these cases, it appeared at codon 251. The aflatoxin specific-mutation of p53 at codon 249 was rare in the present study.

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