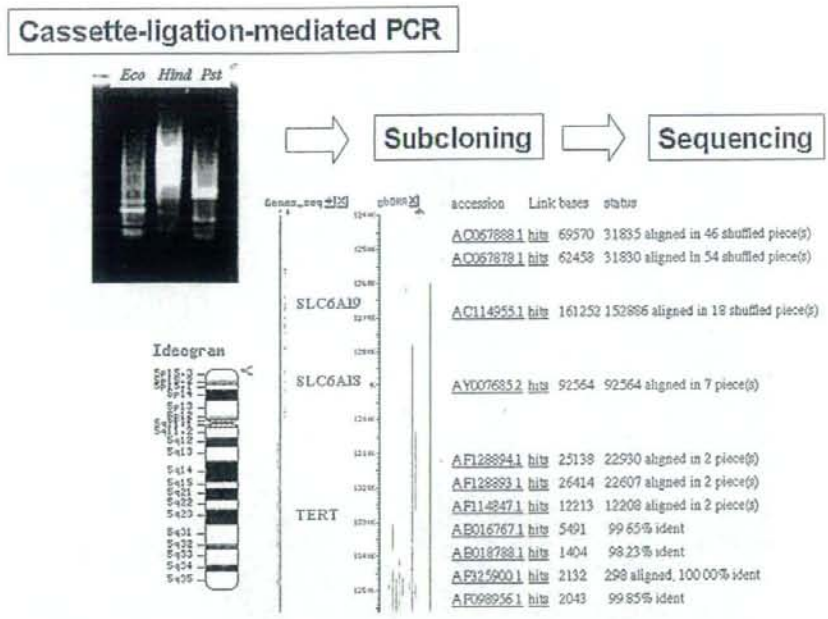

Fig. 4. Cassette-ligation-mediated polymerase chain reaction (PCR). HBV DNA is integrated into the telomerase reverse transcriptase (*hTERT*) gene located on chromosome 5p15. *Ident*, Identical

and HBV DNA and remission of hepatitis, because the oncogenic potential due to occult HBV infection or the integration of HBV DNA is considered to continue.[29]

Itsubo et al.[30] reported a case of recurrent HCC associated with HCV infection due to transfusion during the first liver resection. The first liver resection had been performed 18 years previously for HCC associated with HBV infection. In our patient, serum anti-HCV was positive and HCV RNA was negative in 1995. Although the HCV infection in the patient reported by Itsubo et al.[30] might have been caused by the transfusion during the first operation, it was transient because HCV RNA was negative and the levels of AST and ALT were within the reference ranges. Thus, the recurrent HCC was not directly caused by the HCV infection. However, an HCV superinfection might have played a role in the spontaneous HBsAg clearance in our patient.[31]

The rare case that we have reported indicates that long-term follow up is necessary for chronic HB, even after the clearance of serum HBsAg and HBV DNA and remission of hepatitis, because the oncogenic potential due to occult HBV infection or the integration of HBV DNA is considered to continue.

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Frequent detection of hepatitis B virus DNA in hepatocellular carcinoma of patients with sustained virologic response for hepatitis C virus

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Short title: HCC with sustained virologic response

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ABSTRACT

Hepatocellular carcinoma (HCC) develops several years after the eradication of hepatitis C virus (HCV) by interferon therapy. Risk factors for the development of HCC are only partly understood. To elucidate the role of occult hepatitis B virus (HBV) infection in hepatocarcinogenesis in patients with sustained virologic response, the prevalences of HBV-related makers were examined. Study group comprised 16 patients with sustained virologic response (group A) and 50 with HCV (group B). Anti-HBc and anti-HBs in serum were examined by enzyme-linked immunoassay. HBV DNA in liver was examined by nested polymerase chain reaction, using primers specific for genes encoding for HBx, HBsAg, HBcAg, and HBV cccDNA. Sequence of the amplified HBV DNA for 'a' determinant of HBsAg was determined in HCC. Anti-HBc was positive in 10 of 16 in group A and 25 of 50 in group B. HBV DNA in liver was detected in 12 of 16 in group A and 21 of 50 in group B ($p = 0.044$). In group A, HBV DNA in liver was detected frequently in patients without cirrhosis and in those with a longer period from the time of HCV eradication to the development of HCC. Mutation in 'a' determinant of HBsAg was found in three HCC of group A. Occult HBV infection may be one of the most important risk factors in hepatocarcinogenesis of Japanese patients with sustained virologic response.

KEY WORDS: anti-HBc, anti-HBs, Interferon, Occult HBV infection

1. Introduction

In Japan, a country endemic for hepatitis B virus (HBV) and hepatitis C virus (HCV), more than 75% of cases of hepatocellular carcinoma (HCC) are attributable to HCV-related chronic liver disease, and nearly 15% are attributable to HBV-related liver disease [Ikai et al., 2007]. Several reports have focused on the clinical role of past HBV infection as indicated by the presence of anti-HBc and the absence of HBsAg in patients with HCV. Marusawa et al. [1999] reported that the prevalence of anti-HBc was high in patients with chronic liver disease, especially in HCC associated with HCV. A retrospective study of 412 patients with HCV showed that the risk of hepatocarcinogenesis increased 2-fold in patients with anti-HBc [Chiba et al., 1996]. Prospective studies have also demonstrated that the presence of anti-HBc is an important risk factor for HCC in patients with HCV [Tanaka et al., 2006; Ikeda et al., 2007]. Conversely, some studies failed to support a role of previous HBV infection in hepatocarcinogenesis [Hiraoka et al., 2003; Stroffolini et al., 2008].

Another important clinical feature is occult HBV infection, defined as the presence of detectable levels of HBV DNA in liver despite the absence of serum hepatitis B surface antigen (HBsAg). The HBV genome is detectable frequently in liver tumors from HBsAg-negative patients with HCV-related liver disease, suggesting that occult HBV infection may contribute to the progression of liver damage and the development of HCC in HCV-positive patients [Cacciola et al., 1999; Tamori et al., 1999, 2003; Squadrito et al., 2006; Pollicino et al., 2004].

Interferon (IFN) has potent antiviral activity against HCV. Complete eradication of HCV by antiviral therapy is associated with a considerable reduction in the incidence of HCC [Yoshida et al., 1999; Tanaka et al., 2000]. Nevertheless, recent studies have shown that HCC develops in 2.5% to 4.2% of patients who have a sustained virologic

response [Toyoda et al., 2000; Ikeda et al., 2005; Kobayashi et al., 2007]. These patients may have had advanced liver fibrosis at the time of HCV eradication, and subclinical tumors might already be present in the liver at the end of IFN therapy [Makiyama et al., 2004]. In some patients with sustained virologic response, however, HCC develops from liver without fibrosis several years after the eradication of HCV by IFN. The etiology of such cases of HCC remains obscure. Delineation of important features of HCC that develop after the elimination of HCV as compared with those established during sustained HCV infection may contribute to a better understanding of the mechanisms involved.

In the present study, resected liver specimens were examined to evaluate the role of previous and occult HBV infection in the development of cancer after the clearance of HCV by IFN treatment. The present results might contribute to a clearer definition of patients at high risk for HCC after the eradication of HCV.

MATERIALS AND METHODS

Patients

Sixteen consecutive patients who underwent surgical resection of HCC in Osaka City University Hospital after eradication of HCV by interferon monotherapy from June 1998 through July 2007 (group A) were studied (Table 1). All patients with sustained virologic response were male in the present study. As a sex-matched control, 50 consecutive HCV-RNA-positive men with HCC during the same period were studied (group B). Serological markers showed that anti-HCV was positive and HBsAg was negative in all patients. One portion of each tissue sample from the 66 patients with HCC was frozen in liquid nitrogen immediately after resection and stored at –80°C until analysis. Total DNA was extracted from these portions by conventional methods as described previously [Tamori et al., 2003]. None of the patients had a history of exposure to aflatoxin B1, insulin administration, hereditary hemochromatosis, autoimmune hepatitis, or primary biliary cirrhosis. The activity of hepatitis and the stage of fibrosis were determined according to a modified version of Desmet's classification in liver tissue specimens obtained before IFN therapy and in noncancerous liver tissue obtained intraoperatively.

Anti-HBc and Anti-HBs in serum

Antibodies to HBsAg (anti-HBs) and anti-HBc in patient sera were tested by enzyme immunoassay and/or radioimmunoassay, using commercially available kits (Dainabott, Tokyo, Japan).

Detection of HBV DNA in serum and liver

DNA was extracted from 100 μl of serum or 10 mg of liver tissue by phenol/chloroform extraction, as described previously [Tamori et al., 2003]. HBV DNA in serum or in liver was amplified with specific primers for genes encoding for HBx, HBsAg, and HBcAg

(sequences of the primers shown in Table 2). Amplification was done in a thermal cycler for 35 cycles: 95°C for 30 sec, 55°C for 60 sec, and 72°C for 60 sec in 40 μl of a reaction buffer containing 30 pmol of the two appropriate primers, four deoxynucleotides each at a concentration of 100 μM, polymerase chain reaction (PCR) buffer, and 2.5 units of Gold Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). The first PCR product (2 μl) was used in a second PCR. To examine HBV covalently closed circular DNA (cccDNA) in liver, extracted DNA was amplified with primers P23, 24, 25, and p26 (Table 2). The amplification procedure and primers have been described previously [Tamori et al., 2005]. The nested PCR analysis was sufficiently sensitive to detect more than 10 copies of HBV DNA [Yotsuyanagi et al. 2000]. Occult HBV infection was diagnosed when 2 or more regions of HBV DNA were amplified, as reported previously [Pollicino et al., 2004].

Sequence of 'a' determinant of HBsAg and HBV genotyping

HBV DNA in liver was amplified with specific primers for HBS 'a' determinant (sequences of the primers shown in Table 2). PCR (an initial incubation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min) was performed in a final volume of 50 μl with a GeneAmp PCR system 9600 (Perkin-Elmer Life Sciences Japan, Tokyo, Japan). Aberrant PCR products were purified with a QIAquick PCR purification kit (Qiagen, Tokyo, Japan) and sequenced with an Applied Biosystems DNA sequencer (Perkin-Elmer) and a Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Tokyo, Japan). HBV genotyping in liver was performed by PCR using type-specific primers [Naito et al., 2001].

Statistical analysis

Statistical analysis was performed with the Statview SE+Graphics program, version 5.0 (SAS Institute, Cary, NC). The Mann-Whitney *U* test was used to compare 2 continuous

variables, and the χ^2 test was used to compare 2 categorical variables. All tests was 2-sided and p value <0.05 was considered to be statistically significant.

Ethical Considerations

This study protocol complied with the ethical guidelines of the Declaration of Helsinki (1975) and was approved by the Ethics Committee of Osaka City University Graduate School of Medicine.

RESULTS

Histological findings in patients with HCC

In group A, 4 patients (25%) had hepatic cirrhosis. In group B, 24 patients (48%) had hepatic cirrhosis. This difference was not significant. Table 1 shows the histological tumor grade in each group. There was no significant difference between the two groups. In patients with sustained virologic response, the period from the end of IFN treatment to the diagnosis for HCC ranged from 13 to 177 months. Histological examinations before and after IFN treatment, performed in 11 of the 16 patients in group A, showed that the staging of hepatic fibrosis improved in 5 patients and the grade of hepatic activity improved in 10 patients (Table 3). In noncancerous liver, there was no fat accumulation.

Anti-HBc, Anti-HBs, and HBV DNA in serum

Anti-HBc was positive in 10 (62.5%) of 16 patients in group A and 25 (50%) of 50 patients in group B ($p = 0.56$). Anti-HBs was detected in 6 (37.5%) of 16 patients in group A and 9 (18%) of 50 patients in group B ($p = 0.2$). HBV DNA was not detected in serum from either patients in group A or those in group B.

HBV DNA in the resected liver

HBV DNA in the resected liver was found in 12 (75%) of 16 patients in group A and 21 (42%) of 50 in group B. The rate of HBV DNA detection was significantly higher in patients with sustained virologic response than in patients with HCV ($p = 0.044$).

In group A, HBV DNA was detected in 7 resected livers from 10 patients with anti-HBc and in 5 resected livers from 6 patients without anti-HBc.

Among patients in group A, HBV DNA was detected in 11 (92%) of 12 patients without cirrhosis and 1 (25%) of 4 patients with cirrhosis ($p = 0.046$). Among patients in group B, HBV DNA was detected in 11 (42%) of 26 patients without cirrhosis and in 10 (42%)

of 24 patients with cirrhosis (Table 4).

HBV cccDNA was detected in 5 (31%) of 16 patients in group A and in 7 (14%) of 50 patients in group B ($p = 0.24$).

Average of the period from the end of IFN treatment to the diagnosis of HCC was 94 months in patients with occult HBV infection and 22 months in those without occult HBV infection. In group A, HBV DNA was detected in all patients in whom HCC developed more than 40 months after the disappearance of HCV (Table 4).

Mutation of the 'a' determinant of HBsAg and HBV genotype in patients with sustained virologic response

In 6 of 12 patients with occult HBV infection, the 'a' determinant area of HBsAg was amplified. Sequencing of the PCR product showed that codon 126 ATT (Ile) had mutated to GTT (Val) in 2 cases. One of them had 2 mutations, which were TCG to TTG at codon 54 and GCT to GGT at codon 157. In the other case, TTC had mutated to TCT at codon 93. No other mutations in the 'a' determinant area were found. HBV genotyping was performed in 12 patients with occult HBV infection. Eight patients were infected with HBV genotype C. In the four other patients, the HBV genotype could not be determined.

DISCUSSION

Previous studies have reported that 43% to 59% of patients with HCC related to HCV are positive for anti-HBc [Marusawa et al., 1999; Ikeda et al., 2007; Stroffolini et al., 2008]. In the present study, anti-HBc and anti-HBs were detected respectively in 50% and 18% of 50 patients with HCV-HCC. As compared with patients who had HCV-HCC, anti-HBc and anti-HBs were detected more frequently in HCC-patients with sustained virologic response. Recent studies show a higher prevalence of anti-HBc in HCC-patients with sustained virologic response [Ikeda et al., 2007; Stroffolini et al., 2008]. The results in the present study were consistent with these reports. The prevalence of anti-HBc is higher among the elderly population in Japan. In the present study, mean ages and age distributions did not significantly differ between the patients with sustained virologic response and the patients with HCV. Taken together, anti-HBc might be one of the specific characteristics of HCC-patients with sustained virologic response.

Occult HBV infection was diagnosed when HBsAg in serum was negative and HBV DNA in serum or liver was positive, irrespective of test results for serum anti-HBc. Previous studies showed a high prevalence of occult HBV infection in patients with HCV and progressive liver disease, particularly HCC [Cacciola et al., 1999; Tamori et al., 1999]. Another report suggested that occult HBV infection is an independent risk factor for carcinogenesis in patients with HCV [Pollicino et al., 2004]. It is controversial whether very small amounts of HBV have carcinogenic potential in patients with HCV. HBV DNA integration into the human genome, one of the most important mechanisms of HBV-related hepatocarcinogenesis [Bréchot, 2004], was suggested to accelerate tumor progression in HBsAg-negative patients with HCV infection [Tamori et al., 2003]. However, a recent study showed that the incidence of HCC in HBsAg-negative patients

with HBV DNA integration was not higher than that in patients without such integration [Toyoda et al., 2008]. The role of HBV DNA integration in patients with HCV infection remains to be clarified. On the other hand, there are few reports about occult HBV infection in patients with HCC in whom HCV was eradicated by IFN treatment. The present study clearly showed that the detection rate for HBV DNA was significantly higher in HCC-patients with sustained virologic response than in HCC-patients with HCV. Stroffolini et al. speculated that the disappearance of HCV might up-regulate HBV replication later on [Stroffolini et al., 2008]. In the present study, HBV cccDNA essential for HBV replication was detected in the liver from patients with occult HBV infection. HBV cccDNA was detected in small amounts, but not significantly more frequently in patients with sustained virologic response than in patients with HCV. It was suggested that HBV is re-amplified or accumulated in the liver of HCC-patients with sustained virologic response. Although HBV DNA integration in HCC of patients with sustained virologic response has been confirmed [Tamori et al., 2005], further studies are needed to elucidate the precise role of occult HBV infection in patients with sustained virologic response.

In the present study, eight patients with occult HBV infection were infected with HBV genotype C. A previous study reported that 610 (84.7%) of 720 Japanese patients with chronic HBV infection had genotype C [Orito et al., 2001]. In Japan, genotype C is predominant in both patients with occult HBV infection and patients with HBsAg. It is speculated that occult HBV infection occurred after the disappearance of HBsAg in patients with HBV infection.

Mutation in 'a' determinant of HBsAg, which contributes to defective HBsAg expression [Chen et al., 1999], was detected in only three of 12 HCC with occult HBV infection. HBV DNA was not detected in serum from any patient in the present study. It

appears that the 'a' determinant mutation does not have a major role in the pathogenesis of occult HBV infection in patients with sustained virologic response. Molecular analysis of the full-length HBV genome in patients with occult HBV infection has shown that viral factors are not responsible for the unique HBV status [Pollicino et al., 2007]. Available evidence suggests that small amounts of HBV, detected only in liver, exist without inducing hepatic injury or that HBV DNA is integrated into hepatocyte nuclei.

Previous studies in Japanese patients indicated that male sex, higher age, and advanced liver fibrosis before IFN therapy are risk factors for HCC in patients with sustained virologic response [Toyoda et al., 2000; Makiyama et al., 2004]. It has been suggested that subclinical tumors are most likely present in liver with severe fibrosis before HCV eradication. In the present study, however, histological findings before IFN therapy showed mild fibrosis (stage 2) in 8 HCC-patients with sustained virologic response. In 5 patients, HCC was diagnosed more than 10 years after the eradication of HCV by IFN. It is doubtful whether neoplastic cells induced by HCV had existed in liver with milder fibrosis and proliferated slowly over such a long period. On the other hand, HBV DNA was detected frequently in HCC-patients without cirrhosis. In addition, HBV DNA was detected in HCC-patients with sustained virologic response that developed more than 40 months after HCV eradication. These results suggest that occult HBV infection is related to carcinogenesis in non-cirrhotic patients with sustained virologic response.

Recently, nonalcoholic steatohepatitis (NASH) is one of the causes of underlying non-viral hepatic cirrhosis and cryptogenic HCC. At the onset of HCC in patients with steatohepatitis, the noncancerous liver is often cirrhotic. In the present study, the noncancerous liver was not cirrhotic in 12 of 16 HCC-patients with sustained virologic response. The frequency of obesity, diabetes mellitus, and alcohol intake, factors related

to steatohepatitis, in HCC patients with sustained virologic response were similar to those in HCC-patients with HCV. These findings suggest that steatohepatitis was not a major risk factor for HCC in patients with sustained virologic response.

In the present retrospective study, HBV DNA in liver was detected frequently in HCC of patients with sustained virologic response compared to in HCV-HCC. On the other hand, a follow up study for 15 patients with sustained virologic response showed that HBV DNA in liver was not detected in two patients in which HCC developed [Tsuda et al., 2004]. However, the number of examined patients in this report was small. A large scale of prospective study is necessary to define the role of occult HBV infection on hepatocarcinogenesis in patients with sustained virologic response.

In conclusion, the above findings provide compelling evidence that continuous existence of HBV DNA in liver may be one of the risk factors for the development of HCC in patients with sustained virologic response in Japan.

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