Effect of occult hepatitis B virus infection on the early-onset of hepatocellular carcinoma in patients with hepatitis C virus infection

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Abstract. Although overt hepatitis B virus (HBV) infection promotes the onset of hepatocellular carcinoma (HCC) in hepatitis C virus (HCV)-infected patients, the effect of occult HBV infection remains unclear. The aim of this study was to investigate the effect of occult HBV infection on the early-onset of HCC in HCV-infected patients. A total of 173 HCC patients with HCV infection were enrolled and classified into 2 groups according to the median age of HCC onset: the early-onset group (n=91; 61.1±5.6 years) and the late-onset group (n=82; 73.8±3.7 years). Independent factors associated with the early-onset of HCC were assessed by multivariate analysis. In the overall analysis, independent risk factors for the early-onset of HCC were the white blood cell count and alanine aminotransferase level, but not the presence of HBV DNA. In a stratification analysis according to albumin levels of ≥3.5 g/dl, the presence of HBV DNA was a significant independent risk factor for the early-onset of HCC (OR 145.18, 95% CI 1.38-15296.61, P=0.036), whereas the presence of antibodies against hepatitis B core antigen was not found to be a risk factor. The presence of HBV DNA was not a risk factor for the early-onset of HCC in the overall analysis. However, its presence was an independent factor for the early-onset of HCC in HCV-infected patients with an albumin level of ≥3.5 g/dl. Thus, occult HBV infection may accelerate hepatocarcinogenesis in HCV-infected patients with relatively low carcinogenic potential.

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Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; BMI, body mass index; WBC, white blood cell; HbA1c, hemoglobin A1c; AST, aspartate aminotransferase; ALT; alanine aminotransferase; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; HOMA, homeostasis model assessment; APRI, AST to platelet ratio index; AUROC, area under the receiver operating characteristic curve analysis; MAPK, mitogen activated protein kinase

Key words: latent HBV infection, hepatoma, liver cancer, oncogenesis, white blood cell

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. It ranks third in men and fifth in women as the cause of death from malignancies in Japan (1). Chronic hepatitis C virus (HCV) infection is the major cause of HCC and accounts for ~60-70% of HCC cases in Japan (2). In addition to hepatic inflammation and subsequent fibrosis, various other factors including aging, obesity and diabetes mellitus are involved in the hepatocarcinogenesis in HCV-infected patients (3-5).

Co-infection of HCV with hepatitis B virus (HBV) is thought to synergistically increase the development of HCC (6). The status of HBV infection is evaluated by the presence of hepatitis B surface antigen (HBsAg), antibodies against hepatitis B core antigen (HBcAb), and HBV DNA. In some cases, HBV DNA can be detected in the serum or liver tissue of patients who are negative for HBsAg, a condition referred to as 'occult HBV infection' (7,8). In Japan, the prevalence of occult HBV infection in HCV-infected patients is reported

Table I. Nucleotide positions and sequences of TaqMan PCR primers and probes.

Primer/Probe	Sequence	Position
S-sense	TGTACAAAACCTTCGGACGGAAA	442-464
S-antisense	TGCGAAAGCCCAGGATGATG	485-504
S-probe	CTGCACTTGTATTCCC	465-480
C-sense	ACTGTGGTTTCACATTTCCTGTCTT	2072-2096
C-antisense	GGCATTTGGTGGTCTGTAAGC	2163-2183
C-probe	CCACACTCCAAAAGAC	2132-2147
X-sense	CTACTGTTCAAGCCTCCAAGCT	1729-1750
X-antisense	GCTCCAAATTCTTTATACGGGTCAATG	1778-1804
X-probe	AAGCCACCCAAGGCAC	1751-1766

Nucleotide positions are based on the sequence of hepatitis B virus subtype adr4 (GenBank accession no. X01587) (29).

to be between 37.7% and 90% (9-11). Occult HBV infection is associated with a poor response to interferon therapy for chronic hepatitis C (12,13) and is also known to accelerate the progression of liver fibrosis, resulting in cirrhosis in patients with HCV infection (9,14,15). Several previous studies have examined the impact of occult HBV infection on the development of HCC in HCV-infected patients, but no clear conclusions have emerged (14,16,17). Moreover, the effects of occult HBV infection on the early-onset of HCC have not been investigated in HCV-infected patients.

Albumin is produced by hepatocytes, and the level of serum albumin is used to evaluate hepatic function (18). Albumin plays a significant role in maintaining colloid osmotic pressure and transports drugs and endogenous substances including bilirubin and unesterified free fatty acids (19). In addition, albumin exerts antioxidative properties (19), and hypoalbuminemia has been shown to be an independent risk factor for mortality among residents of a hyperendemic area of HCV infection in Japan (20). A serum albumin level of ≥3.5 g/dl is an independent predictor of survival in HCC patients (21,22) and in cirrhotic patients with a serum albumin levels of <3.5 g/dl, branched-chain amino acids increase serum albumin levels and subsequently suppress hepatocarcinogenesis (23,24). Thus, the serum albumin level is an important factor in hepatocarcinogenesis.

The aim of this study is to investigate the impact of occult HBV infection on the early-onset of HCC in HCV-infected patients. We also performed a stratification analysis according to the serum albumin level.

Subjects and methods

Subjects. We conducted a retrospective study to investigate the effect of the presence of HBV DNA on the early-onset of HCC in HCV-infected patients. Between 1995 and 2011, 325 patients underwent hepatic resection at the Kurume University Hospital. The inclusion criteria were histologically proven HCC, a positive result for serum anti-HCV, and a negative result for serum HBsAg. Exclusion criteria were the presence of autoimmune hepatitis, primary biliary cirrhosis, and hemochromatosis, no test results for serum HBV DNA, and a histological diagnosis of combined hepatocellular and cholangiocellular carcinoma. Although 214 patients met the inclusion criteria, 41 patients had to be excluded because of one or more of these reasons. The remaining 173 HCC patients with HCV infection were therefore enrolled in this study and classified into 2 groups according to the median age of HCC onset: the early-onset group (n=91; 61.1±5.6 years) and the late-onset group (n=82; 73.8±3.7 years).

The study protocol was approved by the institutional review board, and informed consent for participation in the study was obtained from each subject. None of the subjects were institutionalized.

Data collection. Demographic data were collected at the time of hepatic resection including age, gender, and alcohol intake. Body mass index (BMI) was calculated as body weight in kilograms divided by the square of height in meters (kg/m^2).

Venous blood samples were taken in the morning after a 12-h overnight fast. The presence of serum anti-HCV, HBsAg, and HBcAb was tested using standard clinical methods (Department of Clinical Laboratory, Kurume University Hospital). Blood platelet count, white blood cell (WBC) count, prothrombin time %, plasma glucose levels; hemoglobin A1c (HbA1c) levels, and serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, total bilirubin, insulin, α-fetoprotein (AFP), and des-γ-carboxy prothrombin (DCP) were also measured using standard clinical methods. Insulin resistance was evaluated on the basis of fasting levels of plasma glucose and insulin, according to the homeostasis model assessment for insulin resistance (HOMA-IR), as previously described (25).

The stage of hepatic fibrosis was assessed using the AST-to-platelet ratio index (APRI), which is calculated as the serum AST level (U/l)/upper limit of normal AST (U/l) x100/platelet count ($x10^4$/ml). Patients with APRI values of ≤1.5 were diagnosed as having chronic hepatitis, and patients with APRI values >1.5 were diagnosed as having liver cirrhosis, as previously described (26). The degree of liver cirrhosis was categorized according to the Child-Pugh classification (27). Diabetes mellitus was diagnosed on the basis of fasting blood glucose levels >126 mg/dl or HbA1c levels >6.5%, in accordance with the Diagnostic Criteria for

Table II. Differences in the clinical characteristics between the early-onset and late-onset groups.

Variable	Reference value	Early-onset	Late-onset	P
Number of patients		91	82	
Age (years)		61.1±5.6	73.8±3.7	<0.001
AFP (ng/ml)	<8.7	1876±12163	769±3246	0.588
DCP (mAU/ml)	<40	1083±4120	1071±3845	0.378
Maximal HCC size (mm)	N/A	30.4±19.6	33.2±16.2	0.055
Gender (female/male)	N/A	23/68	20/62	0.893
BMI (kg/m^2)	18.5-22.0	23.6±3.6	22.4±3.2	0.045
Daily alcohol intake (none/0-60 g/>60 g)	N/A	21/42/14	23/36/10	0.676
Platelet count ($x10^4/mm^3$)	13-36	13.8±5.4	13.5±4.6	0.988
WBC count ($/mm^3$)	4000-9000	5009±1526	4420±1210	0.012
AST (U/l)	13-33	56.2±29.5	52.8±27.2	0.412
ALT (U/l)	6-30	62.2±40.9	51.7±31.7	0.104
Albumin (g/dl)	4.0-5.0	3.87±0.45	3.85±0.38	0.520
Prothrombin time (%)	70-130	90.0±11.2	91.7±12.2	0.272
Total bilirubin (mg/dl)	0.3-1.2	0.84±0.35	0.79±0.29	0.346
Chronic hepatitis/Child-Pugh class A/Child-Pugh class B	N/A	40/49/2	36/44/2	0.994
Complication of diabetes mellitus (yes/no)	N/A	30/61	20/62	0.214
Fasting blood glucose (mg/dl)	80-109	119±39	107±31	0.060
Insulin (μU/ml)	5-20	13.1±10.4	9.8±8.0	0.014
HOMA-IR	<2.5	3.05±2.47	2.11±1.03	0.622
HbA1c (%)	4.6-6.2	5.77±0.88	5.50±0.78	0.053
HBcAb positive/negative	N/A	49/42	50/32	0.344
HBV DNA positive/negative	N/A	6/85	3/79	0.385

Values are expressed as the mean ± SE. AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma; BMI, body mass index; WBC, white blood cell; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HOMA-IR, homeostasis model assessment for insulin resistance; HbA1c, hemoglobin A1c; HBcAb, antibody for hepatitis B core antigen; HBV, hepatitis B virus; N/A, not applicable.

Diabetes Mellitus of the Japan Diabetes Society (28), or the use of antidiabetic agents.

Nucleic acid extraction from serum. Total nucleic acid was extracted from 300 μl of plasma using a commercially available kit (High Pure Viral Nucleic Acid kit; Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. The extracted nucleic acid was eluted in 25 μl of elution buffer.

PCR for HBV DNA. Serum HBV DNA was analyzed for the presence of HBs, HBc, and HBx (S, C and X) regions using TaqMan real-time PCR according to the manufacturer's instructions (TaqMan Fast Universal PCR Master mix; Applied Biosystems, Tokyo, Japan). The oligonucleotide primers and probes that were optimized for the HBV subtype adr4 (29) were specific for the S, X and C region sequences are listed in Table I. The full-length HBV DNA (GenBank accession no. X01587) (29) was used as an internal standard in the quantitative real-time detection PCR. We used 8 μl of nucleic acid-containing serum in our study for better sensitivity. The limit of sensitivity of our TaqMan Real-time PCR methods was 4.5 copies/well, and the detection limit of our tests was 45 copies/ml (1.7 log copies/ml). A real-time PCR assay (COBAS TaqMan HBV Auto; Roche Diagnostics) was also performed to detect the core region of HBV DNA (limit of quantification, 1.8 log copies/ml). The presence of HBV DNA was defined as any positivity of S, X or C region.

Statistical analysis. Data are expressed as the absolute value or the mean ± SD. Differences between the early-onset and late-onset groups were analyzed using the Mann-Whitney U test. A logistic regression model with the Firth's correction 30 was used for multivariate stepwise analysis to identify independent variables associated with the early-onset of HCC, as previously described (31,32). All P-values were 2-tailed, and a level of <0.05 was considered statistically significant. All statistical analyses were conducted using SAS version 9.2 (SAS Institute, Cary, NC, USA) or R packages version 2.15.2 (URL http://www.R-project.org/).

Results

Univariate analysis between the early-onset and late-onset groups. AFP levels, DCP levels, and maximal HCC size did not differ between the early-onset and late-onset groups (Table II). Furthermore, although BMI, WBC count, and serum insulin levels were significantly higher in the early-onset group than in the late-onset group, there were no significant differences

Table III. Multivariate stepwise analysis for factors associated with the early-onset of hepatocellular carcinoma.

	Unit	Odds ratio	95% CI	P
HbA1c	1	1.37	0.91-2.07	0.136
BMI	1	1.08	0.98-1.19	0.133
ALT	10	1.10	1.00-1.21	0.045
DCP	20	0.99	0.98-1.00	0.091
WBC count	1000	1.35	1.06-1.73	0.014

All P-values were 2-tailed, and a level of <0.05 was considered statistically significant. HbA1c, hemoglobin A1c; BMI, body mass index; ALT, alanine aminotransferase; DCP, des-γ-carboxy prothrombin; WBC, white blood cell.

in the daily alcohol intake, platelet count, prothrombin time, Child-Pugh classification, presence of diabetes mellitus as a comorbidity, fasting blood glucose level, HOMA-IR value, HbA1c levels, and the serum levels of AST, ALT, albumin, and total bilirubin (Table II). The presence of HBcAb and HBV DNA did not differ either between the early-onset and late-onset groups (Table II).

Multivariate stepwise analysis for early-onset of HCC. Multivariate stepwise analysis showed that the serum ALT level and WBC count were independent risk factors for the early-onset of HCC (OR 1.10; 95% CI 1.00-1.21; P=0.045 and OR 1.35; 95% CI 1.06-1.73; P=0.014, respectively; Table III), but not the presence of HBcAb or HBV DNA.

Stratification analysis according to serum albumin level. Differences in the clinical characteristics between patients with the albumin level of ≥3.5 g/dl and <3.5 g/dl were summarized in Table IV. There were no significant differences in AFP levels, DCP levels, and maximal HCC size between the albumin level of ≥3.5 g/dl and <3.5 g/dl groups (Table IV). In the albumin level of ≥3.5 g/dl group, a significant elevation was seen in platelet count, prothrombin time and the number of patients with chronic hepatitis and a significant depletion was seen in AST level than in the albumin level of <3.5 g/dl group. However, other biochemical parameters and the

Table IV. Differences in the clinical characteristics between patients with the albumin level of ≥3.5 g/dl and <3.5 g/dl.

Variable	Reference value	Albumin level of ≥3.5 g/dl	Albumin level of <3.5 g/dl	P
Number of patients		138	35	
Age (years)		67.8±8.1	67.9±6.7	0.895
AFP (ng/ml)	<8.7	786±3219	3262±18195	0.248
DCP (mAU/ml)	<40	854±3269	1961±5977	0.306
Maximal HCC size (mm)	N/A	30.6±15.9	36.8±23.8	0.171
Gender (female/male)	N/A	35/103	8/27	0.759
BMI (kg/m^2)	18.5-22.0	23.0±3.5	23.0±3.4	0.918
Daily alcohol intake (none/0-60 g/>60 g)	N/A	21/58/38	3/20/6	0.172
Platelet count ($x10^4/mm^3$)	13-36	14.3±4.9	11.3±4.8	0.001
WBC count ($/mm^3$)	4000-9000	4798±1395	4291±1331	0.052
AST (U/l)	13-33	51.2±26.2	67.1±32.6	0.001
ALT (U/l)	6-30	54.9±37.4	63.4±32.7	0.057
Albumin (g/dl)	4.0-5.0	4.02±0.28	3.23±0.20	<0.001
Prothrombin time (%)	70-130	91.6±12.0	88.0±10.0	0.038
Total bilirubin (mg/dl)	0.3-1.2	0.82±0.34	0.80±0.28	0.822
Chronic hepatitis/Child-Pugh class A/Child-Pugh class B	N/A	69/69/0	7/24/4	<0.001
Complication of diabetes mellitus (yes/no)	N/A	38/100	12/23	0.431
Fasting blood glucose (mg/dl)	80-109	112±37	121±49	0.694
Insulin (μU/ml)	5-20	10.1±6.4	17.6±16.3	0.063
HOMA-IR	<2.5	3.12±3.87	4.23±2.23	0.315
HbA1c (%)	4.6-6.2	5.61±0.79	5.68±1.04	0.905
HBcAb positive/negative	N/A	75/63	24/11	0.129
HBV DNA positive/negative	N/A	6/132	3/32	0.315

Values are expressed as the mean ± SE. AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma; BMI, body mass index; WBC, white blood cell; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HOMA-IR, homeostasis model assessment for insulin resistance; HbA1c, hemoglobin A1c; HBcAb, antibody for hepatitis B core antigen; HBV, hepatitis B virus; N/A, not applicable.

Table V. Multivariate stepwise analysis for factors associated with the early-onset of hepatocellular carcinoma in patients with a serum albumin level of ≥3.5 g/dl.

	Unit	Odds ratio	95% CI	P
HBcAb	Positive	0.59	0.27-1.26	0.169
HBV DNA	Positive	145.18	1.38-15296.61	0.036
Prothrombin time	10	0.76	0.54-1.08	0.109
ALT	10	1.08	0.97-1.21	0.145
Albumin	0.1	1.17	1.01-1.36	0.036
DCP	20	0.99	0.98-1.00	0.037
Platelet count	1	0.92	0.84-1.02	0.107
WBC count	1000	1.64	1.15-2.35	0.006

All P-values were 2-tailed, and a level of <0.05 was considered statistically significant. HBcAb, antibody for hepatitis B core antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; DCP, des-γ-carboxy prothrombin; WBC, white blood cell.

Table VI. Multivariate stepwise analysis for factors associated with the early-onset of hepatocellular carcinoma in patients with a serum albumin level of <3.5 g/dl.

	Unit	Odds ratio	95% CI	P
HbA1c	1	1.83	0.75-4.47	0.183
HBV DNA	Positive	0.00	0.00-2.96	0.093
AFP	20	1.39	1.01-1.93	0.045

All P-values were 2-tailed, and a level of <0.05 was considered statistically significant. HbA1c, hemoglobin A1c; HBV, hepatitis B virus; AFP, α-fetoprotein.

presence of HBcAb and HBV DNA did not differ between the albumin level of ≥3.5 g/dl and <3.5 g/dl groups (Table IV).

In patients with a serum albumin level of ≥3.5 g/dl, the WBC count and serum levels of albumin and DCP were identified as independent factors associated with the early-onset of HCC (OR 1.64; 95% CI 1.15-2.35; P=0.006, OR 1.17; 95% CI 1.01-1.36; P=0.036, and OR 0.99; 95% CI 0.98-1.00; P=0.037, respectively; Table V). Although the presence of HBcAb was not found to be a significant risk factor for the early-onset of HCC, the presence of HBV DNA was identified as a significant independent risk factor associated with the early-onset of HCC (OR 145.18; 95% CI 1.38-15296.61; P=0.036; Table VI).

In patients with a serum albumin level of <3.5 g/dl, the serum AFP level was the only significant risk factor found to be associated with the early-onset of HCC (Table V). The presence of HBcAb and HBV DNA was not found to be a significant risk factor for the early-onset of HCC.

Discussion

In the overall analysis, the presence of HBV DNA in serum was not identified as a risk factor for the early-onset of HCC in HCV-infected patients. However, a stratification analysis according to a serum albumin level of ≥3.5 g/dl revealed that the presence of HBV DNA was an independent factor for the early-onset of HCC. These findings suggest that occult HBV infection may accelerate hepatocarcinogenesis in HCV-infected patients with a relatively low carcinogenic potential.

Although co-infection of HCV and HBV is thought to synergistically increase the risk of HCC (6), the overall analysis in this study showed that occult HBV infection was not significantly associated with the early-onset of HCC in HCV-infected patients. Similarly, several studies conducted in Asia have also failed to show any significant effect of occult HBV infection in these patients (33-35). Recently, Lok *et al* (36) performed a nested case-control study using a large number of patients enrolled in the HALT-C cohort and reported no significant difference in the prevalence of occult HBV infection between HCC and non-HCC patients with HCV infection. Taken together, these results suggest that occult HBV infection may not be an intensive promoter of HCC development in the presence of a potent carcinogenic factor such as HCV infection.

In contrast with these previous studies and with our own findings for all patients, a stratification analysis according to a serum albumin level of ≥3.5 g/dl showed that occult HBV infection was an independent risk factor for the early-onset of HCC. In patients with occult HBV infection, it is unclear whether a presence of HBV DNA is due to full-length HBV DNA replicated from covalently closed circular DNA in hepatocytes or fragmented HBV DNA integrated into the hepatocyte genome. However, the *HBx* gene is frequently integrated into cellular genes in HCC (37). The HBx protein upregulates the expression of proto-oncogenes including *c-jun*, *c-fos* and *c-myc*, all of which can promote hepatocarcinogenesis (38,39). In addition, albumin plays a crucial role in the development of various diseases, as it is a major antioxidant (19). In cirrhotic patients with a serum albumin level of <3.5 g/dl, branched-chain amino acids increase serum albumin levels, and this subsequently suppresses hepatocarcinogenesis (23,24). In this study, we found a significant association between occult HBV infection and the early-onset of HCC in patients with a serum albumin level of ≥3.5 g/dl, but not in patients with a serum albumin level of <3.5 g/dl. Taken together, these findings suggest that HBV DNA may promote hepatocarcinogenesis in HCV-infected patients with relatively low carcinogenic potential.

Although we designed this study to investigate the effect of HBV DNA on the early-onset of HCC in HCV-infected patients, we found instead that an elevated WBC count is an independent risk factor for the early-onset of HCC in HCV-infected patients. An elevated WBC count may reflect the consequences or underlying pathogenesis of the early-onset of HCC. One possible explanation is aging, because the WBC count declines in old age (40). Alternatively, an elevated WBC count still within the reference range is known to be associated with the development of various malignancies including gastric, colorectal, endometrial and lung cancers (41,42). The WBC count is a well-validated biomarker of inflammation. Chronic inflammation is a possible risk factor for hepatocarcinogenesis as it leads to the activation of receptors for chemokine and advanced glycation-end products (43,44). Another inflammation marker, C-reactive protein, is reported to be a diagnostic and prognostic marker of HCC (45,46). Taken together, these findings suggest that inflammation may promote the early-onset of HCC in HCV-infected patients.

A limitation of this study is that there were only a small number of HBV DNA-positive patients. Previous studies regarding occult HBV infection had a similar limitation (33,47,48). Since occult HBV infection is not frequently seen in HCV-infected patients with HCC, a multicenter study is needed to confirm our findings.

In conclusion, the presence of HBV DNA in serum was not a risk factor for the early-onset of HCC in HCV-infected patients. However, a stratification analysis based on a serum albumin level of ≥3.5 g/dl revealed that presence of HBV DNA in serum was an independent risk factor for the early-onset of HCC. These findings suggest that occult HBV infection may accelerate hepatocarcinogenesis in HCV-infected patients with relatively low carcinogenic potential.

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