Increasing Incidence of Hepatocellular Carcinoma Possibly Associated with Non-A, Non-B Hepatitis in Japan, Disclosed by Hepatitis B Virus DNA Analysis of Surgically Resected Cases

Michie Sakamoto, Setsuo Hirohashi, Hitoshi Tsuda, Yoshinori Ino, Yukio Shimosato, Susumu Yamasaki, Masatoshi Makuchi, Hiroshi Hasegawa, Massaki Terada, and Yasuhiro Hosoda

Pathology [M. S., S. H., H. T., Y. I., Y. S.] and Genetics [M. T.] Divisions and Department of Surgery [S. Y., M. M., H. H.], National Cancer Center, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104, and Department of Pathology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160 [M. S., Y. H.], Japan

ABSTRACT

At the National Cancer Center Hospital in Japan, the total number of surgically treated hepatocellular carcinomas (HCCs) has been increasing steadily and rapidly over the last 10 years, whereas the number of cases positive for hepatitis B surface antigen in sera (HBsAg) has remained almost stable. Thus, the relative percentage of HCC cases with serum HBsAg has shown a marked decrease. In order to examine whether this increased proportion of HBsAg-seronegative patients carries hepatitis B virus (HBV) DNA in the liver, we extracted DNA from the formalin-fixed and paraffin-embedded cancerous and noncancerous liver tissues of 79 patients with HCC. The HCCs examined included 49 specimens resected during a period from 1970 to 1980 and 30 resected in 1986 and 1987. We were able to detect reliably the presence of HBV DNA by dot-blot hybridization. The presence of HBV DNA in liver tissues showed a good correlation with positivity for serum HBsAg in both examined groups. In total, HBV DNA was detected in 81% (21 of 26) of HBsAg-seropositive cases and in only 8% (4 of 53) of HBsAg-seronegative cases, indicating that the increased number of HBsAg-seronegative cases had no HBV involvement. Among these HBsAg-seronegative HCC patients, 89.7% showed a histology of cirrhosis or chronic active hepatitis in the noncancerous liver and 29.1% had a history of blood transfusion. These results suggest an increasing incidence of non-A, non-B hepatitis-associ-ated HCCs in Japan and the possible transmission of factors by means other than blood transfusion.

INTRODUCTION

It is well known that HBsAg is frequently positive in patients with HCC in areas such as Africa and Southeast and coastal Asia including Japan (1-3). Chronic infection with HBV and chronic liver disease induced by such viral infection are considered to be associated with the development of HCC (4-6). Moreover, the finding of HBV DNA integration into the human cancerous liver DNA in almost all HBsAg-seropositive HCC cases suggests that these integrated viral sequences may play an important role in liver carcinogenesis (7-9). However, recent epidemiological studies have shown a decline in the positivity rate for serum HBsAg among HCC cases in Japan (5, 10, 11), and an increasing incidence of HCCs associated with non-A, non-B hepatitis has been suggested (12, 13), for which no explanation has been given. We have investigated the presence of HBV DNA in specimens of HCC resected at the NCCH and demonstrated a change in the incidence of HBV-related and -unrelated HCCs and the clinical background of the latter cases.

MATERIALS AND METHODS

Cases. The pathological and clinical records of 319 HCC cases treated by surgical resection at the NCCH during a period from January 1976 to September 1987 were reviewed. All histological sections stained with hematoxylin and eosin, routinely prepared for pathological diagnosis, were reviewed, and diagnosis of the tumor was made according to the 1978 WHO classification (15). Hepatoblastomas, cholangiocellular carcinomas, and combined hepatocellular and cholangiocellular carcinomas were excluded from the study. The histological type of the noncancerous liver was divided into two groups, i.e., that showing chronic liver disease and that without such disease. The former group included three categories, chronic hepatitis, precirrhosis, and liver cirrhosis; while the latter group included unremarkable change or nonspecific reactive hepatitis. Serum HBsAg had been measured by a reverse passive hemagglutination test (AUSCELL; Abbott Laboratories, North Chicago, IL), anti-hepatitis B surface antigen by passive hemagglutination test (phytohemagglutinin Test Eisai Kit; Eisai Co., Ltd., Tokyo, Japan), and anti-hepatitis B core antigen by passive hemagglutination test (CORZYMED Disease Kit; Dainabot Co., Ltd., Tokyo, Japan). Data regarding history of blood transfusion (patients with a recorded incidence of transfusion within the previous 10 years were excluded), heavy drinking (more than 86 g ethanol daily for more than 10 years), drug administration, and family history of liver diseases were collected from clinical records.

Sampling of Tissue and DNA Extraction. Among the HCC cases mentioned above, 49 treated during a period from 1970 to 1980 and 30 cases treated in 1986 and 1987 were subjected to the following DNA analysis. Every tissue sample had been routinely fixed in 10% formalin and embedded in paraffin. Paraffin blocks of cancerous and noncancerous areas were selected in each case, and sections 25 μm thick were cut from the blocks with a microtome and collected. DNA was extracted following the method of Goelz et al. (14) as modified by Tsuda et al. (16). The concentration of DNA was measured with a spectrophotometer (17). To create negative controls for DNA without HBV integration, we extracted DNA from the MKN7 cell line (signet ring cell carcinoma of the stomach), the A431 cell line (squamous cell carcinoma of the vulva), the C-Lu65 cell line (giant cell carcinoma of the lung), and formalin-fixed, paraffin-embedded normal human spleen. DNA extracted from two human hepatoma cell lines carrying integrated HBV; PLC/PRF/5 (18) and C-Li21 (established in our laboratory) were used as positive controls.

Dot-Blot Analysis. Ten-μg of each DNA sample was dissolved in 0.4 M sodium hydroxide, and 10 mM Tris (pH 7.4)-1 mM EDTA (pH

Received 5/11/88; revised 9/2/88; accepted 9/15/88.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan.

2 To whom requests for reprints should be addressed.

3 Awardee of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research.

4 The abbreviations used are: HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; NCCH, National Cancer Center Hospital; HBV, hepatitis B virus.

7294
cases; D, HBsAg-seronegative cases. During a period from January 1976 to September 1987. Q. HBsAg-seropositive cases was exposed to Kodak XAR-5 film using intensifying screens.

0.015 M sodium citrate (pH 7.0)-0.1% sodium dodecyl sulfate and 0.65 M sodium chloride, 5 mM EDTA (pH 7.5), 0.1% sodium dodecyl sulfate, 0.1 M piperazine-N,N'-bis-2-ethanesulfonic acid (pH 6.8), and 100 µg/ml denatured salmon testis DNA (Sigma). Filters were washed twice at 65°C for 30 min in 0.1 x 0.15 M sodium chloride-sulfate, and 100 µg/ml denatured salmon testis DNA (Sigma). Filters were washed twice at 65°C for 30 min in 0.1 x 0.15 M sodium chloride-sulfate, and 100 µg/ml denatured salmon testis DNA (Sigma).

DNA on nitrocellulose filters was hybridized to the probe at 42°C for 10 h in 50% formamide, 10% dextran sulfate, 5× Denhardt's solution, 0.1 M piperazine-N,N'-bis-2-ethanesulfonic acid (pH 6.8), 0.65 M sodium chloride, 5 mM EDTA (pH 7.5), 0.1% sodium dodecyl sulfate, and 100 µg/ml denatured salmon testis DNA (Sigma). Filters were washed twice at 65°C for 30 min in 0.1× 0.15 M sodium chloride-0.015 M sodium citrate (pH 7.0)-0.1% sodium dodecyl sulfate and exposed to Kodak XAR-5 film using intensifying screens.

RESULTS

Clinicopathological Data of HCC Cases Treated by Resection at the NCCH. The number of HCC cases treated by surgical resection at the NCCH was found to be increasing year by year, with a progressive increase in the percentage of serum HBsAg-seropositive cases (Fig. 1). As shown in Table 1, the serum HBsAg-seropositive group had a tendency to have a higher male:female ratio (P < 0.05, χ² test) and a higher mean age (P < 0.01, Student's t test) at first operation for HCC than the serum HBsAg-negative group. No significant difference was found between these two groups with regard to associated changes in the liver parenchyma.

Detection of HBV DNA and Its Correlation with Serum HBsAg. The results of dot-blot hybridization using DNAs extracted from formalin-fixed, paraffin-embedded tissues are shown in Fig. 2. The intensity of the hybridization signal in each case was compared with that of the negative control material. In each case, DNAs extracted from both cancerous and noncancerous areas were examined, but no definite correlation with regard to the intensity of hybridization existed between the two, and some cases were positive in only one of the two types of tissue (Fig. 2).

HBV DNA was detected in 21 of 49 HCC cases treated by resection during the period from 1970 to 1980 (Table 2). Of 23 HBsAg-seropositive cases, 18 (78%) were positive for tissue HBV DNA, whereas only 12% (3 of 26) of HBsAg-seronegative cases were positive (Fig. 2B, cases 1–3).

In another 30 HCC cases treated by resection in 1986 and 1987, 3 of 3 HBsAg-seropositive cases were positive for tissue HBV DNA, while only 1 of 27 HBsAg-seronegative cases was positive (Table 2; Fig. 3).

When the results of the two studies were combined, HBV DNA was detected in 81% (21 of 26) of HBsAg-seropositive cases and 8% (4 of 53) of seronegative cases.

Among four serum HBsAg-negative but tissue HBV DNA-positive patients, one had a brother and a sister with positive serum HBsAg and a grandmother with liver cirrhosis, and another had a brother with positive serum HBsAg.

Serum HBsAg-negative Cases. More information about the serum HBsAg-negative cases (treated during a period from 1981 to 1987) is shown in Table 4. A past history of blood transfusion was recorded in 29.1% of patients (59 of 203) and heavy alcoholic intake in 17.6% (37 of 210). Only a few patients had a long history of administration of several drugs for hypertension, diabetes mellitus, or other conditions. Two patients had a history of amebic dysentery. Histological evidence of schistosomiasis caused by Schistosoma japonica was found in one patient. Family history of liver disease was rarely shown. Among serum HBsAg-positive cases treated during the same period, a past history of blood transfusion and heavy alcoholic intake were recorded in 15.9% (7 of 44) and 8.7% (4 of 46) of patients, respectively.

DISCUSSION

Our previous study (16) demonstrated the usefulness of DNA extracted from stored formalin-fixed and paraffin-embedded tissues for retrospective analysis on gene amplification. In the present study we were able to confirm its usefulness for the study of viral infection by obtaining good correlation between

Table 1  Serum HBsAg-positive and -negative cases of HCC treated by surgical resection at the NCCH during a period from January 1976 to September 1987

<table>
<thead>
<tr>
<th>Chronic liver disease</th>
<th>Total cases</th>
<th>Male</th>
<th>Female</th>
<th>M:F ratio</th>
<th>Mean age (yr)</th>
<th>Total</th>
<th>Chronic hepatitis</th>
<th>Pre-cirrhosis</th>
<th>Liver cirrhosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum HBsAg positive</td>
<td>76</td>
<td>57</td>
<td>19</td>
<td>3.0</td>
<td>50.5</td>
<td>67</td>
<td>20</td>
<td>11</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>243</td>
<td>207</td>
<td>36</td>
<td>5.75</td>
<td>58.7</td>
<td>218</td>
<td>44</td>
<td>46</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>319</td>
<td>264</td>
<td>55</td>
<td>4.8</td>
<td>56.7</td>
<td>285</td>
<td>64</td>
<td>57</td>
<td>164</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.

Fig. 1. Serum HBsAg in cases of HCC treated by resection at the NCCH during a period from January 1976 to September 1987. □, HBsAg-seropositive cases; ▬, HBsAg-seronegative cases.

Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 1988 American Association for Cancer Research.
serum HBsAg and HBV DNA in the liver tissue.

Integration of HBV DNA in the genome of HCC was demonstrated by Southern blot hybridization and was shown to be almost invariably present in HBsAg-seropositive patients (7–9). Hino et al. (21) found HBV DNA integration in 89% (8 of 9) of HBsAg-seropositive patients and in 13% (2 of 15) of HBsAg-seronegative patients, figures which are similar to those presented here. However, previous results reported for HBsAg-seronegative patients have been inconsistent, the frequency of integration varying from 0% (22) to 30% (8) or more, and the possibility of technical artifacts has been suggested (23). In this study, we determined the positivity by comparison with negative controls showing no HBV DNA integration or free HBV particles and were able to obtain reliable data even with HBsAg-seronegative patients. The presence of four serum HBsAg-negative but tissue HBV DNA-positive patients may indicate the higher sensitivity of our present dot-blot analysis in comparison with serological examination of HBsAg, at least in some cases. On the other hand, we obtained a few false-negative cases in which serum HBsAg was positive but HBV DNA was undetectable by dot-blot analysis. These false negatives might be caused by loss of signal from fixed tissue, low levels of viral replication, or focal HBV DNA integration in a few hepatocytes. DNA samples prepared from liver tissues included not only host genomic DNA but also free viral DNA, which may be present in hepatocytes, blood, or other compartments. Therefore, dot-blot analysis alone is insufficient to prove integration of HBV DNA, and positive cases will include HCC with HBV DNA integration or free HBV or both. This may be the reason for the variable pattern of hybridization intensity between DNA from cancerous areas and that from noncancerous areas in positive cases. On the other hand, negative results with dot-blot analysis clearly indicate the absence of HBV DNA integration and dot-blot analysis in recent HCC cases to determine whether the increased proportion of HBsAg-seronegative cases did not contain HBV DNA in the tissues or whether HBsAg-seronegative but HBV DNA-positive cases were increasing. It was found that the former was the case; 96% (26 of 27) of HBsAg-seronegative cases did not possess HBV DNA in the liver tissue, indicating that HBV-unrelated HCCs have been increasing recently.

There remains a possibility that the indications for surgery might have caused a bias in the cases examined. However, our data on male:female ratio, mean age, and associated changes in

### Table 2 Correlation between serum HBsAg and HBV DNA in liver of 49 HCC cases treated by resection during a period from 1970 to 1980

<table>
<thead>
<tr>
<th>HBV DNA</th>
<th>Serum HBsAg</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>16</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>3</td>
<td>23</td>
<td>26</td>
</tr>
</tbody>
</table>

### Table 3 Correlation between serum HBsAg and HBV DNA in liver of 30 HCC cases treated by resection in 1986 and 1987

<table>
<thead>
<tr>
<th>HBV DNA</th>
<th>Serum HBsAg</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>1</td>
<td>26</td>
<td>27</td>
</tr>
</tbody>
</table>

### Table 4 HBsAg-seronegative cases of HCC treated by surgical resection at the NCCH during a period from January 1981 to September 1987

<table>
<thead>
<tr>
<th></th>
<th>Positive cases/ examined cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum anti-hepatitis B surface antigen</td>
<td>40/210 (19.0)%</td>
</tr>
<tr>
<td>Serum anti-hepatitis B core antigen</td>
<td>97/150 (64.7)%</td>
</tr>
<tr>
<td>Transfusion*</td>
<td>59/203 (29.1)%</td>
</tr>
<tr>
<td>Heavy alcohol intake*</td>
<td>37/210 (17.6)%</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.

* Cases for which a history was recorded within the previous 10 years were excluded.

* More than 86 g ethanol daily for more than 10 years.
the liver parenchyma of HCC cases examined in this study are not very different from the data of HCC cases collected throughout Japan by the Liver Cancer Study Group of Japan (10). Moreover, comparison of cases between the HBsAg-positive and -negative groups showed a higher male:female ratio and a higher mean age in the latter, and the same tendency has also been reported in other epidemiological studies (13, 24).

The mechanism of hepatocarcinogenesis is not yet clear, but it has been proposed that chronic liver diseases and HBV infection are major candidates (4–6). Recent clinical studies of HBV seromarkers have suggested an increase of HCC associated with non-A, non-B hepatitis in Japan (12, 13). In the present study, we confirmed that the increased proportion of HBsAg-seronegative cases was unassociated with HBV DNA at the genetic level. In addition, 89.7% of the studied patients had liver cirrhosis or chronic hepatitis. The contribution of alcohol intake to the development of chronic liver disease cannot be neglected in some cases, but histological evidence of alcoholic hepatitis or cirrhosis is not clear in most cases. Although data on hepatitis A virus infection were not available in this study, it is generally accepted that the virus rarely causes chronic liver disease. In addition, the possibility that transient HBV infection in anti-hepatitis B surface antigen in patients with hepatocellular carcinoma. Gastroenterology, 72: 902–909, 1977.

REFERENCES

Increasing Incidence of Hepatocellular Carcinoma Possibly Associated with Non-A, Non-B Hepatitis in Japan, Disclosed by Hepatitis B Virus DNA Analysis of Surgically Resected Cases


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/48/24_Part_1/7294

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.