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

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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-associated 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 serological markers are currently available.

Recently, Goelz et al. (14) devised a method for extracting DNA from formalin-fixed, paraffin-embedded tissues prepared for routine histopathological examination. They showed that the extracted DNA, although not intact, could be used for DNA-DNA hybridization analysis, especially for the study of gene amplification and viral infection in human diseases.

In the present study, we examined retrospectively the presence of HBV DNA in specimens of HCC resected at the NCCH in comparison with the presence of serological markers for HBV in each case. Furthermore, we carried out an epidemiological analysis of HCC patients treated by resection 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 K.K.; Eisai Co., Ltd., Tokyo, Japan), and anti-hepatitis B core antigen by passive hemagglutination test (CORZYME Diagnostic 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 were dissolved in 0.4 M sodium hydroxide, and 10 mM Tris (pH 7.4)–1 mM EDTA (pH...
NON-A, NON-B HEPATITIS-ASSOCIATED HEPATOCELULAR CARCINOMA

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 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 0.65 M sodium chloride, 5 mM EDTA (pH 7.5), 0.1% sodium dodecyl sulfate, 0.1 M piperazine-Avr/V’-bis-2-ethanesulfonic acid (pH 6.8), and 100 µg/ml denatured salmon testis DNA (Sigma). Filters were then baked at 80°C for 2 h under vacuum.

The HBV DNA probe (19) was an approximately 3.2-kilobase BamHI/BamHI fragment of pHBI-1 carrying the entire HBV genome from Dane particles of subtype adr. It was labeled with dCT32P using an oligolabeling system (20).

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-negative cases (Fig. 1). As shown in Table 1, the serum HBsAg-negative 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-positive 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 3; 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

| Serum HBsAg-positive and -negative cases of HCC treated by surgical resection at the NCCH during a period from January 1976 to September 1987 | Chronic liver disease |
|---|---|---|---|
| Sex, mean age, and histological change in the noncancerous liver. | Chronic hepatitis | Precirrhosis | Liver cirrhosis |
| | Total | Male | Female | M:F ratio | Mean age (yr) | Total | Chronic hepatitis | Precirrhosis | Liver cirrhosis |
| Serum HBsAg positive | 76 | 57 | 19 | 3.0 | 50.5 | 67 (88.2)* | 20 | 11 | 36 |
| Serum HBsAg negative | 243 | 207 | 36 | 5.75 | 58.7 | 218 (89.7) | 44 | 46 | 128 |
| Total | 319 | 264 | 55 | 4.8 | 56.7 | 285 (89.3) | 64 | 57 | 164 |

* Numbers in parentheses, percentage.
A. Serum HBsAg(+) B. Serum HBsAg(−) C. Standard

<table>
<thead>
<tr>
<th>Case</th>
<th>HCC</th>
<th>Non-ca</th>
<th>Case</th>
<th>HCC</th>
<th>Non-ca</th>
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![Fig. 2. Detection of HBV DNA by dot-blot hybridization analysis. Each case of group A (serum HBsAg-positive) and group B (serum HBsAg-negative), was examined for DNA from a cancerous area (left lane) and a noncancerous area (right lane). Group C, standard DNAs. Cases 1–5 in group A show positive HBV DNA except for the noncancerous area in case 2. Cases 1–3 in group B are HBV DNA positive but serum HBsAg negative and were treated during a period from 1970 to 1980, the noncancerous areas all being negative. Cases 4 and 5 in group B are negative for both.]

![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>
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<th>Negative</th>
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<td></td>
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![Table 3 Correlation between serum HBsAg and HBV DNA in liver of 30 HCC cases treated by resection in 1986 and 1987]

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<th>Total</th>
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<td>3</td>
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<tr>
<td></td>
<td>Negative</td>
<td>1</td>
<td>26</td>
<td>27</td>
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</table>

Fig. 2. Detection of HBV DNA by dot-blot hybridization analysis. Each case of group A (serum HBsAg-positive) and group B (serum HBsAg-negative), was examined for DNA from a cancerous area (left lane) and a noncancerous area (right lane). Group C, standard DNAs. Cases 1–5 in group A show positive HBV DNA except for the noncancerous area in case 2. Cases 1–3 in group B are HBV DNA positive but serum HBsAg negative and were treated during a period from 1970 to 1980, the noncancerous areas all being negative. Cases 4 and 5 in group B are negative for both.

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

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<td></td>
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<td>27</td>
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</table>

Fig. 3. Dot-blot hybridization analysis of HBV DNA from liver in HCC cases treated by resection at the NCCH in 1986 and 1987. DNA from 30 cases, the DNA from each case being from both cancerous and noncancerous areas, was examined on two filters, one of which is shown as a representative. The two HBV DNA-positive cases exhibited on this filter are (a) a serum HBsAg-negative case and (b) a serum HBsAg-positive case. HBV DNA was detected in both cancerous and noncancerous areas of case a and only in the cancerous area of case b. Standard DNAs, the same as in Fig. 1, are in Lane 3.

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>
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<th>Positive cases/</th>
<th>40/210 (19.0)*</th>
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<td>serum hepatitis B surface antigen</td>
<td>examined cases</td>
<td>97/150 (64.7)</td>
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<tr>
<td></td>
<td>serum hepatitis B core antigen</td>
<td></td>
<td>59/203 (29.1)</td>
</tr>
<tr>
<td></td>
<td>Transfusion*</td>
<td></td>
<td>37/210 (17.6)</td>
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<td></td>
<td>heavy alcohol intake</td>
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* 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.

positive cases. On the other hand, negative results with dot-blot analysis clearly indicate the absence of HBV DNA integration as well as free HBV, indicating almost no contribution of HBV to hepatocarcinogenesis in these cases.

HCC patients show a high positivity rate for serum HBsAg, which has been reported to be about 50% in Japan (3). Recently, however, the figure has steadily declined to less than 30% (5, 10, 11). This tendency of decrease in the positivity rate for serum HBsAg is more marked in HCC patients treated by resection at the NCCH. Therefore, we performed DNA extraction 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
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- and/or anti-hepatitis B core antigen-positive cases had caused hepatocarcinogenesis through a “hit-and-run” mechanism (25) cannot be neglected. However, transient infection alone cannot explain accompanying chronic liver disease. Taking these points into consideration, involvement of non-A, non-B hepatitis virus in these HBV-unrelated HCCs is proposed.

Most previous epidemiological studies of non-A, non-B hepatitis were concerned with posttransfusion non-A, non-B hepatitis (26, 27). In our study, 29.1% of HBsAg-seronegative patients had a history of blood transfusion and its importance as a transmission pathway was thus confirmed. In addition, a further possibility can be proposed that non-A, non-B hepatitis with background factors other than transfusion is present (28, 29) or that not one but several kinds of non-A, non-B viruses exist.

ACKNOWLEDGMENTS

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REFERENCES

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