Hepatitis B and C Viral Infections in Patients with Hepatocellular Carcinoma

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The prevalence of hepatitis B and C virus infections was studied in 70 patients diagnosed as having hepatocellular carcinoma. In addition to viral serological markers, serum hepatitis B virus DNA and hepatitis C virus RNA were determined with a nested polymerase chain reaction assay. Twelve patients (17%) were HBsAg positive, 26 (37%) had antibodies to HBs, HBc or both and 32 (46%) were negative for all hepatitis B virus serological markers. Prevalence of the antibody to hepatitis C virus was 63% (44 patients). Hepatitis B virus DNA was detected in 24 of the 66 tested patients (36%). Twelve of these hepatitis B virus DNA–positive patients were HBsAg negative (seven were positive for antibody to HBs, antibody to HBc or both and five were negative for all hepatitis B virus serological markers). Hepatitis C virus RNA was found in 42 of 68 patients (62%). A high correlation (95%) existed between hepatitis C virus RNA and hepatitis C virus antibodies. Nevertheless, two patients without antibody to hepatitis C virus had serum hepatitis C virus RNA sequences. Coinfection by the two viruses was detected in nine subjects (14%), but no clinical differences were found between these and the rest of the patients. We conclude that nearly 90% (62 of the 70 patients studied) of cases of hepatocellular carcinoma in our geographical area are related to hepatitis virus infections (detected by serological or molecular studies). Hepatitis C is more prevalent than hepatitis B virus in patients with hepatocellular carcinoma, and the infection is still active when the tumor is diagnosed. This fact is probably important in the contribution of hepatitis C virus to the development of hepatocellular carcinoma. (HEPATOLOGY 1992;16:637-641.)

Interest in HCC derives not only from its worldwide incidence but also from evidence implicating hepatitis viruses in its development. Molecular, epidemiological and clinical studies have confirmed the strong association that exists between chronic HBV infection and the occurrence of primary liver tumors (1).

Since the discovery in 1989 of the hepatitis C virus (HCV) (2), the agent responsible for most cases of non-A, non-B hepatitis (NANBH), the prevalence of this virus in various liver diseases has been studied. Thus a growing body of evidence has implicated HCV in the pathogenesis of HCC (3-5). In fact, the carcinogenetic role of HCV appears to be more important than that of the HBV (6). However, detection of HCV antibodies (anti-HCV) does not necessarily indicate the presence of the virus, and neither the real status of the HCV infection nor the relationship between HBV and HCV infections in patients with HCC has been reported until now.

The aim of this study was to analyze the relative role of HBV and HCV infections in the same group of patients with HCC by both serological tests and a polymerase chain reaction (PCR) assay to detect the presence of genomic sequences of the two viruses in serum.

PATIENTS AND METHODS

Patients. Seventy patients with primary liver cancer admitted between November 1986 and June 1991 to the Liver Unit of the University Clinic of Navarra were studied consecutively. The clinical features of these patients are summarized in Table 1. The group comprised 55 men and 15 women; mean age was 61.8 yr (range = 36 to 84 yr). Cirrhosis was found in 65 patients, CAH was found in 1 patient and the remaining 4 patients had otherwise normal livers. Ten patients had histories of blood transfusion (6 to 41 yr before diagnosis of HCC), and 19 had histories of alcohol abuse (> 80 gm/day). The diagnosis of HCC was made clinically in 9 patients and histologically in the other 61 (21 cases by liver biopsy, 39 by fine-needle aspiration and cytology and 1 case at necropsy). The use of clinical material for this study was approved by the local ethics committee. All serum samples were aliquoted and stored at −40° C until they were used.

Viral Markers. HBsAg, HBeAg and antibodies to HBs, HBc and HBe were determined with commercially available RIAs (Abbott Laboratories, Chicago, IL). Anti-HCV was detected in serum by a new second-generation ELISA recently developed by Wellcome Diagnostics (Beckenham, Kent, UK). This assay is based on a recombinant protein (BHC10) that incorporates structural and nonstructural viral antigens (7).

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TABLE 1. Clinical characteristics of patients with HCC

Variable	No. of patients[a]
Sex (M/F)	55/15
Alcohol history (>80 gm/day)	19 (27)
History of blood transfusion	10 (14)
AFP (ng/ml)	
Normal (<5)	17/64 (27)
5-100	16/64 (25)
100-500	17/64 (26)
>500	14/64 (22)
Nontumorous liver histological appearance	
Normal	4 (6)
CAH	1 (1)
Cirrhosis	65 (93)
Child-Pugh class	
A	27/65 (41)
B	22/65 (34)
C	16/65 (25)
Okuda staging	
I	21/65 (32)
II	34/65 (52)
III	10/65 (15)
Tumor diagnosis during follow-up of chronic liver disease	19 (27)

AFP = alpha-fetoprotein.
[a]Numbers in parentheses are percentages.

Oligonucleotide Primers. Amplification was performed with nested PCR for HBV DNA and a reverse-transcription–PCR (RT-PCR) for HCV RNA. Primers for HBV amplification were designed from the S region with the help of a computer program (Ampli-PCR, Cuende JI, Pamplona, Spain) based on the different HBV published sequences. The outer primers were HBV-S1 (5′ AGAATCCTCACAATACCGCA, sense), starting at map position 222, and HBV-S2 (5′ CCCCAATACCACATCATCCA, antisense), starting at map position 757. The inner primers were HBV-S3 (5′ TCCAATCACTCACCAACCTC, sense), starting at map position 324, and HBV-S4 (5′ CCCTACGAACCACTGAACAA, antisense), starting at map position 707 (8). A set of primers described previously (NCR1, NCR2, NCR3 and NCR4) (Garson JA, et al. Lancet 1990;336:878-879, Correspondence) from the highly conserved 5′ non-coding region was used for the HCV amplification.

HBV DNA Extraction and PCR. Two hundred microliters of serum was mixed with 50 μl 2% SDS and 200 μl 1 mg/ml proteinase K and digested at 56° C overnight. DNA was then purified by phenol-chloroform extraction, precipitated with absolute ethanol and resuspended in sterile distilled water. The first round of the amplification reaction was carried out in a total volume of 80 μl containing 50μl of the DNA extracted, 2.5 units of recombinant Taq DNA Polymerase (Ampli-Taq; Perkin-Elmer Cetus, Norwalk, CT), 30 ng of each outer primer (HBV-S1 and HBV-S2), 0.2 mmol/L of each dNTP, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$ and 0.01% (wt/vol) dithiothreitol. Reaction mixtures were overlaid with 100 μl mineral oil to prevent evaporation. After an initial 5-min denaturation step at 95° C, 30 cycles at 95° C for 60 sec, 60° C for 60 sec and 72° C for 90 sec were performed. One microliter of the PCR product was reamplified in the second round of the nested PCR in 50 μl total volume under the same buffer and cycle conditions with the inner primers (HBV-S3 and HBV-S4). Fifteen microliters of the second-round PCR product was analyzed with 2% agarose gel electrophoresis and visualization by UV fluorescence after ethidium bromide staining.

HCV RNA Extraction and RT-PCR. RNA was extracted by the polyethylene glycol/SDS method as described previously (9), with some modifications: 50 μl of serum was brought to a final volume of 1 ml with sterile distilled water, and 500 μl of 30% (wt/vol) polyethylene glycol 8000 in 0.1 mol/L NaCl was added. After incubation on ice for 40 min, the mixture was centrifuged (15,000 *g* for 15 min) and the pellet was resuspended in 330 μl of 1% SDS by agitation. Proteins were removed by phenol extraction, and RNA was precipitated with absolute ethanol overnight at −40° C. The pellet was resuspended in 80 μl of the complementary DNA reaction buffer containing 200 units of cloned Moloney murine leukemia virus reverse transcriptase (BRL, Gaithersburg, MD), 20 units of ribonuclease inhibitor (Pharmacia LKB Biotechnology, Uppsala, Sweden), 5 mmol/L HEPES-HCl (pH 6.9), 50 mmol/L Tris-HCl (pH 7.5), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 10 mmol/L dithiothreitol, 0.125 mmol/L of each dNTP and 0.15 mmol/L of random primers. Reaction mixtures were overlaid with 100 μl mineral oil, incubated first for 45 min at 42° C and followed by 5 min at 95° C and chilled on ice. Twenty microliters of the complementary DNA product was used for nested PCR. The first round of amplification was carried out in a total volume of 50 μl containing 2.5 units of cloned Taq DNA Polymerase, 30 ng of each outer primer (NCR1 and NCR2), 0.2 mmol/L of each dNTP, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$ and 0.01% dithiothreitol. The cycle profile consisted of a 5-min denaturation step at 95° C followed by 35 cycles at 95° C for 60 sec, 50° C for 60 sec and 72° C for 90 sec. One microliter of the first PCR product was amplified in the second round with the inner primers (NCR3 and NCR4) under the same buffer and cycle conditions except for the number of cycles (30 cycles) and the annealing temperature (46° C). Results were analyzed, as in HBV PCR, by electrophoresis of 15 μl of the second-round PCR product.

Precautions and Controls. To avoid possible false-positive results, the recommendations of Kwok and Higuchi (10) were strictly followed. Extraction and amplification were performed in separate rooms; positive displacement pipettes were used; solutions were prepared in a PCR product–free environment, autoclaved and stored in aliquots that were used only once; and DNA samples were added last to each tube. Thirty-one serum samples from anti-HCV–negative subjects vaccinated against HBV were used as negative controls and subjected to the entire process of extraction, reverse transcription (in the case of HCV RNA determination) and amplification, in parallel with the test samples. To detect cross-contamination, "non-DNA" controls (PCR mix without template DNA) were included for every test sample during the amplification process. Each sample was tested at least twice, and most tests were repeated three times to confirm the validity of our results.

RESULTS

Twelve of the 70 patients tested (17%) were HBsAg positive. Antibodies to HBs and HBc were found in 26 (37%) patients; 32 (46%) had no HBV serological markers. HBeAg was detected in two of the HBsAg-positive patients. Anti-HCV was present in 44 (63%) cases. The relationship between HBV and HCV serological data is shown in Table 2. It is noteworthy that none of the HBsAg-positive patients had anti-HCV,

TABLE 2. Prevalence and relationship of anti-HCV and serological markers of HBV infections in patients with HCC

HBV status	Anti-HCV positive (%)	Anti-HCV negative (%)	Total (%)
HBsAg positive	0 (0)	12 (17)	12 (17)
Anti-HBs and/or anti-HBc positive	23 (33)	3 (4)	26 (37)
HBV negative	21 (30)	11 (16)	32 (46)
TOTAL	44 (63)	26 (37)	70

Anti-HBs = antibody to HBs; anti-HBc = antibody to HBc.

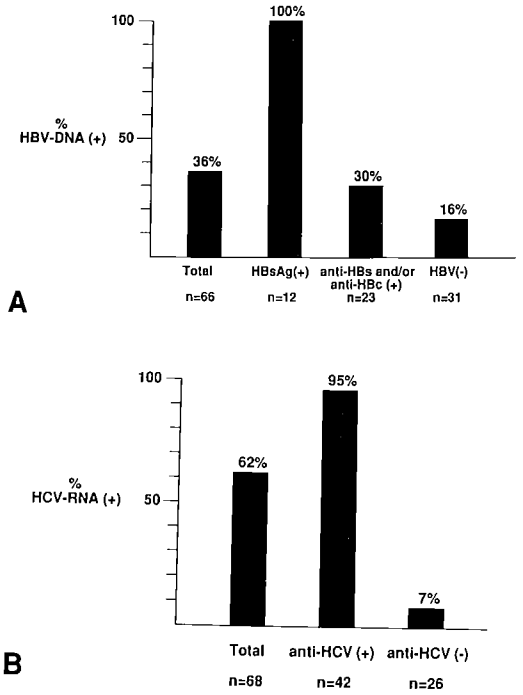

FIG. 1. Results of the detection by PCR and RT-PCR of (A) HBV DNA and (B) HCV RNA in patients with HCC. *HBV(−)* = negative for all HBV serological markers.

whereas 23 of the 26 patients with antibodies to HBs or HBc were anti-HCV positive. No serological markers of HBV or HCV infection were found in 11 patients (16%). Nine of the 10 patients with histories of blood transfusion were anti-HCV positive.

Detection of HBV in Serum. Sera from 66 patients were analyzed for the presence of circulating HBV DNA sequences as detected by amplification of the S region. Serum HBV DNA was positive in 24 patients (36%). This group included all 12 HBsAg-positive patients. Of the remaining HBV DNA–positive cases, seven patients had antibodies to HBs or antibodies to HBc and five were negative for all HBV serological markers (30% and 16%, respectively, of each serological group [Fig. 1A, Table 3]).

Detection of HCV in Serum. Of the 68 patients studied, 42 (62%) had HCV sequences in serum (Fig. 1B, Table 3). HCV RNA was found in 40 of the 42 anti-HCV–positive tested patients (95%). On the other hand, two patients without anti-HCV tested positive on PCR. Interestingly, one of these seronegative patients had had blood transfusions on two different occasions (33 and 10 yr before diagnosis of HCC).

On combination of the results of HBV and HCV testing, coinfection by the two viruses as detected by PCR was found in nine patients (Table 3). All were anti-HCV positive, and six had antibodies to HBs, HBc or both. Of the 11 patients negative for both HBV and HCV serological markers, 1 was positive for serum HBV DNA and another 2 were positive for HCV RNA. Therefore in only 8 of the 70 patients tested (11%) could HCC not be related to HBV or HCV infection by serological study, PCR study or both. Three of these patients with nonviral HCC had alcoholic cirrhosis; another had cirrhosis caused by idiopatic hemochromatosis. In the remaining four patients, the nontumorous liver was histologically normal and no risk factors could be identified.

DISCUSSION

Evidence implicating non-A, non-B agents in the development of HCC appeared before the discovery of HCV, when Ayoola, Odelola and Johnson (11) and Resnick, Stone and Antonioli (12) reported HCC in a patient with community-acquired NANBH and HCC after posttransfusion NANBH (PTNANBH), respectively. Soon, epidemiological studies showed a relationship between PTNANBH and HCC (13-15). These epidemiological associations were confirmed when in 1989 Choo et al. (2, 16) cloned the agent of NANBH and developed a specific serological assay to detect this agent, designated HCV. Since then, several authors have established that the prevalence of anti-HCV in patients with HCC is 60% to 70% (3-5). Furthermore, it has been postulated that the role of HCV could be more important than that of chronic HBV infection (6). Nevertheless, little is known about the biology of HCV infection, and the mechanism by which HCV could contribute to the pathogenesis of HCC remains unknown. Previous studies of PTNANBH have shown the detection of anti-HCV during the transition from acute hepatitis to HCC (17). Moreover, Farci et al. (18) recently described the HCV replication status in five patients with acute and chronic PTNANBH, finding serum HCV RNA in patients with the chronic form throughout the follow-up (10 to 14 yr).

In this study we found that 62% of our patients with HCC (42 of 68 cases tested) had detectable HCV genome sequences in their serum at the time of diagnosis. Moreover, we demonstrated HCV viremia in two patients who received transfusions as long as 40 and 41 yr before development of HCC. Thus (although this is not a direct derivation from our results) it seems that HCV infection remains active for very long periods of time, and persistent viremia could be the rule when HCV infection progresses from acute hepatitis to chronic hepatitis, liver cirrhosis and, finally, HCC. Although demonstration of genome integration (as in HBV infection), has failed until now, it is reasonable to suppose

TABLE 3. Results of serological and PCR testing for hepatitis C and B viruses in patients with HCC

Anti-HCV	HBV markers	No. of patients with markers (%)	HCV RNA (no. positive/no. tested)	HBV DNA (no. positive/no. tested)	HCV RNA/HBV DNA (no. positive/no. tested)
Anti-HCV positive (n = 44; 63%)	HBsAg	0 (0)	0	0	0
	Anti-HBs and/or anti-HBc positive	23 (33)	21/22	6/20	6/18
	HBV negative	21 (30)	19/20	4/20	3/19
Anti-HCV negative (n = 26; 37%)	HBsAg	12 (17)	0/12	12/12	0/12
	Anti-HBs and/or anti-HBc positive	3 (4)	0/3	1/3	0/3
	HBV negative	11 (16)	2/11	1/11	0/11
TOTAL	–	70	42/68	24/66	9/63

Anti-HBs = antibody to HBs; anti-HBc = antibody to HBc.

that continuous liver damage and cirrhosis are the most likely mechanisms by which HCV contributes to the pathogenesis of HCC. Supporting this hypothesis is the fact that all but one of our anti-HCV–positive and/or HCV RNA–positive patients had cirrhosis (the noncirrhotic patient had CAH).

A high correlation between anti-HCV assay results and PCR results has been observed in our series. Forty of the 42 anti-HCV–positive patients (95%) had HCV RNA in their serum; by contrast, only 2 patients exhibited serum HCV sequences in the group of 26 patients negative for antibodies to HCV (7%). In fact, one of these two patients had had blood transfusions on two different occasions (33 and 10 yr before tumor diagnosis). The existence of a fluctuating antibody pattern during long-standing HCV infection (18) could explain the disparity between serological and molecular studies in seronegative/HCV RNA–positive patients. Our results strongly suggest that detection of anti-HCV in patients with HCC implies active HCV infection.

The amplification of HBV has enabled us to detect HBV DNA sequences in serum in 24 of the 66 patients tested (36%). Twelve of the 24 HBV DNA–positive patients were HBsAg negative and 5 were negative for all HBV serological markers. With PCR techniques, low levels of HBV DNA have been reported in patients with HCC and other liver diseases and with no serological markers of active HBV infection (19-22). Recently, Liang et al. (23) demonstrated serum HBV particles in 29% and 31% of HBsAg-negative patients with and without antibodies to HBs and HBc, respectively. Although it is not possible to rule out the detection of integrated HBV DNA sequences from contaminating debris of necrotizing liver cells in the serum, PCR is considered the most sensitive diagnostic assay of HBV replication, and the presence of HBV-DNA in serum correlates with viremia (24). The reliability of the PCR results has been confirmed in experiments inducing HBV or "HBV variant" hepatitis in chimpanzees by inoculation of sera from these HBsAg-negative/HBV DNA–positive patients (25, 26). Thus the role of HBV infection in the development of HCC is not limited to the HBsAg-positive patients. Mechanisms by which HBV may contribute to the pathogenesis of liver cancer include induction of cirrhosis and HBV DNA integration into the hepatocyte genome and modification of cellular gene expression by insertional mutagenesis (27), chromosomal rearrangements (28) or by the transcriptional transactivating activity of the X and pre-S–S regions of the HBV genome (29-31).

Coinfection by HBV and HCV has been found in 9 of the 63 patients (14%) in whom study of the two viruses was possible. It is remarkable that none of these patients was HBsAg positive. In fact, we have not found simultaneous positivities of HBsAg and anti-HCV and/or HCV-RNA in our series. These results are discrepant from previous reports from the United States and Mediterranean countries (32-34) but similar to those obtained in Japan and Taiwan (6, 35, 36). We have no explanation for these differences except for the use in this work of a second-generation HCV ELISA that has probably reduced the number of false-positive results.

Some authors have speculated on possible interactions between HBV and HCV (22, 23) that could influence the natural course of each separate viral disease. In this series no clinical differences suggestive of a more aggressive clinical course were observed between those patients with coinfection and the remaining patients. Thus possible pathogenetic implications of coinfection by the two viruses remain to be established and future studies, including a search for liver HBV DNA, will probably increase the prevalence of coinfection above the 14% reported here.

In conclusion, in our study HCV (62%) was more prevalent than HBV (36%) in serum of patients with HCC. Taking together the serological and PCR results, HBV and HCV are implicated in the development of the tumor in 62 of the 70 patients (89%) studied. Cirrhosis was present in all but one of the patients with virus-related HCC and in four of the eight remaining nonviral tumors (three of alcoholic origin and one due to idiopathic hemochromatosis). Only in four patients (6%) could no risk factors be identified in relation to the development of the tumor. These data emphasize the importance of effective antiviral treatment and programs of HBV vaccination and development of HCV

vaccines, to decrease the incidence of HCC, and the need for an adequate screening program for the early diagnosis of HCC in patients with cirrhosis.

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