administration developed lamivudine resistance within 2 years. However, a negative result of HBV DNA at 6 months does not necessarily guarantee the absence of lamivudine resistance because nearly 30% of such patients developed resistance within 2 years. On the other hand, HBVcrAg levels of less than 4.7 log U/ml at 6 months are a useful indicator of patients who are unlikely to develop lamivudine resistance, because no such patients developed resistance during the follow-up period in the present study. Lower serum HBVcrAg levels may reflect lower levels of cccDNA in hepatocytes because the mRNAs of HBVcrAg are transcribed from the cccDNA (18, 22, 23). This possibility may explain our finding that patients whose HBVcrAg levels decreased sufficiently were unlikely to develop lamivudine resistance, because cccDNA provides the templates for viral and pregenomic messenger RNA (18, 22, 23), which may be a source of lamivudine-resistant strains.

In conclusion, our results suggest that measurement not only of HBV DNA but also of HBVcrAg is useful for predicting the occurrence of lamivudine resistance. HBV DNA measurement is valuable for identifying patients who are at high risk of developing this resistance and HBcrAg measurement is valuable for identifying those who are at low risk.

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# Expression of β-catenin in hepatocellular carcinoma

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## Abstract

**AIM:** The β-catenin has been recognized as a critical member of the Wnt signaling pathway and plays an important role in the generation/differentiation of many tissues. Inappropriate activation of this pathway has been implicated in carcinogenesis. The mechanism underlying the development as well as its prognosis of hepatocellular carcinoma (HCC) has remained unclear. The purpose of this study is to analyze the expression of β-catenin in HCC in relation to histological grades and viral hepatitis backgrounds.

**METHODS:** Thirty-two sections were selected at random from autopsy and surgical cases of HCC. Immuohistologically, the location and positivity of β-catenin expression in HCC was examined.

**RESULTS:** Normal hepatocytes did not express β-catenin. In 78% of HCC β-catenin was expressed at the membrane of the cells, with or without cytoplasmic and/or nuclear expression. The tumor cells with well- and moderately-differentiated grades expressed frequently at the membrane and cytoplasm compared with poorly-differentiated type. Nuclear expression of β-catenin was prone to occur in the tumor cells of poorly-differentiated grade. There were 15% of hepatitis C virus (HCV) backgrounds with nuclear expression. In contrast, there was 38% with nuclear expression in hepatitis B virus (HBV) backgrounds. In nonB-nonC hepatitis, no case expressed nuclear β-catenin.

**CONCLUSION:** The β-catenin expression in HCC cells was heterogenous among types of hepatitis viral infection. Wnt signaling pathway might be deeply involved in less-differentiated HCC and HBV background.



**Key words:** Hepatocellular carcinoma; β-Catenin; Immunohistochemistry

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is a major type of primary liver cancer and one of the rare human neoplasms etiologically linked to viral factors. Chronic infections with the hepatitis B virus (HBV) and the hepatitis C virus (HCV) have been implicated in about 80% of cases worldwide. However, the molecular mechanisms underlying their development are still poorly understood.

HCCs display gross genomic alterations, including DNA rearrangements associated with HBV DNA integration, loss of heterozygosity, and, less importantly, chromosomal amplifications and loss of imprinting[1]. Many genes with somatic mutations have been identified in these tumors. Most frequently involved genes are tumor suppressor genes such as p53, β-catenin, and retinoblastoma genes[2-4].

β-Catenin is a structure protein in the cadherin mediated cell-cell adhesive system as a regulator[5], and plays an important role in the generation/differentiation of tissues as well as in the repair of normal tissues. It is also known to act as a mediator in the Wingless/Wnt signal transduction pathway[6]. In HCC, accumulation of β-catenin was present in the early stage of HCC[7,8]. The previous studies investigated the correlation between β-catenin expression and the differentiation grades of HCC, and prognostic roles of β-catenin expression in HCC[9-11].

The purpose of this study is to investigate the expression of β-catenin in HCC in relation to histological grades and to viral hepatitis backgrounds.

## MATERIALS AND METHODS

Liver tissue sections were obtained at random of 32 sections from 15 autopsy cases and 13 surgical cases. They consisted of 20 males and 8 females. Age ranged from 36 to 86

years with an average of 64. The diagnosis was confirmed histopathologically in all cases, based mainly on examination of sections stained with H&E.

### *Immunohistochemistry*

The sections were stained immunohistochemically by the avidin-biotin complex method for β-catenin antibody (Mouse IgG1, BD Transduction Laboratories - BD Biosciences, SanJose, California, USA) with dilution of 1:200. For immunostaining, the sections were deparaffinized, washed with phosphate-buffered saline (PBS, pH 7.4) in 10 min, soaked in sodium citrate buffer (pH 6.0) and heated in microwave at 97 ℃ in 15 min for antigen retrieval. It was then allowed to cool at room temperature before being immersed into 0.3% $H_2O_2$/methanol to block endogenous peroxidase activity. The sections were pre-incubated with 10% normal bovine serum to prevent non-specific binding. Primary antibodies were incubated for 2 h at room temperature. Secondary antibodies, anti-mouse IgG was applied for 30 min, followed by incubation with avidin-peroxidase for 10 min and visualized with diaminobenzidine (DAB), rinsed and soaked in PBS for 3-5 min, thrice after each step. They were counterstained with Mayer hematoxylin.

For the evaluation of immunohistochemical staining of β-catenin, the constitutional expression on the cell membrane of bile duct at non-cancerous area was used as positive control (Figure 1). The membranous expression (Figure 2) was evaluated as (+) when at least one-third section of it was expressed or strong expression if less than one-third and (-) when it was unexpressed or less than one-third section was weakly expressed. The cytoplasmic expression was evaluated as + when it was stronger than the expression in non-cancerous areas, and (-) when the expression was the same as in non-cancerous area. In the nucleus, the expression (Figure 3) was evaluated as + when it was found clearly in any portion. All the results were observed and agreed by two pathologists (Dr. Ito and Dr. Wen).

## RESULTS

### *Relation between histological grade and hepatitis background*

There were 20 sections with hepatitis C virus background, eight with hepatitis B virus, three with nonB-nonC viral hepatitis, and one section with non-hepatitis infection. Eleven sections were well-differentiated type, 17 were moderately-differentiated type, and four sections were poorly-differentiated type. In well-differentiated cases, their backgrounds consisted of two with viral hepatitis B, six with viral hepatitis C, and three with nonB-nonC viral hepatitis. In moderately-differentiated sections, their backgrounds were three sections with viral hepatitis B, 13 sections with viral hepatitis C, and one section with nonB-nonC viral hepatitis. In poorly-differentiated cases, their backgrounds were three with viral hepatitis B and one section with viral hepatitis C.

### *Immunocytochemical expression of β-catenin*

Most sections (25 of 32 sections: 78%) expressed at the membrane of the cells. Among them, 17 of 32 sections (53%) were only expressed at the membrane (Figure 2). For cytoplasmic expression, there were 9 of 32 cases (28%), but 7 of 32 sections (22%) were only expressed at the cytoplasm and two cases were associated with membranous and/or nuclear expression. There were 7 of 32 (22%) sections with nuclear expression (Figure 3) and there were no cases with nuclear expression alone. Figure 4 shows percentages of immunocytochemical expression pattern in the tumor cells.

### *Relation between hepatitis background and β-catenin expression (Table 1)*

There were eight sections with HBV background which were mainly β-catenin expression of membranous type, in which three sections were both membranous and nuclear expressions. There were 20 sections with HCV background in which six sections were cytoplasmic. Two sections were membranous and nuclear type. One section was membranous and cytoplasmic expression. One section was membrano-cytoplasm-nuclear expression and 10 sections were membranous expression of β-catenin. The sections with nonB-nonC expressed only membranous type. Overall the nuclear localization of β-catenin was highly encountered in HBV background.

### *Relation with the grade of HCC histology of β-catenin expression (Table 2)*

For well-differentiated grade, there were five sections with membranous expression, three sections with cytoplasmic expression, two sections with membrano-nuclear expression, and one section with membrano-cytoplasmic expression. For

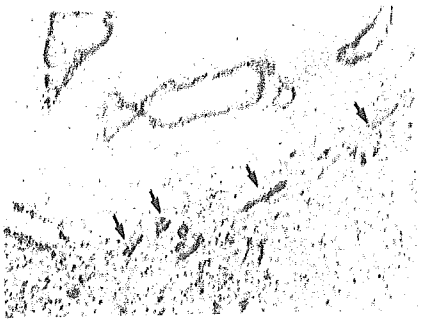

**Figure 1** Constitutive expression of β-catenin in normal bile ducts. Membranous expression is conspicuous in normal bile ducts and newly formed bile ducts (arrow heads), but no expression is observed in normal hepatocytes.

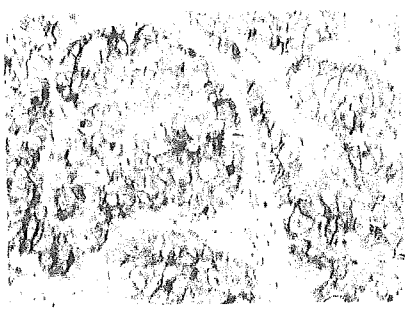

**Figure 2** Membranous expression. Membranous expression is conspicuous in tumor cell membranes. This case is moderately-differentiated HCC.

moderately-differentiated grade, there were 11 sections with membranous expression, four sections with cytoplasmic expression, and two sections with membrano-nuclear expressions. For poorly-differentiated grade, there was one section with membranous expression, two sections with membrano-nuclear expression, and one section with membrano-cytoplasm-nuclear expression.

There were 7 of 32 sections (22%) which expressed the nuclear type, in which three sections were encountered in poorly-differentiated grade, two sections in moderately-differentiated grade, and two sections in well-differentiated grade. In each differentiation grade, nuclear expression was highly detected in poorly-differentiated type (3 of 4 sections, 75%) compared with well (2 of 11 sections, 18%) and moderately-differentiated types (2 of 17 sections, 12%).

**Table 1** β-Catenin expression and viral hepatitis background

| | M (%) | M+N (%) | M+C (%) | M+N+C (%) | C(%) |
|---|---|---|---|---|---|
| HBV | 49 (4/8) | 38 (3/8) | 13 (1/8) | | |
| HCV | 50 (10/20) | 10 (2/20) | 5 (1/20) | 5 (1/20) | 30 (6/20) |
| NonB-nonC | 100 (3/3) | | | | |

M: membranous, M+N: membranous and nuclear, M+C: membranous and cytoplasmic, M+C+N: membranous, cytoplasmic, and nuclear, C: cytoplasmic.

**Table 2** β-Catenin expression and histological grade

| | M (%) | M+N (%) | M+C (%) | M+N+C (%) | C (%) |
|---|---|---|---|---|---|
| Well | 46 (5/11) | 18 (2/11) | 9 (1/11) | | 27 (3/11) |
| Moderate | 65 (11/17) | 12 (2/17) | | | 23 (4/17) |
| Poor | 25 (1/4) | 50 (2/4) | | 25 (1/4) | |

M: membranous, M+N: membranous and nuclear, M+C: membranous and cytoplasmic, M+C+N: membranous, cytoplasmic, and nuclear, C: cytoplasmic.

## DISCUSSION

Prevalence and localization of β-catenin nuclear expression and mutation varies among previous reports[7,9-15]. Also prognostic implication of β-catenin expression in hepatocarcinogenesis is inconsistent[9-11,14,15]. Some reports suggest better prognosis in cases with nuclear expression and mutation[10,16], but most reports indicate tumor progression and tumor cell proliferation[9,11,15].

In HCC, accumulation of β-catenin was present in the early stage of HCC. Most authors have described that β-catenin is strongly expressed on the membrane of HCC cells[7,9-11]. Suzuki *et al.* showed that β-catenin expression in nodule-in-nodule HCC is 41.7% at the membrane and 41.7% in the cytoplasm of the cells[7]. Inagawa *et al.* noted that about 61% exhibited increased membranous and/or cytoplasmic expression[9]. Hey-Chi Hsu *et al.* examined 366 cases of multifocal HCCs and stained 282 cases immunochemically for β-catenin, in which 212 cases (57.9%) expressed at the cell membrane alone and 70 cases (19.1%) expressed scattered nuclear expressions[10]. We have documented 78% β-catenin expression at the membrane with or without cytoplasmic and/or nuclear expression, in which 53% of them expressed only at the membrane of the cells. The tumor cells with well- and moderately-differentiated grade expressed at the membrane and cytoplasm for β-catenin. Nuclear expression of β-catenin occurred in moderately- and poorly-differentiated grades, although having no section with nuclear expression alone. In viral infection background, HBV-infected HCC expressed nuclear translocation in high prevalence. These findings suggest that nuclear expression of β-catenin is likely to be induced in less-differentiated type and HBV backgroud. Nuclear expression of β-catenin implies more important roles in view of tumor progression than membranous and cytoplasmic expression. β-catenin can enter the nucleus by binding the T-cell factor and lymphoid-enhancer factor family of DNA binding proteins, and regulates transcription of target genes, such as cyclin D1 and c-myc. Both c-mys and cyclin D1 are involved in the transition between the G1-S check-point of the cell cycle and do so by influencing the activity of retinoblastoma tumor-suppressor pRB[17,18].

Cyclin D1 is a major regulator of the progressing of cells into proliferation stage of the cell cycle. Increased β-catenin levels may promote neoplastic conversion by triggering cyclin D1 gene expression and consequently uncontrolled progression into the cell cycle. Activation of cyclin D1 and disruption of the Rb pathway are also commonly involved in liver tumorigenesis[18]. In our cases cyclin D1 was expressed only in poorly-differentiated HCCs (data not shown). In less-differentiated HCCs, cell proliferation might be induced via β-catenin/cyclin D1 signal pathway[10,19]. But the prevalent type of HCC, well- and moderately-differentiated type, does not seem to be involved

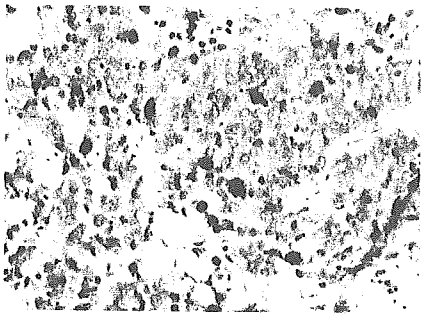

**Figure 3** Nuclear expression. Nuclear expression is encountered in the poorly-differentiated HCC (arrows). Most cells co-expressed β-catenin in the cytoplasm.

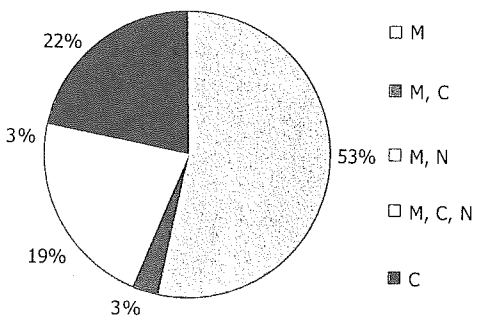


**Figure 4** Localization of β-catenin expression in cell level. M: membranous, M+C: membranous and cytoplasmic, M+N: membranous and nuclear, M+N+C: membranous, cytoplasmic, and nuclear, C: cytoplasmic.

in cyclin D1 overexpression. One study suggests that without cyclin D1 activation the β-catenin-related cell proliferation exists in hepatocarcinogenesis[9,20]. And the study on embryonic development indicated that β-catenin might not regulate cyclin D1 during liver development[21].

Statistical analysis clearly showed a distinction between the gene expression profiles of HCV and HBV-related HCC[22]. HBV-associated HCC exhibited involvement of different cellular pathways, those controlling apoptosis, p53 signaling and G1/S transition. In HCV-related HCC a more heterogenous pattern with an over-expression of the TGF-beta induced gene was identified. It is still unclear as to whether the gene expression profile in HCV or HBV-related HCC exhibits a degree of specificity[22].

By mutational analysis of β-catenin gene, Hey-Chi Hsu *et al.* indicated that mutation plays a more important role in the tumorigenesis of non-HBV-related HCC than in HBV-related HCC and different types of β-catenin mutations reflect different etiologies of carcinogenesis in specific tissue[10]. In this study, viral hepatitis backgrounds were 63% of HCV and 25% of HBV. This prevalence is similar to that of previous studies. There are differences of expression pattern for β-catenin between B and C viral hepatitis backgrounds. Nuclear translocation was highly observed in HBV background. These results suggest that the expression of β-catenin is influenced by virus infections and carcinogenesis of HBV and HCV infections are different. Integrated hepatitis B virus (HBV) DNA is present in many HCC, suggesting that HBV has a direct oncogenic effect through interaction with transformation-associated genes. The HBX protein of hepatitis B virus is thought to contribute to the development of carcinoma by disruption of intercellular adhesion. β-catenin was tyrosine-phosphorylated in a Src-dependent manner in HBX-expressing cells. Tyrosine phosphorylation of β-catenin by Src kinase results in an increase in its free cytosolic pool. Recent study suggests that HBX induces the stabilization and subsequent nuclear translocation of β-catenin by activating Src kinase and GSK3β suppression.

The overall cytological expression pattern was not consistent with previous reports, suggesting that ethnical and district differences are significant in Wnt signaling pathway in hepatocarcinogenesis. More studies are warranted to understand the expression of β-catenin which effects histological grade and viral hepatitis background in HCC better.

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# Infectious Source Factors Affecting the Severity of Sexually Transmitted Acute Hepatitis due to Hepatitis B Virus Genotype C

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**Key Words**

Hepatitis B virus · Acute hepatitis · Fulminant hepatitis · Infectious source · Sexual transmission · Precore mutation · Core promoter mutation

**Abstract**

***Objective:*** The aim of this study was to identify clinical features and virological aspects of infectious sources that are related to the severity of sexually transmitted acute hepatitis B virus (HBV) infection in patients, especially in cases of genotype C. ***Methods:*** Nineteen patients with acute HBV infection, 10 classified with severe acute hepatitis (SH) (prothrombin time; PT <40%) and 9 with typical acute hepatitis (AH) (PT >40%), and their infectious sources (all were sexual partners) were studied. Infectious source factors were analyzed in relation to the severity of hepatitis in the patients' partners. ***Results:*** The nucleotide homology of HBV-DNA between each pair was ≥98.9%. Sixteen were infected with HBV genotype C. Among the 16 infectious sources, age, numbers with elevated alanine aminotransferase (ALT, 7/9 vs. 1/7), anti-HBe positivity (8/9 vs. 1/7) and core promoter mutations at nt 1762 (7/9 vs. 1/7), nt 1764 (8/9 vs. 1/7) and precore mutation at nt 1896 (8/9 vs. 1/7) were significantly higher in the sources of SH than in those of AH. ***Conclusion:*** Higher age, elevated ALT, anti-HBe positivity and core promoter/precore mutations were possible risk factors for an infectious source of the severe form of sexually transmitted acute hepatitis due to HBV genotype C.



## Introduction

Hepatitis B virus (HBV) is one of the most common endemic viruses in the world, with more than 300 million people chronically infected. HBV causes a variety of liver diseases, including self-limiting acute hepatitis, fulminant hepatitis, chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Acute HBV infection induces acute hepatitis, and 1–2% of those patients develop fulminant hepatitis.

Both vertical and horizontal means are known for HBV transmission, and the former can be well prevented by injection with immunoglobulin containing a high titer of antibody to the hepatitis B surface antigen (HBsAg) combined with HBV vaccine to newborns [1]. Among the infectious routes of horizontal HBV transmission, post-transfusion hepatitis has decreased dramatically by the

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screening of donated blood [2, 3], and sexual transmission or transmission from partners has become the most common route in adults, in both western and eastern countries [4–9]. To diminish the occurrence of fulminant hepatitis B in adults, it is important to understand the risk factors of the infectious source that have a relationship with the severity of sexually transmitted hepatitis in patients; however, those factors are not well known. The purpose of this study was to determine infectious source factors that are related to the severity of sexually transmitted hepatitis in patients.

## Patients and Methods

*Patients and Infectious Sources*

Nineteen patients with acute HBV infection and their sexual partners (which included spouses), who were proven to be positive for HBsAg, were enrolled. All patients and infectious sources were Japanese living in the western part of Japan. The patients with acute HBV infection included all available patients with known infectious sources attended in our hospitals between 1995 and 2003. Patients with acute HBV infection were divided into 2 groups according to the level of prothrombin time (PT), as PT or level of clotting factors are important markers for estimating the severity of liver disease [10, 11]. The severe acute hepatitis (SH) group was composed of 10 patients who showed severe liver dysfunction with prolonged PT (PT activity percentage <40%), and the self-limited typical acute hepatitis (AH) group was composed of 9 patients who had a PT activity percentage greater than 40%. Among the 10 patients in the SH group, 6 were diagnosed with fulminant hepatitis which was defined as severe liver dysfunction, showing PT of less than 40%, with the presence of hepatic encephalopathy within 8 weeks from the first appearance of symptoms [12], while the other 4 were diagnosed with acute hepatitis severe type that was defined as acute hepatitis, with PT of less than 40%, without hepatic encephalopathy. The ratio of SH to AH was high in this study, because all patients with SH and their sexual partners or spouses were cooperative and willing to be involved in this study, whereas some patients with AH and some of their sexual partners with AH rejected to be involved in this study.

The diagnostic criteria for acute HBV infection were positivity for IgM type antibody to anti-hepatitis B core (HBc) and for anti-HBc with a low titer (<90% in 200-fold diluted serum). Only patients with acute HBV infection who were negative for IgM type antibody to hepatitis A virus (IgM-anti-HA), antibody to hepatitis C virus (anti-HCV) and HCV-RNA were included in this study.

Sexual partners (n = 19) who were proven to be positive for serum HBsAg and suspected of being the infectious sources were the main subjects of this study. Age, sex, diagnosis, liver function tests, HBV markers and HBV sequences of the core promoter to the precore region were analyzed. Serological and biochemical tests were performed between 1 and 4 weeks from the onset of acute HBV infection. Diagnoses were based on the results of biochemical and serological tests, except for 5 subjects who were diagnosed by histological examination (3 with chronic hepatitis, 1 with liver cirrhosis, 1 with hepatocellular carcinoma). Subjects with normal liver function test results were diagnosed as asymptomatic carriers, whereas those with elevated transaminase and delay in an indocyanine green clearance test without signs of portal hypertension were diagnosed with chronic hepatitis.

Acute phase serum samples (taken less than 2 weeks after onset) from patients with acute HBV infection were obtained and stored at −80°, until used. Serum samples from the sexual partners were obtained within 4 weeks from the onset of acute HBV infection and were also stored at −80°.

The purpose of this study was explained to all patients or their families as well as the sexual partners or spouses. Written informed consent was obtained from all of the subjects who were involved in this study.

*Serological Markers*

HBsAg (AxSYM HBsAg, Dainabot, Tokyo, Japan), anti-HBc (AxSYM HBc, Dainabot), IgM-anti-HBc (AxSYM HBc-M, Dainabot), hepatitis B e antigen (HBeAg)(AxSYM HBeAg, Dainabot), antibody to HBeAg (anti-HBe)(AxSYM HBeAb, Dainabot), IgM-anti-HA (AxSYM HA-M, Dainabot), anti-HCV (Ortho Diagnostics, Tokyo, Japan) and HCV-RNA (Amplicor TM HCV, Roche Diagnostics, Mannheim, Germany) were assayed, using commercial kits. Viral load was estimated by the level of HBV-DNA or serum DNA polymerase activity. Quantification of HBV-DNA was done either by the transcription-mediated amplification (TMA) method (GEN-PROBE Inc., San Diego, Calif., USA), a branched DNA probe assay (Daiichi-Kagaku, Tokyo, Japan) or the solution hybridization method (Toray Industries, Inc., Tokyo). Serum DNA polymerase activity was measured using Kaplan's method, with some modifications [13]. As this was a retrospective study, the assays of the viral load were not identical among the patients who had been admitted to several different hospitals; moreover, the amount of stored sera were not enough.

*Sequencing of Core Promoter and Precore Region*

DNA was extracted from sera. Briefly, 50 µl of each serum sample was incubated with lysis buffer containing proteinase K. DNA was extracted using a phenol-chloroform solution and precipitated with ethanol, and HBV-DNA was amplified by polymerase chain reaction (PCR). For the amplification of the core promoter and precore regions, a semi-nested PCR was performed using primers P1, F3 and P4 (P1: 5′-AAGGACTGGGAGGAGTTGGGGGA-3′, nt 1725–1747; P4: 5′-GATACAGAGCAGAGGCGGTGT-3′, nt 2015–1995; F3: 5′-GTCAGAAGGCAAAAAAGAGAG-3′, nt 1966–1946; P1 and P4 for first round PCR, and P1 and F3 for second round PCR). Direct sequencing was done using a commercially available kit (Big-Dye Terminator Cycle Sequencing FS Ready Reaction Kit, Applied Biosystems, Alameda, Calif., USA) with P1 and F3 used as the sequencing primers [14].

The accuracy of the sequences was ensured by identification of the sequence data of the genome obtained by the sense sequencing primer (P1) and that obtained by the anti-sense sequencing primer (F3).

*HBV Genotyping*

The HBV genotype was determined, based on the restriction fragment length polymorphism patterns of the S gene sequence [15].

*Statistical Analysis*

Statistical analyses were performed using Wilcoxon's Rank test and Fisher's exact test. p values of less than 0.05 were considered statistically significant.

**Table 1.** Clinical features of patients with acute hepatitis B virus infection[a]

| Patient | Diagnosis | Sex/age | T. bil mg/dl | ALT IU/l | PT % | HBeAg/Ab | Viral load | | Genotype | Mutation | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | DNA-P, cpm | HBV-DNA | | nt 1762T | 1764A | 1896A |
| SH1 | FH | M/51 | 23.6 | 1,296 | 9 | –/+ | 10 | | C | – | – | – |
| SH2 | FH | M/44 | 14.9 | 7,126 | 29 | –/+ | 10 | | C | – | + | + |
| SH3 | FH | M/40 | 33.4 | 4,895 | 19 | –/+ | 3 | | C | + | + | + |
| SH4 | FH | F/25 | 7.3 | 4,664 | 26 | –/+ | NE | | C | + | + | + |
| SH5 | FH | F/44 | 15.5 | 4,775 | 14 | –/+ | 1 | | C | + | + | + |
| SH6 | AHs | M/24 | 8.9 | 10,880 | 18 | –/+ | 119 | | C | + | + | + |
| SH7 | AHs | F/29 | 10.8 | 4,908 | 22 | –/+ | 56 | | C | + | + | + |
| SH8 | AHs | F/27 | 7.9 | 2,139 | 36 | –/+ | NE | | C | + | + | + |
| SH9 | AHs | F/26 | 6.4 | 2,850 | 30 | –/+ | 2 | | C | + | + | + |
| AH1 | AH | M/25 | 24.4 | 2,170 | 55 | –/+ | 980 | | C | – | – | – |
| AH2 | AH | M/22 | 2.1 | 3,040 | 90 | +/– | NE | | C | – | – | – |
| AH3 | AH | M/41 | 16.8 | 1,082 | 64 | –/+ | | <0.7 mEq/ml | C | – | – | – |
| AH4 | AH | F/40 | 4.9 | 797 | 98 | –/+ | | 5.6 LGE/ml | C | – | – | – |
| AH5 | AH | M/26 | 9.6 | 4,080 | 68 | –/+ | 222 | | C | – | + | + |
| AH6 | AH | F/28 | 1.6 | 2,892 | 54 | –/+ | | 6.3 LGE/ml | C | – | – | – |
| AH7 | AH | M/26 | 2.5 | 3,039 | 75 | –/+ | NE | | C | – | – | – |

[a] SH = Severe acute hepatitis; FH = fulminant hepatitis; AHs = acute hepatitis severe type; AH = typical acute hepatitis; T. bil = total bilirubin; ALT = alanine aminotransferase; PT = prothrombin time; DNA-P = DNA polymerase; NE = not examined; LGE = log genome equivalents.

**Table 2.** Clinical features of infectious sources of patients with acute HBV infection[a]

| Infectious sources | Transmitted patient | Clinical diagnosis | Sex/age | T. bil mg/dl | ALT IU/l | HBeAg/Ab | Viral load | | Genotype | Mutation | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | DNA-P, cpm | HBV-DNA | | nt 1762T | 1764A | 1896A |
| IS1 | SH1 | ASC | F/53 | 0.5 | 16 | –/+ | 25 | | C | – | – | – |
| IS2 | SH2 | CH | F/40 | 0.6 | 54 | –/+ | 232 | | C | – | + | + |
| IS3 | SH3 | CH | F/49 | 0.7 | 64 | –/+ | 4.5 | | C | + | + | + |
| IS4 | SH4 | CH | M/23 | 0.4 | 33 | –/+ | NE | | C | + | + | + |
| IS5 | SH5 | LC | M/57 | 2.2 | 96 | –/+ | 6 | | C | + | + | + |
| IS6 | SH6 | CH | F/21 | 0.1 | 79 | +/– | 9,846 | | C | + | + | + |
| IS7 | SH7 | CH | M/22 | 0.8 | 129 | –/+ | 4 | | C | + | + | + |
| IS8 | SH8 | LC | M/38 | 0.7 | 70 | –/+ | | 7.2 LGE/ml | C | + | + | + |
| IS9 | SH9 | HCC | M/45 | 0.5 | 114 | –/+ | 68 | | C | + | + | + |
| IS10 | AH1 | ASC | F/22 | 0.5 | 22 | +/– | 1,500 | | C | – | – | – |
| IS11 | AH2 | ASC | F/20 | 0.3 | 34 | +/– | NE | | C | – | – | – |
| IS12 | AH3 | ASC | F/28 | 0.3 | 11 | +/– | | >3,800 mEq/ml | C | – | – | – |
| IS13 | AH4 | ASC | M/39 | 0.3 | 24 | +/– | | 510 pg/ml | C | – | – | – |
| IS14 | AH5 | CH | F/19 | 0.5 | 20 | –/+ | 222 | | C | + | + | + |
| IS15 | AH6 | CH | M/28 | 0.3 | 549 | +/– | | 220 mEq/ml | C | – | – | – |
| IS16 | AH7 | ASC | F/22 | 0.5 | 10 | +/– | | 8.5 LGE/ml | C | – | – | – |

[a] IS = Infectious source; SH = severe acute hepatitis; AH = typical acute hepatitis; ASC = asymptomatic carrier; CH = chronic hepatitis; LC = liver cirrhosis; HCC = hepatocellular carcinoma; T. bil = total bilirubin; ALT = alanine aminotransferase; DNA-P = DNA polymerase; NE = not examined; LGE = log genome equivalents.

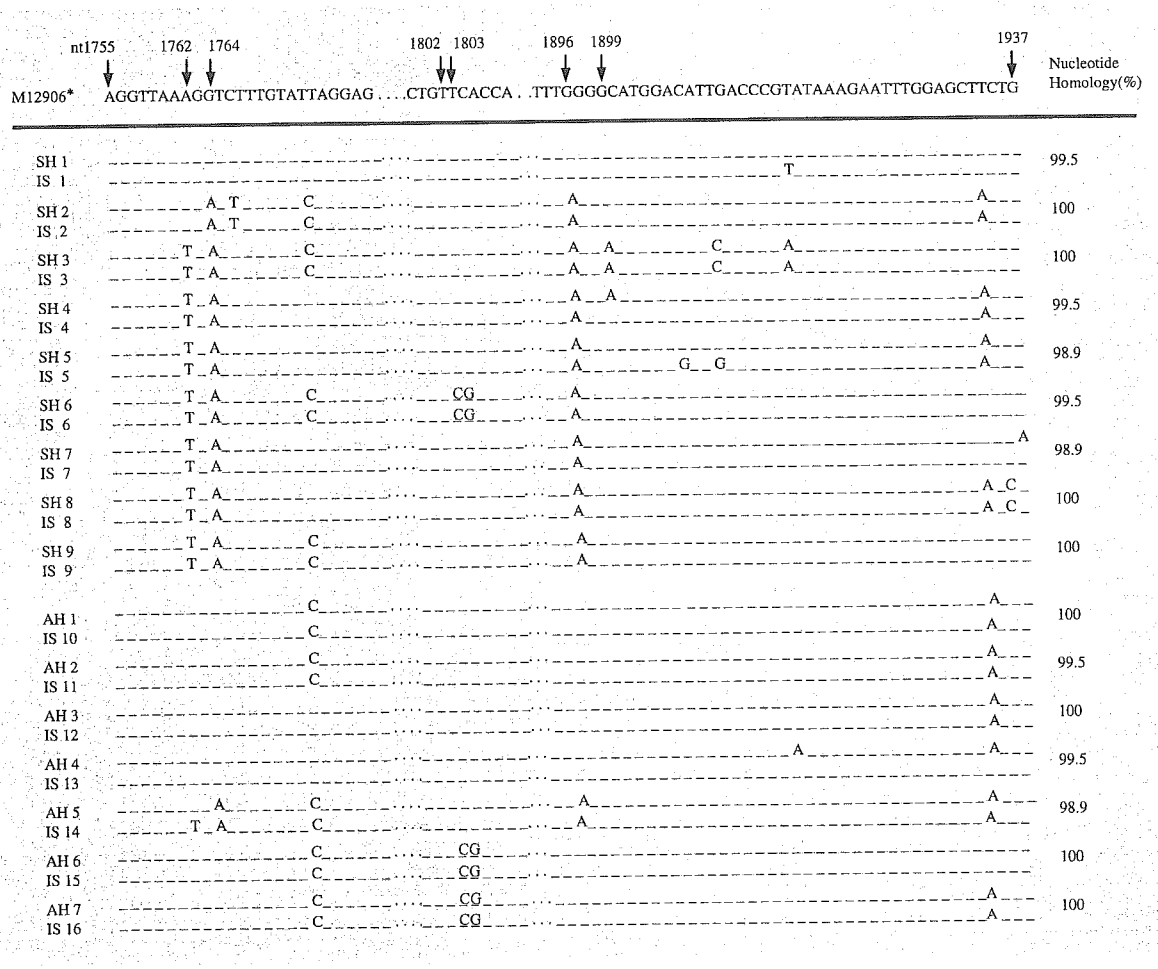


**Fig. 1.** Comparison of HBV nucleotide sequence in patients with acute HBV infection and their infectious sources. SH = Severe acute hepatitis; AH = typical acute hepatitis; IS = infectious source. * From Kobayashi and Koike [49].

## Results

*Features of Patients with Acute HBV Infection*

Sixteen patients with acute HBV infection were infected with genotype C, 2 with genotype D and 1 with genotype A. Table 1 shows clinical features of 16 patients with acute HBV genotype C infection, with data from the early stage of onset of hepatitis. There were no differences for age and sex distribution between the SH and AH groups. One patient with fulminant hepatitis (SH2) died, 1 with fulminant hepatitis (SH3) received a liver transplant and the other 14 were alive at the time of this study. Among those with genotype C HBV infection, core promoter mutations (nt 1762T, nt 1764A) and a precore mutation (nt 1896A) were significantly higher in patients with SH (7/9 vs. 0/7, $p < 0.05$; 8/9 vs. 1/7, $p < 0.01$; 8/9 vs. 1/7, $p < 0.01$, respectively).

*Features of Infectious Sources*

Among all 19 infectious sources, 2 were infected with genotype D, 1 had genotype A and 16 had genotype C. Five of them were spouses and 14 were sexual partners. The HBV genotype was identical in all 19 pairs. Table 2 shows data of the infectious source subjects with genotype

**Table 3.** The comparison of clinical features of infectious sources of patients with acute genotype C HBV infection[a]

| | Infectious sources of patients with | | p value |
|---|---|---|---|
| | SH | AH | |
| Number | 9 | 7 | |
| Age, median (range), years | 40 (21–57) | 22 (19–39) | 0.037 |
| Sex, M/F | 5/4 | 2/5 | 0.358 |
| Diagnosis | | | 0.035 |
| ASC | 1 | 5 | |
| CH, LC, HCC | 8 | 2 | |
| T. bil | | | 0.999 |
| Within normal range, < 1.2 mg/dl | 8 | 7 | |
| Elevated ≥ 1.2 mg/dl | 1 | 0 | |
| ALT | | | 0.041 |
| Within normal range (<40 IU/l) | 2 | 6 | |
| Elevated (≥ 40 IU/l) | 7 | 1 | |
| HBeAg/anti-HBe state | | | 0.009 |
| HBeAg + | 1 | 6 | |
| Anti-HBe + | 8 | 1 | |
| Mutation in core promoter region | | | |
| nt 1762T | 7 | 1 | 0.041 |
| nt 1764A | 8 | 1 | 0.009 |
| Mutation in precore region | | | |
| nt 1896A | 8 | 1 | 0.009 |

[a] ASC = Asymptomatic carrier; CH = chronic hepatitis; LC = liver cirrhosis; HCC = hepatocellular carcinoma; T. bil = total bilirubin; ALT = alanine aminotransferase; SH = severe acute hepatitis; AH = typical acute hepatitis.

C infection. Among the 9 sources of SH, 1 was diagnosed as an asymptomatic carrier and the other 8 had chronic liver diseases (5 with chronic hepatitis, 2 with liver cirrhosis, 1 with hepatocellular carcinoma). Of the 7 sources of AH, 5 were asymptomatic carriers and 2 had chronic hepatitis.

*Comparison of HBV between Partner Pairs*

Nucleotide sequences of all subjects infected with genotype C between nt 1755 and nt 1937 (183 bases) were analyzed, and their sequences are partially shown in figure 1. A comparison of the nucleotide sequences revealed that the nucleotide homology between each pair was ≥98.9%, and the same nucleotide substitutions at the same positions were seen in each pair. Therefore, the sexual partners were confirmed as the respective infectious sources.

*Features of Infectious Sources Infected with Genotype C*

A comparison of clinical features between 9 infectious sources of SH and 7 infectious sources of AH is shown in table 3. Statistical differences were observed for age, diagnosis, ALT, HBeAg/Ab and mutations at nt 1762, nt 1764 and nt 1896. Age ($p < 0.05$), ratio of patients with chronic liver diseases ($p < 0.05$), elevated ALT ($p < 0.05$), anti-HBe ($p < 0.01$) and the mutations at nt 1762T ($p < 0.05$), nt 1764A ($p < 0.01$) and nt 1896A ($p < 0.01$) were significantly higher in the infectious sources of SH than in those of AH. Among 6 of the infectious sources diagnosed as asymptomatic carriers, 1 was a source of SH and positive for anti-HBe, while the other 5 were infectious sources of AH and positive for HBeAg. Multivariate analysis was not possible in this study because the number of patients was restricted.

## Discussion

Previous studies concerning factors related to the severity of acute HBV infection were mainly performed by analyzing virological aspects of patients. Many reports, mainly from Asia, have shown that the G to A mutation at nt 1896 in the precore region that induces translational

stop codon, and the A to T mutation at nt 1762 and the G to A mutation at nt 1764 in the core promoter region are frequently found in patients with fulminant hepatitis [16–21]. These mutations in the precore and core promoter regions are related to a reduction of the HBeAg by the translational and transcriptional levels, respectively. The mechanisms involved with severe liver damage and these mutations are under investigation in relation to viral replication, viral gene expression and localization of viral proteins [22–27]. On the other hand, there are many reports from western countries showing that the frequencies of these mutations were not high among fulminant hepatitis patients and that they were not related to the severity of hepatitis [28–32]. It has been reported that the frequencies of these mutations are different among HBV genotypes [33–36]. The differences of frequency of these mutations in fulminant hepatitis in western and eastern countries may be related to the difference of HBV genotype distribution throughout the world, as genotypes B and C are major genotypes in East Asia, and genotypes A and D are major in the US and Europe [33, 34, 37–39].

Several investigators have studied infectious source factors in cases of infant patients with fulminant hepatitis B, and some results have indicated that anti-HBe positivity or mutant HBV with a precore mutation (nt 1896G to A) in the mother has a relation with fulminant hepatitis in the child; however, some reports are contradictory [40–44]. In cases of adult patients with acute HBV infection, only a few reports have discussed the virological aspects of the infectious sources. Aye et al. [45] studied 7 adult patients with acute HBV infection and 7 HBV carriers who were their infectious sources, and found that all 5 infectious sources with acute hepatitis B were positive for HBeAg without precore mutant HBV and 4 of the 5 were healthy carriers, whereas 2 of 2 infectious sources of fulminant hepatitis were patients with chronic hepatitis and 1 was positive for anti-HBe with precore mutated HBV. Further, Yotsumoto et al. [46] and Tanaka et al. [47] noted that infectious sources of adult patients with fulminant hepatitis were positive for anti-HBe with a precore mutation. However, there are no known reports that have performed statistical analysis of the risk factors in infectious sources in relationship to the severity of hepatitis in partner patients.

In the present study, we analyzed the clinical features and viral genomes of infectious sources infected with genotype C HBV, which is the main genotype found in our region. It has been proven that the infectious source factors that related to the transmission of SH were higher age, anti-HBe, chronic liver diseases, ALT abnormality and mutations in core promoter and precore regions. It is interesting that all of these factors were related to the immune pressure to HBV in the chronically infected host. Therefore, it is suspected that HBV strains that are able to replicate under host immune pressure may have a relationship to SH in the receivers. HBeAg-positive asymptomatic carriers are thought to be at the immune tolerance stage in the course of chronic HBV infection. All patients infected by an HBeAg-positive asymptomatic carrier were diagnosed with AH, and no infectious sources with SH were diagnosed as an HBeAg-positive asymptomatic carrier. One infectious source of SH was diagnosed as ASC; however, this source was positive for anti-HBe, and it may be better to diagnose this case as an inactive HBsAg carrier state [48]. These results also support the suspicion that replication-competent HBV strains under immune pressure have a relation with SH in receivers. These results of adult acute HBV infection were not inconsistent with reports of infant cases with fulminant hepatitis B, suggesting the importance of anti-HBe and the precore mutant in mothers.

The present results confirmed that core promoter or precore mutations are related to the severity of acute genotype C HBV infection, as seen from the analyses of HBV in patients with acute HBV infection and their infectious sources. However, all but one infectious sources in this study were infected with either mutant type HBV (nt 1762T, nt 1764A and nt 1896A) or wild type HBV (nt 1762A, nt 1764G and nt 1896G); therefore, it was not possible to analyze and compare the impact of the nature of these 3 mutations with the severity of hepatitis in infected patients.

Because of the restricted number of patients in this study, statistical analysis could only be done for genotype C. Risk factors relating to the severity of hepatitis might be different among different genotypes. This issue should be clarified in the future.

In conclusion, among the partners of patients chronically infected with genotype C HBV, those who were diagnosed with chronic liver diseases, positive for anti-HBe and infected with core promoter (nt 1762T, nt 1764A) or precore (nt 1986A) mutants were identified as risky infectious sources of sexually transmitted severe hepatitis.

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# Risk factors for development of hepatocellular carcinoma in patients with chronic hepatitis C after sustained response to interferon

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***Background.*** Interferon (IFN) is expected to prevent the progression of hepatitis C virus infection to cirrhosis and the development of hepatocellular carcinoma (HCC), but there have been several reports of the development of HCC after a sustained response to IFN. Our aim was to elucidate the incidence and clinical features of, and risk factors for, HCC in sustained responders to IFN, taken for the treatment of chronic hepatitis C. ***Methods.*** We designed a retrospective cohort study conducted at 16 major Hospitals. The subjects were a total of 1056 patients showing sustained responses, 29 of whom developed HCC. ***Results.*** The incidence of HCC per 100 person-years was 0.56 (95% confidence interval, 0.35–0.76) in sustained responders. By the Cox proportional hazard model, we found that older age, higher serum aspartate aminotransferase level, and lower platelet count before IFN therapy were independent risk factors associated with the development of HCC. A risk index of HCC development, based on the coefficients of these risk factors, was used to classify patients into three groups, with low, intermediate, and high risk. The incidence rates of HCC for these three groups were 0.11, 0.44, and 1.98 per 100 person-years, respectively. The median period to the development of HCC was 4.6 years (range, 1.4–9.0 years), and there were no other specific clinical features of the HCC that developed in these patients. ***Conclusions.*** This study suggests that the risk of development of HCC is not completely eliminated in sustained responders to IFN. These findings may be useful in determining a follow-up strategy after a sustained response to IFN.

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**Key words:** hepatitis C virus, hepatocellular carcinoma, interferon, sustained response

## Introduction

Hepatitis C virus (HCV) infection is one of the most common causes of chronic hepatitis, and it is also a major risk factor for hepatocellular carcinoma (HCC).[1,2] Chronic hepatitis C is often asymptomatic and mild, but may slowly progress to liver cirrhosis and eventually to HCC.[3–5] Therefore, it has been assumed that eradication of HCV would provide the most effective means of preventing HCC.

Currently, interferon (IFN) represents the mainstay of treatment for chronic hepatitis C.[5–9] IFN therapy can lead to a decrease in serum transaminase activity, and to the disappearance of serum HCV RNA in patients with chronic hepatitis C. These patients appear to benefit by the prevention of progression to cirrhosis and HCC.[5,7,10–14] However, HCC can still occur in patients who are treated successfully with IFN, i.e., those showing a sustained response to the therapy.[5,10–25] The incidence and clinical features of HCC, and the risk factors for carcinogenesis, have not yet been investigated, although they have been documented in individuals and in small numbers of patients.[5,10–25] We investigated a large cohort of patients showing a sustained response to IFN therapy given for chronic hepatitis C. Our aims were to assess the incidence of HCC in these patients and to discover the clinical variables that may be associated with the development of HCC. Our study also focused on the clinical features of HCC. We designed a multicenter retrospective cohort study, because a single-institution study would have provided inadequate numbers of sustained responders who developed HCC.

## Patients and methods

### *Patients*

This study was conducted at 16 major hospitals belonging to the Japanese Society of Gastroenterology, Kyushu Division. A large cohort of sustained responders to IFN therapy given for chronic hepatitis C, in whom HCC had, or had not, been detected, was assembled consecutively by means of data collection instruments. All sustained responders included in the study were positive for HCV RNA before IFN therapy, and were followed up for more than 1 year after termination of IFN therapy, during the period July 1988 to August 2001. Sustained response was defined as the presence of HCV RNA negativity (determined by using qualitative HCV RNA assay) more than 6 months after the termination of IFN therapy. Diagnosis of HCC was based either on histological examination or on typical computed tomographic and/or angiographic findings at each institution. Patients were excluded if HCC was detected within 1 year after the termination of IFN therapy, because in such cases it was highly likely that the cancer had been present at the end of the IFN therapy. In Japan, at the time of the study, the standard schedule was 6–10 MU IFN-α every day for the first 2–4 weeks and then the same dose given three time a week for the following 20–22 weeks, or 6 MU IFN-β every day for 6–8 weeks.

During the study period at the 16 hospitals, a total of 3504 patients with chronic hepatitis C had received IFN therapy and had been followed up for more than 1 year thereafter, and a sustained response was obtained in 1091 (31.1%) of them. Among the sustained responders, 30 patients (2.7%) developed HCC. By means of the data collection instrument, we requested individual clinical data before IFN therapy for all sustained responders, as well as clinical data at the time of diagnosis of HCC for patients who had developed HCC. The clinical data for all 1091 sustained responders identified were obtained from the 16 hospitals (8 university hospitals and 8 regional hospitals) listed in the appendix. Of these patients, 35 were excluded from the analysis because of the development of HCC within 1 year after IFN therapy (1 patient) or insufficient clinical records before commencement of IFN therapy (34 patients). The final study population comprised a total of 1056 patients showing sustained response to IFN therapy given for chronic hepatitis C, 29 of whom had developed HCC.

## Methods

To identify risk factors for the development of HCC in sustained responders to IFN therapy, we used univariate analysis and multivariate analysis to investigate 23 variables before IFN therapy for their relationship to the development of HCC. These variables were chosen by considering possible factors involved in the development of HCC, as indicated by previous investigations,[1–5,10–25] or suggested from our own clinical experience. Each variable, which was classified as host-related or treatment-related, was divided into one of two subgroups on the basis of clinically meaningful values. HCV RNA load was determined quantitatively by competitive reverse-transcription polymerase chain reaction (RT-PCR), branched-DNA probe assay, or Amplicor-HCV monitor assay.[26–28] When the serum HCV RNA level was more than $10^6$ equivalents/ml by branched DNA assay, more than $10^6$ copies/ml by competitive RT-PCR, or more than $10^5$ copies/ml by Amplicor-HCV monitor assay, it was designated as a high viral load; an HCV RNA level of $10^5$ copies/ml by the Amplicor-HCV monitor assay has already been demonstrated to correspond to approximately $10^6$ equivalents/ml by the branched DNA probe assay or $10^6$ copies/ml by competitive RT-PCR.[26–28] HCV subtype was classified by either the method of Okamoto et al.,[29] or Tanaka et al.'s method.[30] Genotypes 1a and 1b corresponded to serological group 1, and genotypes 2a and 2b corresponded to serological group 2, according to the Simmonds et al.[31] classification.[31] The data from liver biopsies that were done within 6 months before IFN therapy were included in this study. Assessments of the staging of liver fibrosis and the grade of inflammatory activity were based on the classification of Desmet and colleagues,[32] in which staging is defined as follows: F0 (no fibrosis), F1 (fibrous portal expansion), F2 (bridging fibrosis), F3 (bridging fibrosis with architectural distortion), and F4 (cirrhosis), and grading is defined as follows: A0 (no activity), A1 (mild activity), A2 (moderate activity), and A3 (severe activity).

To elucidate the clinical features of HCC that developed in sustained responders, 17 variables at the time of diagnosis of HCC were investigated. Number of tumors, maximum tumor size, portal vein invasion, hepatic vein invasion, and bile duct invasion were examined by ultrasonography, computed tomography, and/or angiography. The period to the development of HCC was measured from the day of termination of IFN therapy to the day when HCC was first diagnosed by imaging modalities, such as ultrasonography or computed tomography. The follow-up period for the detection of HCC after termination of IFN therapy was defined as the interval during which checks for HCC were done using tumor markers and/or imaging modalities.

### *Statistical analysis*

Follow up ended with the last recorded visit before August 31, 2001. Incidences were calculated in person-

**Table 1.** Patient characteristics of 1056 sustained responders to interferon therapy given for chronic hepatitis C

| | | Number of patients |
|---|---|---|
| **Host-related variables** | | |
| Age (years) | Median (range) | 50 (11–76) |
| Sex | Male | 711 (67%) |
| History of blood transfusion | Positive | 266 (27%) |
| Alcohol abuse[a] | Positive | 78 (8%) |
| Smoking habit[b] | Positive | 248 (38%) |
| HCV viral load | High ($\geq 10^6$) | 159 (21%) |
| HCV serologic group | Group 1 | 372 |
| | Group 2 | 466 |
| Hepatitis B surface antigen | Positive | 17 (2%) |
| **Treatment-related variables** | | |
| Interferon type | α | 829 (79%) |
| | β | 166 (16%) |
| | α + β | 61 (6%) |
| Total amount of interferon (MU) | Median (range) | 480 (42–1740) |
| Treatment period (weeks) | Median (range) | 22 (2–56) |
| Prior interferon therapy | Positive | 87 |

HCV, hepatitis C virus
[a] Alcohol intake, ≥80 g/day × 5 years
[b] Smoking habit, ≥20 cigarettes/day for ≥10 years

years; incidence curves of HCC were calculated by the Kaplan-Meier method; and differences in survival were evaluated by log rank tests. Hazard ratios and trend *P* values were calculated by treating the categories as ordinal variables. The Cox proportional hazard model was used to determine the most significant variables related to the development of HCC. All patients were then assigned a risk index value for the development of HCC, as follows: the value of each factor in the final model was multiplied by its corresponding regression coefficient, and these values were totaled to obtain the risk index for each patient. Stratification of the patients was conducted on the basis of this risk index. All *P* values were two-tailed and were considered significant when less than 0.05.

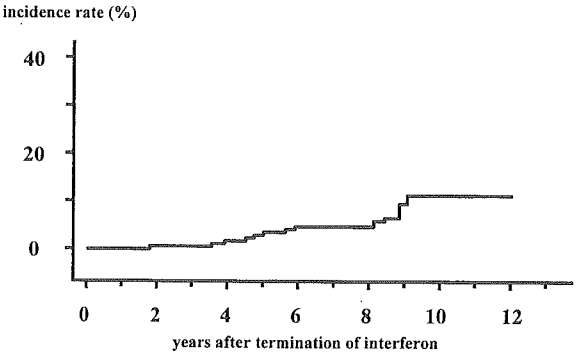


**Fig. 1.** Cumulative incidence of hepatocellular carcinoma in 1056 sustained responders to interferon therapy given for chronic hepatitis C

## Results

### *Patient characteristics*

Table 1 summarizes the patient characteristics of the 1056 sustained responders to IFN therapy given for chronic hepatitis C. The median age was 50 years (range, 11–76) years, and there were 711 men and 345 women ( sex ratio, 2.1:1). Hepatitis B surface antigen was positive in 17 patients (2%). The HCV serological group was group 1 in 372 patients and group 2 in 466 patients, and thus a higher proportion of patients were in serological group 2. A total of 829 patients (79%) received IFN-α, 166 patients (16%) received IFN-β, and 61 patients (6%) received both. The median dose and duration of IFN administration were 480 MU and 22 weeks, respectively. No patients received peginterferon or combination therapy with ribavirin, and 87 patients (8%) received more than two cycles of IFN therapy.

### *Incidence of HCC*

Twenty-nine of the 1056 sustained responders developed HCC, with a median follow-up period of 4.7 years. The incidence of HCC per 100 person-years was 0.56 (95% confidence interval, 0.35–0.76), and the incidences of HCC at 3, 5, 7, and 10 years after the termination of IFN therapy were 0.5%, 3.3%, 4.9%, and 11.1%, respectively (Fig. 1).

*Univariate analyses*

On univariate analysis (Table 2), age more than 60 years, positive smoking habit, platelet count less than $15 \times 10^4/mm^3$, aspartate aminotransferase (AST) more than 100 IU/l, prothrombin time less than 80%, and higher fibrosis stage (incidence of HCC per 100 person-years: F0, 0.00; F1, 0.27; F2, 0.47; F3, 0.62; F4, 1.31) were significant risk factors associated with the development of HCC. Alcohol abuse, total bilirubin, albumin, alanine aminotransferase, virological variables (viral load, serological group), tumor markers (alpha-fetoprotein, protein induced by vitamin K absence or antagonist-II), and treatment-related variables (treatment period, IFN type, total amount of IFN) were not significant risk factors.

*Multivariate analyses*

All variables whose $P$ values were less than 0.20 on the univariate analyses were entered into the multivariate analyses (Table 3). However, history of blood transfusion, smoking habit, prothrombin time, and indocyanine green retention rate at 15 min (ICG R15) were not included in the model because inadequate data were available. Multivariate regression analysis, which assessed the independent predictive importance of each variable studied for the development of HCC, showed that older age, higher serum AST level, and lower platelet count were significantly related to the development of HCC.

*Risk groups based on the regression model*

For the clinical application of these findings, a risk index was calculated based on the regression coefficients derived from the three variables identified by multivariate analysis. The index equation was as follows: $1.14 \times$ (0, age $\leq$ 60 years; 1, age $>$ 60 years) + $1.13 \times$ (0, AST $\leq$ 100 IU/l; 1, AST $>$ 100 IU/l) + $1.02 \times$ (0, platelet count $\geq 15 \times 10^4/mm^3$; 1, platelet count $< 15 \times 10^4/mm^3$). The risk index was $\ln[hi(t)/h_0(t)]$, where $hi(t)/h_0(t)$ was the relative risk of the development of HCC for the $i$-th patient. The index values ranged from 0.00 to 3.29. The patients were then classified into three groups according to the risk index, as follows: low risk, risk index less than 1.00 (equivalent to patients with none of the three risk factors); intermediate risk, risk index from 1.00 to 2.00 (equivalent to patients with one of the three risk factors); and high risk, risk index greater than 2.00 (equivalent to patients with two or more of the three risk factors). The incidence curves for the three groups are shown in Fig. 2. The incidence rates of HCC per 100 person-years (95% confidence interval) in the low-, intermediate-, and high-risk groups were 0.11 (0.00–0.26), 0.44 (0.11–0.77), and 1.98 (1.09–2.87), respectively. There was a significant difference in survival time among the three groups ($P < 0.0001$).

*Clinical features of HCC*

The characteristics of the 29 patients in whom HCC developed after sustained response are shown in Table 4. All patients were HCV RNA-negative (determined by using qualitative HCV RNA assay), at the time of diagnosis of HCC. Twenty-five patients (86%) were aged 60 years or more, and 24 patients (83%) were men. Among the 13 patients in whom liver biopsy was done at the time of diagnosis of HCC, A0, A1, and A2 histological activity was observed in 5 (38%), 6 (46%), and 2 (15%) patients, respectively. F0, F1, F2, F3, and F4 histological stages were observed in 1 (8%), 1 (8%), 7 (54%), 2 (15%), and 2 (15%) patients, respectively. The median period from the termination of IFN therapy to the development of HCC was 4.6 years (range, 1.4–9.0 years), and there were 11 patients (38%) in whom HCC was detected more than 5 years after the termination of IFN therapy. The periods and methods of medical follow-up examination after the end of IFN therapy varied among the patients, and 8 patients did not receive a sufficient post-treatment medical examination. Among them, HCC of 5 cm or more in size was detected in 5 patients (63%).

## Discussion

IFN is already widely used as a standard therapeutic modality for chronic hepatitis C.[5–9] It is generally assumed that eradication of HCV by IFN halts the progression of the disease and prevents clinical complications, including the development of HCC.[5,7,10–14] However, there have been reports of several patients in whom HCC developed after successful IFN therapy.[5,10–25] The incidence and clinical features of HCC, the risk factors for the disease, and the mechanism of carcinogenesis in these patients have not been fully elucidated, because the development of HCC is very rare in sustained responders to IFN therapy. This prompted us to perform a multicenter retrospective cohort study to gather clinical data on such patients.

Of all 1056 sustained responders to IFN therapy in the 16 hospitals in the study, 29 developed HCC, with a median period to development of 4.7 years, and the incidence of HCC was 0.56 (95% confidence interval, 0.35–0.76) per 100 person-years. This value was consistent with the results of previous studies of small numbers of sustained responders to IFN who developed HCC.[5,11–14,20,21–25] This rate was considerably lower than that in IFN-refractory patients or HCV-positive pa-

**Table 2.** Univariate analysis of 1056 sustained responders in relation to development of HCC

| Variables | | No. of patients | No. of patients developing HCC | Incidence (95% CI) (/100 person-years) | Hazard ratio (95% CI) | *P* value (log rank) |
|---|---|---|---|---|---|---|
| **Host-related variables** | | | | | | |
| Age | ≤60 years | 840 | 13 | 0.32 (0.14–0.49) | — | |
| | >60 years | 216 | 16 | 1.43 (0.73–2.13) | 4.23 (2.04–8.80) | 0.001 |
| Sex | Male | 711 | 24 | 0.67 (0.40–0.94) | — | |
| | Female | 345 | 5 | 0.30 (0.04–0.57) | 0.47 (0.18–1.23) | 0.12 |
| History of blood transfusion | Positive | 266 | 11 | 0.80 (0.33–1.28) | — | |
| | Negative | 723 | 16 | 0.45 (0.23–0.67) | 0.60 (0.28–1.30) | 0.19 |
| Alcohol abuse[a] | Positive | 78 | 2 | 0.53 (0.00–1.26) | — | |
| | Negative | 946 | 26 | 0.56 (0.34–0.77) | 1.05 (0.25–4.42) | 0.95 |
| Smoking habit[b] | Positive | 248 | 14 | 1.16 (0.55–1.77) | — | |
| | Negative | 405 | 7 | 0.36 (0.09–0.62) | 0.30 (0.12–0.75) | 0.009 |
| HCV viral load | High (≥$10^6$) | 159 | 1 | 0.15 (0.00–0.45) | — | |
| | Low (<$10^6$) | 593 | 11 | 0.42 (0.17–0.66) | 2.68 (0.35–20.77) | 0.35 |
| HCV serological group | Group 1 | 372 | 5 | 0.27 (0.03–0.52) | — | |
| | Group 2 | 466 | 10 | 0.47 (0.18–0.76) | 1.78 (0.60–5.26) | 0.30 |
| Hepatitis B surface antigen | Positive | 17 | 0 | 0.00 | | |
| | Negative | 1008 | 27 | 0.54 (0.34–0.75) | [c] | 0.56 |
| Platelet count (×$10^4$/mm$^3$) | ≥15 | 568 | 7 | 0.27 (0.07–0.46) | — | |
| | <15 | 358 | 21 | 1.15 (0.66–1.65) | 3.95 (1.68–9.30) | 0.002 |
| Total bilirubin (mg/dl) | ≥1.0 | 207 | 8 | 0.75 (0.23–1.27) | — | |
| | <1.0 | 824 | 21 | 0.52 (0.30–0.75) | 0.37 (0.32–1.65) | 0.45 |
| Albumin (g/dl) | >4.0 | 564 | 17 | 0.59 (0.31–0.87) | — | |
| | ≤4.0 | 396 | 8 | 0.42 (0.13–0.72) | 0.78 (0.34–1.80) | 0.56 |
| Aspartate aminotransferase (IU/l) | >100 | 196 | 13 | 1.26 (0.57–1.94) | — | |
| | ≤100 | 844 | 16 | 0.39 (0.20–0.58) | 0.35 (0.17–0.73) | 0.005 |
| Alanine aminotransferase (IU/l) | >100 | 459 | 17 | 0.73 (0.38–1.07) | — | |
| | ≤100 | 591 | 12 | 0.42 (0.18–0.66) | 0.63 (0.30–1.32) | 0.22 |
| Prothrombin time (%) | ≥80 | 493 | 9 | 0.39 (0.14–0.65) | — | |
| | <80 | 158 | 10 | 1.19 (0.45–1.93) | 2.72 (1.10–6.74) | 0.03 |
| ICG R15 (%) | ≥10 | 322 | 9 | 0.52 (0.18–0.86) | — | |
| | <10 | 274 | 1 | 0.08 (0.00–0.23) | 0.18 (0.02–1.44) | 0.11 |
| Alpha-fetoprotein (ng/ml) | >20 | 66 | 2 | 0.58 (0.00–1.39) | — | |
| | ≤20 | 554 | 16 | 0.58 (0.30–0.87) | 1.10 (0.25–4.81) | 0.78 |
| PIVKA–II (AU/ml) | >0.063 | 42 | 0 | 0.00 | | |
| | ≤0.063 | 235 | 8 | 0.66 (0.20–1.12) | | 0.63 |
| Histological activity grade | A0 (No) | 12 | 0 | 0.00 | | |
| | A1 (Mild) | 309 | 6 | 0.40 (0.08–0.73) | | |
| | A2 (Moderate) | 359 | 11 | 0.64 (0.26–1.01) | | |
| | A3 (Severe) | 169 | 5 | 0.61 (0.07–1.14) | 1.28 (0.74–2.21) | 0.39 |
| Histological fibrosis stage | F0 (No) | 26 | 0 | 0.00 | | |
| | F1 (Mild) | 405 | 5 | 0.27 (0.03–0.50) | | |
| | F2 (Moderate) | 301 | 7 | 0.47 (0.12–0.82) | | |
| | F3 (Severe) | 170 | 6 | 0.62 (0.12–1.11) | | |
| | F4 (Cirrhosis) | 97 | 4 | 1.31 (0.03–2.60) | 1.56 (1.03–2.36) | 0.03 |
| **Treatment-related variables** | | | | | | |
| Treatment period (weeks) | ≥24 | 472 | 17 | 0.73 (0.38–1.08) | | |
| | <24 | 584 | 12 | 0.41 (0.18–0.65) | 0.56 (0.27–1.16) | 0.11 |
| Interferon type | α | 829 | 25 | 0.61 (0.37–0.85) | — | |
| | β | 166 | 4 | 0.55 (0.01–1.10) | 0.99 (0.34–2.86) | 0.98 |
| | α + β | 61 | 0 | 0.00 | [c] | |
| Total amount of interferon (MU) | >500 | 491 | 10 | 0.42 (0.16–0.68) | — | |
| | ≤500 | 534 | 16 | 0.60 (0.31–0.89) | 1.34 (0.61–2.95) | 0.47 |
| Prior interferon therapy | Positive | 87 | 2 | 0.46 (0.00–1.10) | — | |
| | Negative | 955 | 27 | 0.57 (0.36–0.79) | 1.17 (0.28–5.00) | 0.82 |

HCC, hepatocellular carcinoma; CI, confidence interval; HCV, hepatitis C virus; ICG R15, indocyanine green retention rate at 15 min; PIVKA II, protein induced by vitamin K absence or antagonist-II; —; reference category

[a] Alcohol intake ≥80 g/day + 5 years

[b] Smoking habit, ≥20 cigarettes/day for ≥10 years

[c] not estimated

tients who did not receive IFN therapy, which has been reported to be 1.4%–7% yearly,[4–7,10–13,21–24] and it was obvious that IFN therapy decreased the risk of HCC in sustained responders. However, the incidence of HCC gradually increased over a period of at least 9 years after the termination of IFN therapy (Fig. 1). This suggests that the risk of HCC is not completely eliminated in patients who have a sustained response to IFN therapy,

at least for up to 9 years following cessation of the treatment.

Identification of the risk factors for the development of HCC in sustained responders is important, so that high-risk patients can be screened carefully for early detection of HCC and given potentially curative treatments such as hepatic resection; such patients generally have a good hepatic reserve after the elimination of HCV. Among the variables we investigated, multivariate analysis showed age to be an independent risk factor. As the patient ages, the period of HCV infection becomes longer, and the liver becomes more severely cirrhotic. Therefore, advanced age may simply represent the progression of associated liver disease. These findings are compatible with previous reports of the development of HCC in patients with chronic hepatitis C.[11–14,20–22]

Serum AST level and platelet counts were also independent risk factors in the present study. Some studies have reported that increased AST level and decreased platelet count are correlated with the progression of liver fibrosis,[33–34] which has been reported to be one of the most important risk factors for the development of HCC in patients with chronic hepatitis C.[5,11–13,21] Progression of liver fibrosis may reduce the clearance of AST,[35] leading to increased serum AST levels.[36] This progression is also associated with decreased production of thrombopoietin by hepatocytes[37] and progressive hypersplenism with worsening portal hypertension;[38] and, hence, reduced platelet production and increased platelet destruction. Moreover, in the present study, these factors were strongly associated with histological stage (Pearson's correlation coefficient; $P < 0.0001$). Therefore, increased AST level and decreased platelet count may reflect more progressive liver fibrosis.

For the clinical application of these findings, we proposed a risk index based on the independent risk factors. Patients were classified into three groups, with low, intermediate, and high risk ($P < 0.0001$ for difference in survival time among the three groups; Fig. 2). This index can be easily calculated, because it is based on variables obtained during routine laboratory examinations before IFN therapy is begun. This index, therefore, may be helpful in assessing the risk of development of HCC after sustained response to IFN therapy, although it is also important to validate this risk index by applying it to other populations of patients. Patients in the high-risk group (incidence rate, 1.98 per 100 person-years) may benefit from regular diagnostic imaging for the early detection of HCC.

In the analysis of the clinical features of HCC there were no specific findings. The period to the development of HCC after IFN therapy (median, 4.6 years; 1.4–9.0 range, years) was variable. HCC developed even in two patients whose liver showed improvement to mild fibrosis (stage F0 or F1) and in five patients whose liver improved to no activity (A0) after IFN therapy. The follow-up periods and methods for the detection of HCC after the termination of IFN therapy varied among the patients, and in some patients HCC was detected at far more advanced stages than in others, because of insufficient follow up after IFN therapy. This finding may suggest the need for regular follow up by diagnostic imaging, even after sustained response to IFN therapy for chronic hepatitis C, especially in the high-risk group.

Our study involved some uncertainties. First, because the study was retrospective, many data items were missing from the replies to the data collection instrument, and we had to ignore unmeasured or unrecorded data when conducting the statistical analyses. In the multivariate analysis, therefore, only variables whose $P$ values were less than 0.20 on the univariate analysis were entered. Also, history of blood transfusion, smoking habit, prothrombin time, and ICG R15, whose $P$ values were lower than 0.20, had to be excluded from the model because of missing data; these factors were potentially significant on multivariate analysis. Secondly, we sought information on serum hepatitis B virus DNA

**Table 3.** Significant risk factors identified in 1056 sustained responders, as determined by multivariate analysis with the Cox proportional hazard model

| Variable | Hazard ratio (95% confidence interval) | $P$ value |
|---|---|---|
| Age | 3.13 (1.32–7.42) | 0.01 |
| Aspartate aminotransferase | 3.10 (1.31–7.31) | 0.01 |
| Platelet count | 2.78 (1.07–7.20) | 0.04 |

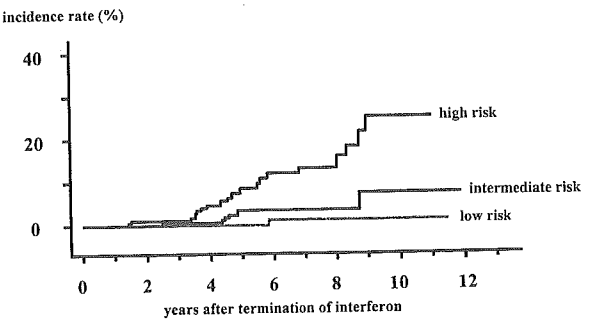


**Fig. 2.** Cumulative incidence of hepatocellular carcinoma for the three groups determined by a risk index based on the results of multivariate analysis. Low risk (risk index < 1.00); intermediate risk (risk index from 1.10 to 2.00); high risk (risk index, ≥2.00)