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Survey of Hepatitis B Virus Co-infection in Hepatitis C Virus-infected Patients Suffering from Chronic Hepatitis and Hepatocellular Carcinoma in Japan

More than 90% of primary hepatomas in Japan are thought to be related to hepatitis B virus (HBV) and hepatitis C virus (HCV) infection. Hepatitis B surface antigen (HBsAg) and antibody to hepatitis B core antigen (anti-HBc) in serum are used as a serological marker for chronic hepatitis (CH) and as a test for past HBV infection, respectively. Preliminary data recently obtained in Japan or internationally indicated that HBsAg-negative HCV-related CH or hepatocellular carcinoma (HCC) patients often showed positivity for anti-HBc and the presence of HBV DNA, suggesting co-infection of HBV.[1–3] This was unexpected, as such patients had generally been considered to be free of HBV infection. Anti-HCV positivity was frequently found in blood donors presenting the HBsAg negative and the anti-HBc positive serological markers.[4] This disposition was also observed during the screening of blood donors with anti-HBc, which was previously used as a surrogate marker for the prevention of post-transfusion nonA nonB hepatitis.[5] We therefore surveyed the presence of HBV DNA in HBsAg negative/anti-HCV positive and anti-HBc positive CH or HCC patients in Japan, to try to clarify whether there is any involvement of HBV in the development of HCC in HCV-infected patients.

Anti-HBc positivity was detected by using the commercially available standard assay system on 64-fold and/or 200-fold diluted sera from patients. A survey for HBV DNA in liver samples of patients was performed by nested polymerase chain reaction (PCR) analysis using primers for HBV DNA,[1, 2] and by Southern blot analysis using whole and/or a specific segment of HBV DNA as a probe. Nested PCR reaction was carried out as described previously.[1] Briefly, each sample was subjected to nested PCR reaction in a 50 μl standard reaction mixture with combinations of primers to amplify each HBV gene as follows; HBs gene: *HBS-1*/*HBS-R1* for 1st reaction and *HBS-11*/*HBS-R11* for nested PCR, X gene: *X-Pr*/*XCD-R1* for 1st reaction and *XCD-1*/*XCD-R11* for nested PCR, HBc gene: *HBC-1*/*HBC-R1* for 1st reaction and *HBC-1*/*HBC-R11* for nested PCR, X gene to HBc gene: *XCD-1*/*HBC-R1* for 1st PCR and *XCD-2*/*HBC-R11* for nested PCR. In HCV-infected CH and HCC patients with anti-HCV positive and HBsAg negative serological markers, anti-HBc positivity was found to be 41.7% in 1501 CH patients, and 58.3% in 816 HCC patients. In control, non-hepatic patients with anti-HCV negative/HBsAg negative markers, positivity of HBcAb was 18.3% in older patients (average age 64) and 11.8% in younger patients (average age 44), indicating that anti-HBc positivity in anti-HCV positive/HBsAg negative CH and HCC patients is two to three times higher than in control non-hepatic patients. These results suggested that silent HBV infection (HBsAg negative infection) frequently occurred in HCV-related CH and/or HCC patients.

The presence of HBV DNA in the liver biopsy samples of CH and HCC patients was analyzed by a quantitative nested PCR method[1] using primers that enabled us to judge the state of HBV DNA in the cell.[6] huH2-2 cell DNA was used as a single copy control of HBV DNA that can be detected up to 10^{-4} fold dilution, but not at 10^{-5} fold dilution in the nested PCR.[1] When 17 CH and 62 HCC biopsy tissue samples with anti-HCV positive/HBsAg negative/anti-HBc positive markers (out of a total of 43 HCV-related CH and 149 HCV-related HCC patients) were tested, HBV DNA was detected in 58.8% (10/17) and 56.4% (35/62) of CH and Southern blot-negative HCC samples, respectively (Table I), although the copy number of HBV DNA in these samples was found to be as low as 0.1 copy per cell.

Previous studies indicated that integration of HBV DNA into hepatic cells of HBV-infected patients frequently occurred between the 3′ end of the X gene and the contiguous core antigen gene, resulting in the split-up of these two genes in the HBV genome.[7] Therefore, only closed-circular (CC) molecules without nicks or gaps in one strand can be detected using primers that amplify the HBV DNA connecting the two regions. Our analysis suggested the presence of CC HBV DNA in some cases.[1]

The clonality of HBV DNA was further tested in 65 HCC DNA samples from anti-HCV-positive patients by means of a Southern blot analysis that was sufficiently sensitive to detect 0.1

Table I. Analysis of HBV DNA from Liver Tissues of Patients with Hepatic Diseases

	HBsAg	Anti HCV	Anti HBc		% of nested PCR	% of Southern blot analysis
No. of chronic hepatitis tissues analyzed 43	negative 41	positive 41	positive	17	58.8 (10/17)	
			negative	24	41.7 (10/24)	
No. of hepato-carcinoma tissues analyzed	positive 21	negative 21	positive	21	100	95
	negative 149	positive 149	positive	93	56.4 (35/62)	10.8 (7/65)
			negative	45	58.3 (7/12)	2.3 (1/43)
			not determined	11	44.4 (4/9)	0 (0/10)

copy of HBV DNA per cell. Clonal integration was observed in 10.8% of the HCC DNA analyzed (Table I), and the copy number of HBV DNA was about 1 per cell or in some cases even less than that.

All these data indicated the presence of HBV DNA in the liver samples of HBsAg negative/anti-HCV-positive and anti-HBc positive CH and HCC patients. If these HBV DNAs were present in the closed circular form, they could serve as templates for DNA replication and the expression of viral genes.[8] Consequently, they would constitute a serious problem from the clinical point of view, although involvement of HBV DNA in the development of HCV-related HCC has not been demonstrated yet. It should be noted that even in the tissue samples from patients carrying anti-HBc negative and anti-HCV positive/HBsAg negative serological markers, who are thought to be free of past HBV infection, HBV DNA was detected by nested PCR assay in 41.7% (10/24) and 58.3% (7/12) of CH and HCC cases, respectively (Table I). These observations imply the existence of certain cases of past HBV infection that escaped detection by the standard assay system widely employed today. Thus, a more sensitive assay system for anti-HBc detection should be established without delay, in order to prevent transfusion of infected blood or transplantation of infected liver from HBsAg negative individuals carrying low levels of anti-HBc antibody that are undetectable under current assay conditions.

In addition, an epidemiological case-control study reported that the risk of developing HCC (odds ratio) was increased by 2.6-fold in anti HBc-positive CH patients.[9] Furthermore, the presence of HBV DNA was surveyed in DNA samples obtained from HBsAg negative/anti-HBc positive blood donors or from donor/recipients of liver transplantation cases. HBV DNA was detected with high frequency in both the blood and the tissues of each individual case surveyed.[10]

Infectious HBV was strongly suggested to be present in the hepatic tissue of anti-HBc positive/HBsAg negative individuals, irrespective of whether HCV was present or not. Although the possibility of an interaction between these two viruses in the development of HCC has not been tested yet, a possible role of past exposure to HBV in virus-related hepatocarcinogenesis should not be overlooked.

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