Identification of Integrated Hepatitis B Virus DNA Sequences in Human Hepatocellular Carcinomas in Korea

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The rate of HBV DNA integration into the genome of hepatocellular carcinoma (HCC) cells varies greatly among HCC tissues from different geographical districts. Because of the potential etiological significance of identifying HBV DNA integration in HCC tissues from Korea, we investigated 13 aseptically obtained HCC surgical specimens by Southern blot analysis. Among 13 HCCs, eight (61.5%) contained integrated HBV DNA. Of eight HBsAg-positive patients, six (75%) had integrated HBV DNA in their HCCs while two (40%) out of five HBsAg-negative patients had integrated HBV DNA. Those two HBsAg-negative patients with integrated HBV DNA were all anti-HBs-positive. However, it was not demonstrated in the patient without any HBV serological markers. These findings highly suggest a prominent etiologic role of HBV in the development of HCC. On the other hand, it also strongly favors a multifactorial etiology of human HCC in Korea.

Key Words: Hepatocellular carcinoma, HBV, HBV DNA, DNA integration, Korea.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers throughout the world (Bosch et al., 1989). Epidemiological evidence indicates that hepatitis B virus (HBV) infection is associated with high-risk development of HCC (Bosch et al., 1989; Beasley et al., 1981). Its incidence varies geographically, from the Southeast Asian countries and Sub-Saharan Africa as high incidence areas, to western and northern Europe as well as North America as the low incidence areas (Popper et al., 1987). In the high incidence areas, almost all cases are associated with evidence of HBV infection, as indicated by hepatitis B surface antigen (HBsAg) and/or antibody to hepatitis core antigen (anti-HBc). Furthermore, molecular

hybridization analyses identified integrated HBV DNA in the cellular genome of most HCC tissues from HBsAg carriers (Brechot et al., 1981; Chen et al., 1982; Hino et al., 1985; Koshy et al., 1981; Shafritz and Kew, 1981). In contrast, the most important associated factor with HCC in areas of low HBsAg carrier rate was suggested to be alcohol abuse (Popper et al., 1987).

Recently, integrated HBV DNA has also been detected in patients with HBsAg-negative alcoholic cirrhosis (Brechot et al., 1982). However, HBV DNA integration in HBsAg-negative patients with HCCs is still a matter of controversy because other investigators could not demonstrate molecular evidence for a role of HBV DNA in the development of HCC in alcoholic liver disease (Walter et al., 1988). Comparative epidemiological studies may clarify why there are inconsistencies among data from different geographical districts (Hino et al., 1985; Harris and Sun, 1984). These inconsistencies may originate in part from possible false-positive results from bacterial contamination of human tumor samples, especially those obtained at autopsy (Barbacid, 1984; Hino et al., 1984).

We investigated the integration of HBV DNA into the genomic DNA of HCC from Korean patients using

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aseptically obtained surgical samples to determine the etiologic role of HBV in the development of HCC in Korea.

MATERIALS AND METHODS

The HCC tissues were obtained surgically from 13 Korean patients and then immediately frozen in dry ice and stored at -70°C until extraction of total cellular DNA. The sera which had been collected and stored at -20°C before surgery were tested for HBsAg, anti-HBc, anit-HBs, and HBeAg, using commercially available radioimmunoassay kits (AUSRIA-II, CORAB, AUSAB, and Abbott-HBe; Abbott-Laboratories, Chicago, Ill., respectively).

Total genomic DNA was extracted from frozen liver tissues by proteinase K digestion, phenol/chloroform extraction and subsequent RNase digestion according to Maniatis *et al.* (1982). Samples of 20ug of high-molecular weight DNAs were digested with Eco R1 and Hind III, fractionated by electrophoresis in a 0.7% agarose gel, and then transferred onto a nitrocellulose filter. The filter was prehybridized and hybridized with nick-translated HBV probe (Nick translation kit, Boeringer Mannheim, Mannheim, West Germany and ATCC #45020 pAM6, Rockville, Md., respectively) in conditions described earlier (Lee *et al.*, 1988) with a modification: 0.5% nonfat milk solution was substituted for Denhart's solution. The specific activities of HBV DNA probes were $1-2 \times 10^8$ (cpm/ug). After hybridization, the filter was washed several times with $1 \times \text{SCC}$ at 43°C and exposed to two to four days on Kodak X-omat XPR-1 film for autoradiography.

RESULTS

Clinical, serological, and integrated HBV DNA findings in 13 patients with HCC are given in Table 1. Eight patients were positive for HBsAg. One patient was negative for all HBV serological markers; therefore, 12 patients in this study had present or past HBV infection. Out of 13 patients, eight (61.5%) had integrated HBV DNA in their HCCs; the number of integrated HBV DNAs were 1 in 4, 2 in 3 and 3 in 1 patients. A representative picture of Southern blot analysis is shown in Fig. 1.

The relationship between the serological profile and the rate of HBV DNA integration is depicted in Fig. 2. Two female patients with anit-HBs in their sera also had integrated HBV DNAs in HCCs.

DISCUSSION

The rates of HBV DNA integration into HCC genome show wide geographic variation. They also differ greatly from one investigator to another: In five previously published studies, which investigated the HBV DNA integration rates in HBsAg-positive patients with HCC, they were 100%, 100%, 100%, 75%, and 82.6%, respectively. The studies above were done by Shafritz and Kew (1981) who investigated seven HCCs obtained at postmortem examination from South African black males; by Brechot *et al.* (1981) who studied with HCCs obtained at autopsy from the Ivory Coast; by Chen *et al.* (1982) who used five HCCs obtained surgically from Taiwan; by Koshy *et al.*, (1981) who tried

Table 1. Clinical, serological, and integrated HBV findings in 13 Korean patients with hepatocellular carcinoma

Case No.	Sex/Age	HBV Markers				Integrated HBV DNA (No.)*
		HBsAg	anit-HBs	anti-HBc	HBeAg	
1	M/59	+	-	+	+	+(2)
2	M/59	+	-	+	-	+(1)
3	M/35	+	-	+	-	+(2)
4	M/43	+	-	+	-	+(2)
5	M/45	+	-	+	-	+(3)
6	M/53	+	-	+	-	+(1)
7	M/56	+	-	+	-	-
8	M/55	+	-	+	-	-
9	M/56	-	-	+	-	-
10	M/62	-	-	+	-	-
11	M/50	-	-	-	-	-
12	F/51	-	+	+	-	-(1)
13	F/62	-	+	+	-	+(1)

*DNA integration (No.) indicates the number of bands found on the autoradiogram after digestion with Hind III.

Fig. 1 A representative autoradiogram of integrated HBV DNA sequences in undigested (UND), Eco R1 digested, and Hind III digested DNA from Case No. 3 by Southern blot analysis.

Fig. 2 Relationship between HBV serological profile and the rate of HBV DNA integration

to detect in four HCCs from Senegal and West Germany; and by Hino et al. (1985) who used 23 HCCs obtained mainly at autopsy. The difference among the rates of HBsAg-negative HCCs was more striking: The rates reported by Shafritz and Kew (1981), Brechot et al. (1981), Koshy et al. (1981) and Hino et al. (1985) were 0%, 100%, 0%, and 1.7%, respectively.

The HBV DNA integration rate of the present study (75%) in HBsAg-positive patients with HCC was relatively low in comparison with 100% by Shafritz and Kew (1981), brechot et al. (1981), and Chen et al. (1982), but was comparable with 75% by Koshy et al. (1981) and 82.6% by Hino et al. (1985). Our integration rate of 40% in HBsAg-negative patients was between

100% by Brechot and 0% by Koshy et al. (1981) and Shafritz and Kew (1981) and 1.7% by Hino et al. (1985). We included in the present study only aseptically obtained surgical samples; thus, we minimized the possibility of false-positive results. It is evident that most if not all patients with HCC in Korea had integrated HBV DNA. To further clarify the role of HBV in the development of HCC, application of the polymerase chain reaction (PCR) technique (Saiki et al., 1988), which was recently developed to amplify specific gene sequences in the order of 10^5 would be a great help in maximizing sensitivity.

It is of great interest that all of two anti-HBs positive patients had integrated HBV DNA in their HCCs. This finding is consistent with that of Korba et al. (1987) in that integrated WHV DNA was present in HCC tissues from anti-WHS-positive woodchucks. These data suggest that any previous infection by hepadna viruses may have been a risk factor for the development of HCC, and also demonstrate that the hepadna virus can persist in infected host for prolonged periods after serological recovery from active infection.

rates of DNA integration, it is now clear from previous reports (Chen et al., 1982; Ganem and Vamus, 1987; Hino et al., 1985; Koshy et al., 1981; Shafritz and Kew, 1981) and the present study that HBV is the most important etiologic agent in the development of human HCC; however, it is not a sole etiologic factor. Therefore, we suggest that comparative epidemiologic analysis of other causative factors of HCC—for instance, aflatoxin B1 which has been proven as a potent hepatocarcinogen in animals (Essingman et al., 1982) and recently suggested as a possible human hepatocarcinogen (Lee et al., 1989) and hepatitis C virus, a specific assay of which has been recently developed (Kuo et al., 1989)—be carried out in order to clarify the inconsistencies among data from different areas.

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