学と薬学 **47**：651-661，2002.

10) Takahashi K, Okamoto H, Kishimoto S et al：Demonstration of a hepatitis C virus-specific antigen predicted from the putative core gene in the circulation of infected hosts. J Gen Virol **73**：667-672, 1992.

11) Aoyagi K, Ohue C, Iida K et al：Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J Clin Microbiol **37**：1802-1808, 1999.

12) Tanaka E, Ohue C, Aoyagi K et al：Evaluation of a new enzyme immunoassay for hepatitis C virus (HCV) core antigen with clinical sensitivity approximating that of genomic amplification of HCV RNA. Hepatology **32**：388-393, 2000.

13) 飯野四郎，小俣政男，熊田博光 他：分岐DNAプローブ法によるC型肝炎ウイルスRNA定量法の検討．医学と薬学 **30**：327-334，1993.

*　　　*　　　*

ELSEVIER

Available online at www.sciencedirect.com
SCIENCE DIRECT

Hepatology Research 34 (2006) 130–134

HEPATOLOGY RESEARCH

http://www.elsevier.com/locate/hepres

Increasing hepatitis C virus-associated hepatocellular carcinoma mortality and aging: Long term trends in Japan

Naota Taura [a,b], Hiroshi Yatsuhashi [a,*], Keisuke Hamasaki [b], Kazuhiko Nakao [c], Manabu Daikoku [a], Toshihito Ueki [a], Koji Yano [a], Takehiro Matsumoto [a], Hiromi Ishibashi [a], Katsumi Eguchi [b]

[a] *Clinical Research Center, National Nagasaki Medical Center, Kubara 2-1001-1, Omura, Nagasaki 856-8562, Japan*
[b] *The First Department of Internal Medicine, Nagasaki University School of Medicine, Sakamoto 1-7-1, Nagasaki 852-8501 Japan*
[c] *Health Research Center, Nagasaki University, Sakamoto1-7-1, Nagasaki 852-8501, Japan*

Received 13 June 2005; received in revised form 10 November 2005; accepted 28 November 2005

Abstract

Background: The incidence of hepatocellular carcinoma (HCC) in Japan has been increasing. The aim of the study was to determine the epidemiological trends in HCC mortality in Japan.
Methods: We reviewed the medical records of all patients whose death was caused by liver disease between 1981 and 2000 at two hospitals. The courses of death were separated based on presence or absence of HCC when death ensued. Additionally, cohorts of patients with HCC were analyzed in 5-year time periods.
Results: The number of deaths from hepatitis C virus (HCV)-associated HCC steadily increased 2.6 times from 49 to 128 during observation period. The mean age at death from HCV-associated HCC from 1996 to 2000 was significantly higher than that in the period from 1981 to 1985 ($p<0.0001$).
Interpretation: Deaths from HCV-associated HCC increased from 1981 to 2000, consistent with the aging of the population in Japan.
© 2005 Elsevier Ireland Ltd. All rights reserved.

Keywords: Hepatitis C virus; Hepatocellular carcinoma; Epidemiological

1. Introduction

Hepatocellular carcinoma (HCC) affects approximately half a million people each year worldwide, making it the fifth most common malignancy in men and the ninth most common in women [1–7]. Recently, a trend of increasing rates of HCC has been reported from several developed countries in North America, Europe and Asia [1–9], and the incidence of primary liver cancer in Japan has been increasing over the past four decades [10,11]. HCC often develops in patients with liver cirrhosis caused by hepatitis C virus (HCV), hepatitis B virus (HBV) or excessive alcohol consumption.

Of the hepatitis viruses that cause HCC, HCV is more common than HBV in Japan [12–15]. Although the age-adjusted incidence rates of HCC have been increasing during the period of rising HCC mortality, the temporal and demographic features of survival for HCC patients in Japan are unknown. Hence, we have analyzed these trends over time, using information from two independent databases that deal with HCC in Japan.

2. Patients and methods

We reviewed the medical records of all patients who died from liver disease and received medical care between 1981 and 2000 at the Liver Disease Center, National Nagasaki Medical Center and at The First Department of Internal

* Corresponding author. Tel.: +81 957 52 3121; fax: +81 957 53 6675.
E-mail address: yatsuhashi@nmc.hosp.go.jp (H. Yatsuhashi).

1386-6346/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.hepres.2005.11.007

Medicine, Nagasaki University School of Medicine. A total of 1001 patients were studied. All the patients were followed-up after diagnosis until death in one of the two hospitals and we were able to confirm their date of death and that death had occurred after severe liver disease.

All patients were entered into this study because sera were stored at −80 °C. These sera were used to assay HBV or HCV infection. A diagnosis of chronic HCV infection was based on the presence of anti-HCV antibody and HCV-RNA detected by polymerase chain reaction (PCR), whereas diagnosis of chronic HBV infection was based on the presence of hepatitis B surface antigen (HBsAg) or anti-hepatitis B core antigen (anti-HBc) reactivity. Diagnosis of HCC was based on histological findings or on characteristic images in dynamic computed tomography, dynamic magnetic resonance imaging and hepatic angiography. Demographic information, including age at death, sex and year of death, was collected from the patients' chart. Excessive alcohol consumers (an alcohol consumption of >50 g/day for 5 years) were not including in this study.

The courses of death were separated into those occurring with or without HCC when death ensued. Additionally, the patients with HCC were analyzed in 5 yearly intervals (1981–1985, 1986–1990, 1991–1995 and 1996–2000). Patients were classified according to 5-year age groups, and by HBV or HCV infection, and the number of patients in each age group with HBV- or HCV-associated HCC was calculated in each time period.

The SAS computer program for Windows was used to perform statistical analysis of the data, using analysis of variance (ANOVA).

3. Results

A total of 1001 patients died at the Liver Disease Center, National Nagasaki Medical Center and at The First Department of Internal Medicine, Nagasaki University School of Medicine from 1981 to 2000. The patients with HBV-associated HCC were 73.7% (210 of 285) in HBV-related disease and the patients with HCV-associated HCC were 84.5% (381 of 451) in HCV-related disease. There were 653 patients with HCC died. The mean time during followed-up were 2.5 years. The proportion of patients diagnosed with HBV-associated HCC was 32% (210 of 653), whereas 58% (381 of 653) had HCV-associated HCC, and an additional 2% (12 of 653) had HCC associated with both viruses (Table 1).

From 1981 to 2000, 210 patients died of HBV-associated HCC, whereas 381 died of HCV-associated HCC. Table 2 shows the number and the mean age at death from HBV- or HCV-associated HCC during the 5-year periods 1981–1985, 1986–1990, 1991–1995 and 1996–2000. The number of deaths from HBV-associated HCC was not changed within the range from 49 to 58 during the four 5-year periods: 54 (1981–1986), 49 (1986–1990), 49 (1991–1995) and 58 (1996–2000), and the mean age at death was not also statistically significantly different among the periods: 55.4 ± 9.9 (1981–1985), 55.6 ± 10.3 (1986–1990), 55.5 ± 10.6 (1991–1995) and 59.3 ± 10.2 (1996–2000). In contrast, the number of deaths from HCV-associated HCC steadily increased 2.6 times from 49 to 128 during same observation period: 49 (1981–1986), 90 (1986–1990), 114

Table 1
Course of death from 1981 to 2000

	HBV	HCV	Overlap	Others	Total
HCC (%)	210 (32)	381 (58)	12 (2)	50 (8)	653 (100)
Chronic liver failure	47	35	1	36	119
GI bleeding	8	17	1	13	39
Other disease	3	5	0	16	24
Acute liver failure	10	1	3	19	33
Other cancer	7	12	0	114	133
Total (%)	285 (28)	451 (45)	17 (2)	248 (25)	1001 (100)

HCC, hepatocellular carcinoma; GI bleeding, gastrointestinal bleeding; HBV, hepatitis B virus; HCV, hepatitis C virus; overlap, both HBV and HCV positive; other, both HBV and HCV negative.

Table 2
Mean age of KBV associated HCC deaths

Year	1981 - 1985	1986 - 1990	1991 - 1995	1996 - 2000	total
Number	54	49	49	58	210
Mean age (y.o.)	55.4	55.6	55.5	59.3	56.8
SD	9.9	10.3	10.6	10.2	10.3

NS NS NS
NS NS
NS

Mean age of HCV-associated HCC deaths

Year	1981 - 1985	1986 - 1990	1991 - 1995	1996 - 2000	total
Number	49	90	114	128	381
Mean age (y.o.)	60.0	63.0	64.1	67.0	64.3
SD	8.1	7.0	7.2	7.9	7.8

NS NS 0.0267
0.0176 0.0016
< 0.0001

HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; S.D., standard deviation; NS, not significant.

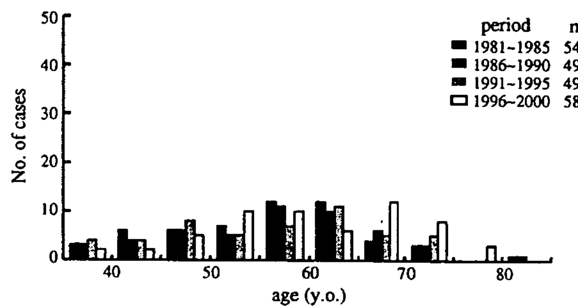

Fig. 1. Age distribution of the total number of deaths from hepatitis B virus-associated hepatocellular carcinoma from 1981 to 2000. There was no change of number of patients and age distribution of patients who died from hepatitis B virus-associated hepatocellular carcinoma during the four time periods.

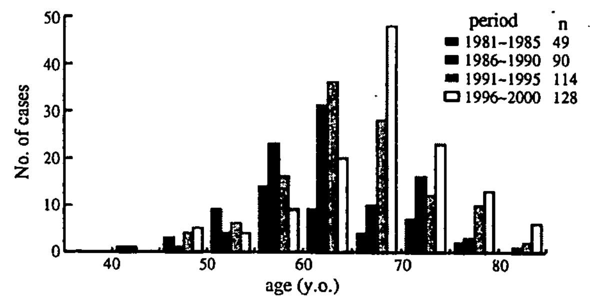

Fig. 2. Age distribution of the total number of deaths from hepatitis C virus-associated hepatocellular carcinoma from 1981 to 2000. The number of death from HCV associated HCC has increased 2.6 times during recent 20 years and this increase was provided by a close association with older shift of age distribution.

(1991–1995) and 128 (1996–2000). In addition, the mean age at death from HCV-associated HCC also increased over time. The mean age at death from 1996 to 2000 (67.0 ± 7.9 years old) was significantly higher than that from 1981 to 1985 (60.0 ± 8.1) (p < 0.0001), 1986 to 1990 (63.0 ± 7.0) (p = 0.0016) and 1991 to 1995 (64.1 ± 7.2) (p = 0.0267), respectively.

Fig. 1 shows the age distribution for deaths from HBV-associated HCC during the four 5-year periods. There was no change of number of patients and age distribution for deaths from HBV-associated HCC during these periods. In contrast, Fig. 2 shows the age distribution for deaths from HCV-associated HCC during the four 5-year periods. The number of patients with HCV-associated HCC aged more than 60 years in 1981–1985, 1986–1990, 1991–1995 and 1996–2000 were 22, 61, 88 and 110 patients, respectively. Fig. 2 indicated that the number of death from HCV associated HCC has increased during recent 20 years and this increase was provided by a close association with older shift of age distribution.

Table 3 shows the age distribution of HCC deaths in 5-year period (1981–1985, 1986–1990, 1991–1995 and 1996–2000). The number of patients with HCV-associated HCC obviously had an increase in the ratio of patients aged more than 60 years (p < 0.0001): 18.6% (1981–1985), 37.9% (1986–1990), 51.2% (1991–1995) and 54.4% (1996–2000). There was a significant difference of age distribution in the patients with HCV-associated HCC between aged more than and less than 60 years old in each 5-year period (p < 0.0001). In contrast, there was no difference in the age distribution of patients with other types of during these periods.

Fig. 3 shows the ratio between HCV-associated deaths and HBV-associated HCC deaths in 5-year period (1981–1985, 1986–1990, 1991–1995 and 1996–2000). The ratio between HCV-associated HCC and HBV-associated HCC increased and reached a plateau during the observation period: 0.9 (1981–1985), 1.8 (1986–1990), 2.3 (1991–1995) and 2.2 (1996–2000) (1981–1985 versus 1991–1995, p = 0.0030; 1981–1985 versus 1996–2000, p = 0.0042). Above all, the ratio of patients aged more than 60 years old increased during the observation period: 1.1 (1981–1985), 3.0 (1986–1990), 4.2 (1991–1995) and 3.8 (1996–2000) (1981–1985 versus

Table 3
Age distribution of HCC deaths in 5-year period

Age (y.o.)	1981–1985, no. (%)	1986–1990, no. (%)	1991–1995, no. (%)	1996–2000, no. (%)	p-Value
HBV					
<60	34 (28.8)	29 (18.0)	28 (16.3)	29 (14.4)	NS
>60	20 (17.0)	20 (12.5)	21 (12.2)	29 (14.4)	
HCV					
<60	27 (22.9)	29 (18.0)	26 (15.1)	18 (8.9)	<0.0001
>60	22 (18.6)	61 (37.9)	88 (51.2)	110 (54.4)	
Overlap					
<60	1 (0.9)	3 (1.9)	2 (1.2)	1 (0.5)	NS
>60	0	2 (1.2)	0	3 (1.5)	
Other					
<60	5 (4.2)	2 (1.2)	4 (2.3)	2 (1.0)	NS
>60	9 (7.6)	15 (9.3)	3 (1.7)	10 (4.9)	
Total	118 (100)	161 (100)	172 (100)	202 (100)	

HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; overlap, both HBV and HCV positive; other, both HBV and HCV negative.

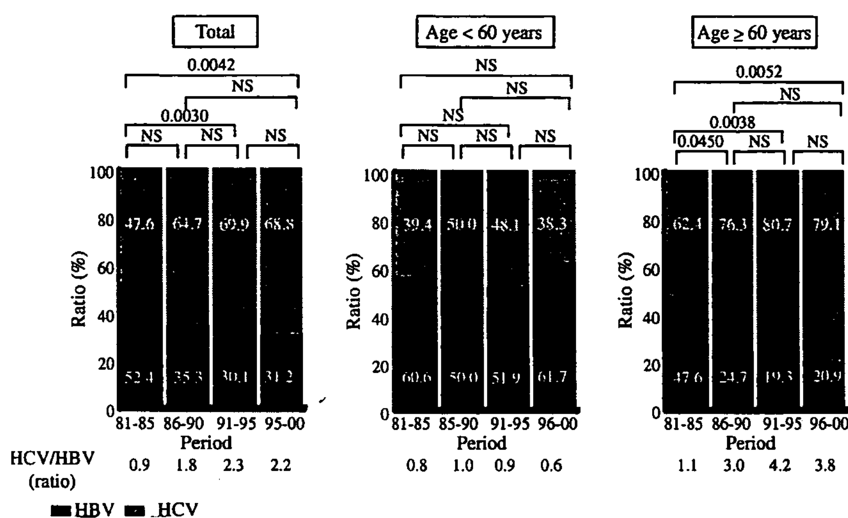

Fig. 3. Ratio between hepatitis C virus-associated hepatocellular carcinoma deaths and hepatitis B virus-associated hepatocellular carcinoma from 1981 to 2000. The ratio between HCV-associated HCC and HBV-associated HCC increased and reached a plateau during the observation period.

1986–1990, $p=0.0450$; 1981–1985 versus 1991–1995, $p=0.0038$; 1981–1985 versus 1996–2000, $p=0.0052$). In contrast, there was no difference in the ratio of patients aged more than 60 years old of during these periods.

4. Discussion

HCC accounts for approximately 6% of all human cancers. It is estimated that half a million cases occur annually worldwide, making HCC the fifth most common malignancy in men and the ninth in women [1–7,9]. The age-adjusted mortality rate from HCC has increased over the past decades in Japan [16], and in the current study more than 90% of deaths from HCC were HBV- and/or HCV-related and the number of deaths from HCV-associated HCC apparently increased 2.6 times from 1981 to 2000, and the mean age of deaths from HCV-associated HCC also significantly rose. During the same period, the number and the age distribution of deaths from HBV-associated HCC remained unchanged. The increase in the number of deaths from HCV-associated HCC seemed to be closely associated with the shift of age distribution of HCV infected population between 1981 and 2000. Although our data had the limitations of applying the findings from two hospitals to a general population, Kiyosawa described that deaths due to HCC in Japan have continued to increase in males, particularly in those older than 60 years of age between 1982 and 2003. This also suggests that the average age of diagnosis of HBV-related HCC was similar in all three time periods. In contrast, the average age of patients with HCV-related HCC rose from 61.6 years in 1982 to 63.1 years in 1990 and 67.8 years in 2003 [11]. The research group for population-based cancer registration in Japan described that incidence of HCC in Japan have continued to increase and reached a plateau in males and female from 1975 to 1999. Above all, the age distribution incidence and incident rate of HCC reached a peak older than 65 years old in males and female [17]. And, this study suggested that the ratio between HCV-associated HCC and HBV-associated HCC increased and reached a plateau from 1981 to 2000, especially more than 60 years old. Where did these findings and difference of HCC development between HCV and HBV, which were considered to be both oncogenic virus after long-term persistent infection with inflammation and fibrotic change in the liver but popular hepatitis virus infections in Japan, come from?

The simple reason may be explained as follows. From 1981 to 2000, mortality from a variceal hemorrhage in cirrhotic patients has declined [9,18]. Long term nutritional supplementation with oral branched-chain amino acids has been useful in the prevention of progressive hepatic failure, and improvement of surrogate markers and perceived health status in advanced cirrhosis has occurred [19,20]. Additionally, many new treatments and techniques have been introduced for HCC, including transcatheter arterial embolization, percutaneous ethanol injection therapy, microwave coagulation therapy, radiofrequency ablation, systemic chemotherapy and advance surgical techniques. However, these advances of medical treatment cannot explain the difference between HBV-associated HCC and HCV-associated HCC.

Alternatively, well considered reasons of the recent rapid increase of the number of patients who died from HCV-associated HCC in Japan, were shown in the current two studies. First, Hamada et al. recently reported that the majority of HCC patients develop HCC when they are aged over 60 years old, regardless of the timing of HCV infection. This result was obtained by the long-term observation of the patients infected by post-transfused HCV infection [21]. This also suggests that HCC has increased among patients over 60 years old with HCV infection and such phenomenon has never been observed nor reported till now in patients with HBV infection.

Second, the chronically HCV-infected population is aging in Japan. Yoshizawa et al. reported that age-specific prevalence rates for the presence of anti-HCV antibody among ~300,000 voluntary blood donors from Hiroshima in 1999 clearly increased with the age, reaching the highest rate of 7% in individuals who were more than 70 years old [11,22]. In a word, HCV infected people become older with years in Japan and they were regarded as a high risk for HCC. Then, the number of deaths from HCV-associated HCC has been increased recent 20 years in Japan.

El-Serag et al. reported that an increase in the number of cases of HCC affecting mainly younger age groups has occurred in the United States (U.S.) over the past two decades [23,24]. HCV infection accounts for most of the increase in the number of cases of primary liver cancer [4,6,7,9,25], while the rates of primary liver cancer associated with alcoholic cirrhosis and HBV infection have remained unchanged [4,6,9]. Tanaka et al. reported that HCV was introduced into the U.S. population around 100 years ago and was widely disseminated between 1954 and 1978 [26]. Most HCV-infected patients in the U.S. were born between 1940 and 1965 [27,28], and are therefore younger than HCV-infected Japanese patients. Hence, the burden of disease associated with HCV infection will probably increase in the U.S. during the next 10–20 years, as has occurred in Japan, as this cohort reaches an age at which complications of chronic liver disease typically occur [1–7,26]. The current study suggests that increased HCV-associated HCC will occur in the U.S. over the next two to three decades.

In conclusion, we found that the number of patients with HCV-associated HCC in Japan has increased, consistent with aging of the population, but the number of patients with HBV-associated HCC has remained unchanged over the last 20 years.

References

[1] El-Serag HB. Hepatocellular carcinoma and hepatitis C in the United States. Hepatology 2002;36:S74–83.
[2] El-Serag HB. Hepatocellular carcinoma: an epidemiologic view. J Clin Gastroenterol 2002;35:S72–8.
[3] El-Serag HB. Hepatocellular carcinoma: recent trends in the United States. Gastroenterology 2004;127:S27–34.
[4] El-Serag HB, Everhart JE. Diabetes increases the risk of acute hepatic failure. Gastroenterology 2002;122:1822–8.
[5] El-Serag HB, Hampel H, Yeh C, Rabeneck L. Extrahepatic manifestations of hepatitis C among United States male veterans. Hepatology 2002;36:1439–45.
[6] El-Serag HB, Mason AC. Risk factors for the rising rates of primary liver cancer in the United States. Arch Intern Med 2000;160:3227–30.
[7] Hassan MM, Frome A, Patt YZ, El-Serag HB. Rising prevalence of hepatitis C virus infection among patients recently diagnosed with hepatocellular carcinoma in the United States. J Clin Gastroenterol 2002;35:266–9.
[8] McGlynn KA, Tsao L, Hsing AW, Devesa SS, Fraumeni Jr JF. International trends and patterns of primary liver cancer. Int J Cancer 2001;94:290–6.
[9] El-Serag HB, Everhart JE. Improved survival after variceal hemorrhage over an 11-year period in the Department of Veterans Affairs. Am J Gastroenterol 2000;95:3566–73.
[10] Kiyosawa K, Tanaka E. Characteristics of hepatocellular carcinoma in Japan. Oncology 2002;62:5–7.
[11] Kiyosawa K, Umemura T, Ichijo T, et al. Hepatocellular carcinoma: recent trends in Japan. Gastroenterology 2004;127:S17–26.
[12] Hamasaki K, Nakata K, Tsutsumi T, et al. Changes in the prevalence of hepatitis B and C infection in patients with hepatocellular carcinoma in the Nagasaki prefecture. Jpn J Med Virol 1993;40:146–9.
[13] Kato Y, Nakata K, Omagari K, et al. Risk of hepatocellular carcinoma in patients with cirrhosis in Japan. Analysis of infectious hepatitis viruses. Cancer 1994;74:2234–8.
[14] Shiratori Y, Shiina S, Imamura M, et al. Characteristic difference of hepatocellular carcinoma between hepatitis B- and C-viral infection in Japan. Hepatology 1995;22:1027–33.
[15] Shiratori Y, Shiina S, Zhang PY, et al. Does dual infection by hepatitis B and C viruses play an important role in the pathogenesis of hepatocellular carcinoma in Japan? Cancer 1997;80:2060–7.
[16] Wada I, Hara T, Kajihara S, et al. Population-based study of hepatitis C virus infection and hepatocellular carcinoma in western Japan. Hepatol Res 2002;23:18–24.
[17] Japan* T.R. G. f. P.-b. C. R. i. Cancer incidence and incidence rates in Japan in estimates based on. Jpn J Clin Oncol 2004;34:352–6.
[18] McCormick PA, O'Keefe C. Improving prognosis following a first variceal haemorrhage over four decades. Gut 2001;49:682–5.
[19] Marchesini G, Bianchi G, Rossi B, et al. Nutritional treatment with branched-chain amino acids in advanced liver cirrhosis. J Gastroenterol 2000;35:7–12.
[20] Marchesini G, Bianchi G, Merli M, et al. Nutritional supplementation with branched-chain amino acids in advanced cirrhosis: a double-blind, randomized trial. Gastroenterology 2003;124:1792–801.
[21] Hamada H, Yatsuhashi H, Yano K, et al. Impact of aging on the development of hepatocellular carcinoma in patients with posttransfusion chronic hepatitis C. Cancer 2002;95:331–9.
[22] Yoshizawa H. Hepatocellular carcinoma associated with hepatitis C virus infection in Japan: projection to other countries in the foreseeable future. Oncology 2002;62:8–17.
[23] El-Serag HB, Anand B, Richardson P, Rabeneck L. Association between hepatitis C infection and other infectious diseases: a case for targeted screening? Am J Gastroenterol 2003;98:167–74.
[24] Rabeneck L, El-Serag HB, Davila JA, Sandler RS. Outcomes of colorectal cancer in the United States. No change in survival (1986–1997). Am J Gastroenterol 2003;98:471–7.
[25] Nair S, Shiv Kumar K, Thuluvath PJ, Shivakumar KS, Shiva Kumar K. Mortality from hepatocellular and biliary cancers: changing epidemiological trends. Am J Gastroenterol 2002;97:167–71.
[26] Tanaka Y, Hanada K, Mizokami M, et al. Inaugural article: a comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. Proc Natl Acad Sci USA 2002;99:15584–9.
[27] Alter MJ, Kruszon-Moran D, Nainan OV, et al. The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. N Engl J Med 1999;341:556–62.
[28] Armstrong GL, Alter MJ, McQuillan GM, Margolis HS. The past incidence of hepatitis C virus infection: implications for the future burden of chronic liver disease in the United States. Hepatology 2000;31:777–82.

Copyright © Blackwell Munksgaard 2005

Liver International
DOI: 10.1111/j.1478-3231.2005.01200.x

Clinical Studies

Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance

Tanaka E, Matsumoto A, Suzuki F, Kobayashi M, Mizokami M, Tanaka Y, Okanoue T, Minami M, Chayama K, Imamura M, Yatsuhashi H, Nagaoka S, Yotsuyanagi H, Kawata S, Kimura T, Maki N, Iino S, Kiyosawa K, HBV Core-Related Antigen Study Group. Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance.
Liver International 2006: 26: 90–96. © Blackwell Munksgaard 2005

Abstract: *Objective:* The clinical usefulness of hepatitis B virus core-related antigen (HBVcrAg) assay was compared with that of HBV DNA assay in predicting the occurrence of lamivudine resistance in patients with chronic hepatitis B. *Patients:* Of a total of 81 patients who were treated with lamivudine, 25 (31%) developed lamivudine resistance during a median follow-up period of 19.3 months. *Results:* The pretreatment positive rate of HBe antigen, or pretreatment levels of HBVcrAg or HBV DNA did not differ between patients with and without lamivudine resistance. Levels of both HBVcrAg and HBV DNA decreased after the initiation of lamivudine administration; however, the level of HBVcrAg decreased significantly more slowly than that of HBV DNA. The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months of treatment than in the remaining 25 patients. The cumulative rate of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. Lamivudine resistance did not occur during the follow-up period in the 19 patients whose HBVcrAg level was less than 4.6 log U/ml at 6 months of treatment, while it did occur in 50% of the remaining patients within 2 years. *Conclusion:* These results suggest that measurement of HBV DNA is valuable for identifying patients who are at high risk of developing lamivudine resistance, and that, conversely, measurement of HBVcrAg is valuable for identifying those who are at low risk of lamivudine resistance.

Eiji Tanaka,[1] Akihiro Matsumoto,[1] Fumitaka Suzuki,[2] Mariko Kobayashi,[2] Masashi Mizokami,[3] Yasuhito Tanaka,[3] Takeshi Okanoue,[4] Masahito Minami,[4] Kazuaki Chayama[5], Michio Imamura[5], Hiroshi Yatsuhashi[6], Shinya Nagaoka[6], Hiroshi Yotsuyanagi[7], Sumio Kawata[8], Tatsuji Kimura[9], Noboru Maki[9], Shiro Iino[10], Kendo Kiyosawa[1], and HBV Core-Related Antigen Study Group

[1]Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan, [2]Department of Research Institute for Hepatology, Toranomon Hospital, Minato-ku, Tokyo, Japan, [3]Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Science, Nagoya, Japan, [4]Department of Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan, [5]Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan, [6]Clinical Research Center, National Nagasaki Medical Center, Omura, Japan, [7]Department of Internal Medicine, Division of Gastroenterology and Hepatology, St. Marianna University, Kawasaki, Japan, [8]Department of Gastroenterology, School of Medicine, Yamagata University, Yamagata, Japan, [9]Advanced Life Science Institute, Inc., Wako, Japan, [10]Kiyokawa Hospital, Tokyo, Japan

Key words: chronic hepatitis B – HBV core-related antigen – HBV DNA – lamivudine resistance

Eiji Tanaka, MD, Department of Medicine, Shinshu University School of Medicine, Asahi 3-1-1, Matsumoto 390-8621, Japan.
Tel: +81-263-37-2634
Fax: +81-263-32-9412
e-mail: etanaka@hsp.md.shinshu-u.ac.jp

Received 19 June 2005,
accepted 8 August 2005

Kiyomi Yasuda (Kiyokawa Hospital, Tokyo, Japan); Hitoshi Togashi and Takatumi Saito (Department of Gastroenterology, School of Medicine, Yamagata University); Masataka Tsuge (Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan); Rumiko Nakao (Clinical Research Center, National Nagasaki Medical Center, Omura, Japan); Chiaki Okuse and Hideaki Takahashi (Department of Internal Medicine, Division of Gastroenterology and Hepatology, St. Marianna University, Kawasaki, Japan).

Lamivudine, a nucleoside analogue that inhibits reverse transcriptases, was first developed as an anti-viral agent against human immunodeficiency virus (HIV). It was later also found to be effective against hepatitis B virus (HBV) because HBV is a member of the Hepadnaviridae family of viruses, which use reverse transcriptases in their replication process (1, 2). Lamivudine was found to inhibit the replication of HBV, reduce hepatitis, and improve histological findings of the liver in long-term treatment (3–5). Furthermore, it has been shown that lamivudine treatment improves the long-term outcome of patients with chronic hepatitis B (6, 7). However, there are a number of problems with lamivudine therapy, such as relapse of hepatitis because of the appearance of YMDD mutant viruses and the reactivation of hepatitis after discontinuation of the treatment (8–11).

The concentration of HBV DNA in serum decreases and usually becomes undetectable during lamivudine administration, but it rapidly increases when HBV becomes resistant to lamivudine. Thus, the measurement of HBV DNA is useful for monitoring the anti-viral effects of lamivudine. However, a negative result of HBV DNA in serum does not necessarily indicate a good outcome of lamivudine therapy, because lamivudine resistance may occur even if HBV DNA levels remain undetectable during therapy (11–13). Recently, a chemiluminescence enzyme immunoassay (CLEIA) was developed in our laboratory for the detection of hepatitis B virus core-related antigen (HBVcrAg) (14, 15). The assay reflects the viral load of HBV in a similar manner to that used in assays, which detect HBV DNA. HBVcrAg consists of HBV core and e antigens; both proteins are transcribed from the precore/core gene and their first 149 amino acids are identical (16–18). The HBVcrAg CLEIA simultaneously measures the serum levels of hepatitis B core (HBc) and e (HBe) antigens, using monoclonal antibodies, which recognize common epitopes of these two denatured antigens. In the present study, we analyzed the clinical significance of the HBVcrAg assay in monitoring the anti-viral effects of lamivudine treatment.

Patients and methods

Patients

A total of 81 patients with chronic hepatitis B, who received lamivudine therapy, were enrolled in the present study. These were 58 men and 23 women with a median age of 49 years (range 24–79 years). The 81 patients were selected retrospectively from six medical institutions in Japan (Shinshu University Hospital, Toranomon Hospital, Nagoya City University Hospital, Kyoto Prefectural University Hospital, Hiroshima University Hospital, National Nagasaki Medical Center). Eight to 25 patients who met the following three criteria were selected consecutively in each institution: the first, a daily dose of 100 mg lamivudine was administered for at least 6 months in a period from 1999 to 2004; the second, histologically confirmed for chronic hepatitis without liver cirrhosis; and the third, serum samples at several time points available for testing. All patients were naive for lamivudine therapy. Chronic hepatitis B was defined as positive hepatitis B surface (HBs) antigen for more than 6 months with elevated levels of serum transaminases. The HBV genotype was A in two patients, B in three and C in 76. Serum HBV DNA was detectable in all patients, and HBe antigen was positive in 51 (63%) of the 81 patients just before lamivudine administration. The median follow-up period was 19 months with a range from 6 to 50 months. Follow-up of patients ended when lamivudine administration was discontinued. Written informed consent was obtained from each patient.

The occurrence of lamivudine resistance was defined as a rapid increase in serum HBV DNA levels with the appearance of the YMDD mutations during lamivudine administration. Using this criteria, resistance appeared in 27 (33%) of the 81 patients. The median period from the start of lamivudine administration to the occurrence of resistance was 12 months with a range from 4 to 37 months.

Serological markers for HBV

HBs antigen, HBe antigen and anti-HBe antibody were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd., Tokyo, Japan). Six major genotypes (A–F) of HBV can be detected using the method reported by Mizokami et al. (19), in which the surface gene sequence amplified by polymerase chain reaction (PCR) is analyzed by restriction fragment length polymorphism. The YMDD motif, that is, lamivudine resistant mutations in the active site of HBV polymerase, was detected with an enzyme-linked mini-sequence assay kit (HBV YMDD Mutation Detection Kit, Genome Science Laboratories Co., Ltd., Tokyo, Japan) (20).

Serum concentration of HBV DNA was determined using Amplicor HBV monitor kit (Roche, Tokyo, Japan), which had quantitative range from 2.6 to 7.6 log copy/ml. Sera containing

over 7.0 log copy/ml HBV DNA were diluted 10- or 100-fold with normal human serum and re-tested to obtain the end titer.

Serum concentrations of HBVcrAg were measured using the CLEIA method reported previously (10, 11). Briefly, 100 μL serum was mixed with 50 μL pretreatment solution containing 15% sodium dodecylsulfate and 2% Tween 60. After incubation at 70 °C for 30 min, 50 μL pretreated serum was added to a well coated with monoclonal antibodies against denatured HBc and HBe antigens (HB44, HB61 and HB114) and filled with 100 μL assay buffer. The mixture was incubated for 2 h at room temperature and the wells were then washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies against denatured HBc and HBe antigens (HB91 and HB110) were added to the well, and the mixture was incubated for 1 h at room temperature. After washing, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA) was added and the plate was incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and the HBVcrAg concentration was determined by comparison with a standard curve generated using recombinant pro-HBe antigen (amino acids, 10–183 of the precore/core gene product). The HBVcrAg concentration was expressed as units/ml (U/ml) and the immunoreactivity of recombinant pro-HBe antigen at 10 fg/ml was defined as 1 U/ml. In the present study, the cutoff value was tentatively set at 3.0 log U/ml. Sera containing over 7.0 log U/ml HBVcrAg were diluted 10- or 100-fold in normal human serum and re-tested to obtain the end titer.

Statistical analysis

The Mann–Whitney U-test and Wilcoxon signed-ranks test were utilized to analyze quantitative data, and Fisher's exact test was used for qualitative data. A log-rank test was used to compare the occurrence of lamivudine resistance. Statistical analyses were performed using the SPSS 5.0 statistical software package (SPSS, Inc., Chicago, IL). A P-value of less than 0.05 was considered to be statistically significant.

Results

Table 1 shows a comparison of the clinical and virological backgrounds of the 27 patients who showed lamivudine resistance and the 54 patients who did not. Median age, gender distribution and median follow-up period did not differ between the two groups, and the positive rate of HBe antigen was similar. Both HBV DNA and HBVcrAg levels at the beginning of lamivudine administration were similar between the two groups; however, both HBV DNA and HBVcrAg levels at 6 months after the start of lamivudine administration were significantly lower in the lamivudine resistance negative group than in the positive group. ALT level was normal at the beginning in eight (15%) of the 54 patients without lamivudine resistance and in two (7%) of the 27 patients with it ($P > 0.2$).

Table 1. Comparison of the clinical and virological backgrounds of patients who showed lamivudine resistance and those who did not

Characteristics	Appearance of lamivudine resistance		
	Negative ($n = 54$)	Positive ($n = 27$)	P
Age (years)*	47.0 (24–79)	50.6 (34–67)	0.140†
Gender (male %)	74%	67%	>0.2‡
Follow-up period (months)*	16 (6–50)	21 (9–43)	>0.2†
HBV genotype (A/B/C)	2/2/50	0/1/26	>0.2‡
HBe antigen (positive %)	59%	70%	>0.2‡
ALT (IU/ml)*			
Initial	85 (22–713)	95 (20–1140)	>0.2†
At 6 months	27 (11–115)	30 (15–92)	>0.2†
HBV DNA (log copy/ml)*			
Initial	7.0 (3.5–9.1)	7.3 (4.2–9.2)	>0.2†
At 6 months	<2.6 (<2.6–4.8)	3.3 (<2.6–6.6)	<0.001†
HBVcrAg (log U/ml)*			
Initial	6.2 (<3.0–8.8)	7.3 (4.4–9.1)	0.073†
At 6 months	5.2 (<3.0–6.7)	5.8 (4.7–8.4)	<0.001†

HBe antigen, hepatitis B e antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; HBVcrAg, HBV core-related antigen. *Data are expressed as median (range). †Mann–Whitney U test. ‡χ^2-test.

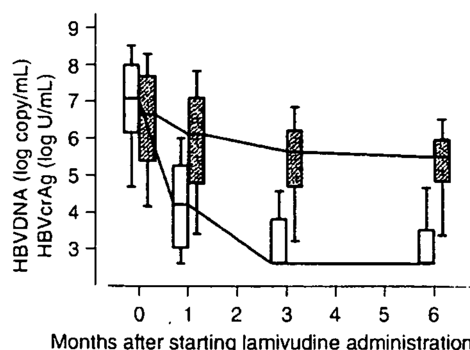

Fig. 1. Changes in the median levels of hepatitis B virus core-related antigen (HBVcrAg) and hepatitis B virus (HBV) DNA during lamivudine administration. The box plots show the 10th, 25th, 50th, 75th and 90th percentiles, with the open boxes indicating HBV DNA and shaded boxes indicating HBVcrAg. The median amount of decrease from the baseline in HBVcrAg levels was significantly smaller (Wilcoxon signed-ranks test) than that in HBV DNA level at 1 (2.80 log copy/ml vs. 0.27 log U/ml, $P < 0.001$), 3 (3.60 log copy/ml vs. 0.83 log U/ml, $P < 0.001$) and 6 months (3.90 log copy/ml vs. 1.15 log U/ml, $P < 0.001$) after the initiation of lamivudine administration.

Figure 1 shows changes in HBV DNA and HBVcrAg levels during lamivudine treatment in all patients. The level of HBV DNA decreased rapidly and became undetectable at 3 months after treatment was initiated. On the other hand, although HBVcrAg levels decreased continuously, the median amount of decrease from the base-line was significantly lower than that in HBV DNA levels at 1, 3 and 6 months after starting lamivudine administration (Wilcoxon signed-ranks test, $P<0.001$ at all analyzed points in time).

Changes in HBV DNA and HBVcrAg levels during lamivudine administration are compared in Fig. 2 between the 27 patients who showed lamivudine resistance and the 54 patients who did not. Serum HBV DNA levels were found to decrease rapidly and become undetectable within 6 months in 45 (83%) of the 54 patients without lamivudine resistance. On the other hand, only 11 (41%) of the 27 patients with lamivudine resistance showed a similar rapid decrease, and the HBV DNA levels of the remaining patients stayed above the detection limit during the follow-up period. HBVcrAg levels decreased but did not reach levels lower than 4.7 log U/ml (5000 U/ml) in the 27 patients with lamivudine resistance. In 19 (35%) of the 54 patients without lamivudine resistance, on the other hand, the levels decreased to levels below 4.7 log U/ml within 6 months after the start of lamivudine administration. The level of HBVcrAg increased rapidly as did the level of HBV DNA when lamivudine resistance occurred.

The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 logcopy/ml at 6 months after the initiation of treatment than in the remaining 25 patients (Fig. 3). The cumulative occurrence of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. There was no occurrence of lamivudine resistance during the follow-up period in the 19 patients whose HBVcrAg levels were less than 4.6 log U/ml at 6 months after the initiation of lamivudine therapy (Fig. 3). On the other hand, lamivudine resistance occurred in 50% of the remaining patients within 2 years.

Discussion

The HBVcrAg assay is a unique assay, which measures the amounts of e and core antigens

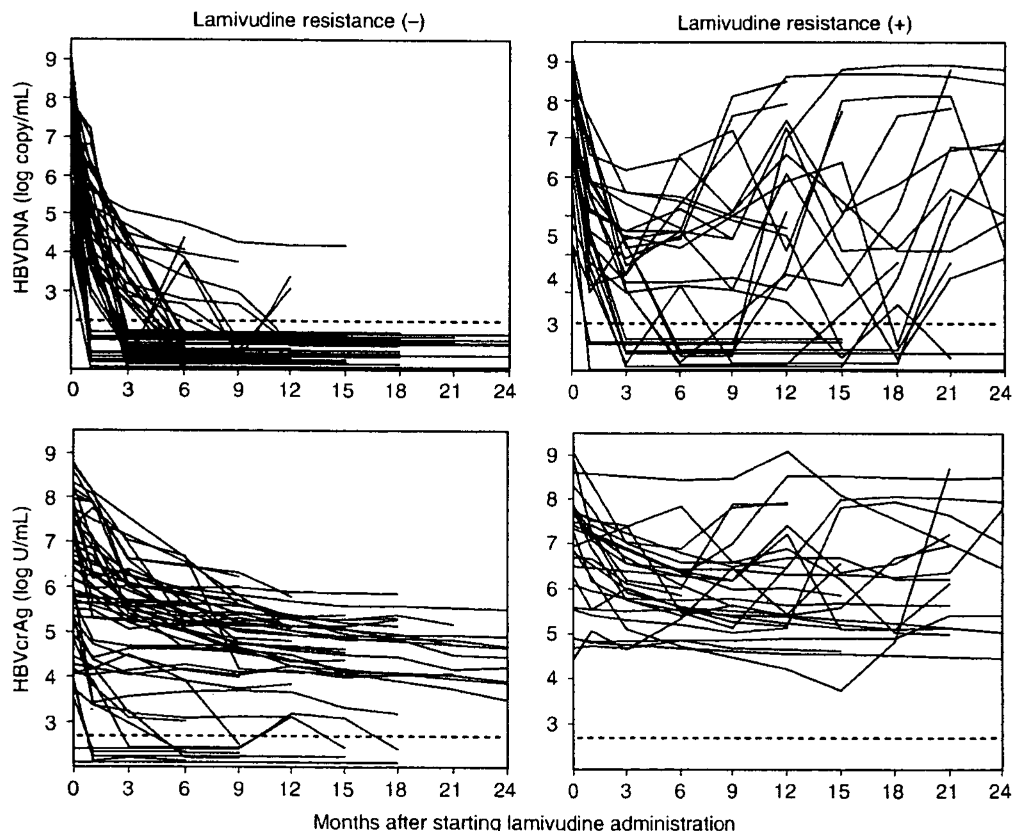

Fig. 2. Comparison of changes in serum hepatitis B virus (HBV) DNA and serum HBV core-related antigen (HBVcrAg) levels between patients who showed lamivudine resistance and those who did not. The broken lines indicate the detection limit of each assay.

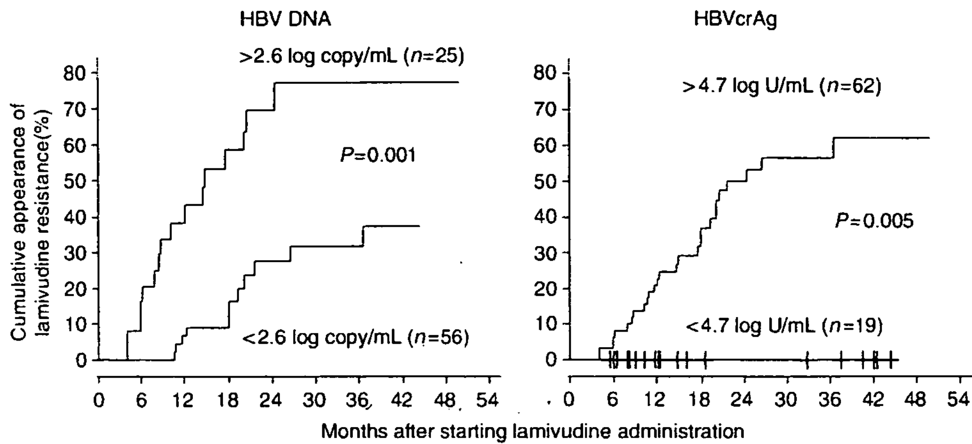

Fig. 3. Comparison of the cumulative occurrence of lamivudine resistance between patients who showed hepatitis B virus (HBV) DNA levels of less than the detection limit (2.6 log copy/ml) at 6 months after starting lamivudine administration and those who did not (left figure), and similarly between patients who showed HBV core-related antigen (HBVcrAg) levels of less than 4.7 log U/ml and those who did not (right figure).

coded by the core gene of the HBV genome with high sensitivity and a wide quantitative range. Serum HBVcrAg levels reflect the viral load in the natural course because these levels correlate linearly with those of HBV DNA (14, 15). On the other hand, the character of HBVcrAg is somewhat different from that of HBV DNA in patients undergoing anti-viral therapies such as lamivudine. That is, HBVcrAg levels decrease significantly more slowly than those of HBV DNA after the initiation of lamivudine administration.

HBV is an enveloped DNA virus containing a relaxed circular DNA genome, which is converted into a covalently closed circular DNA (cccDNA) episome in the nucleus of infected cells (18, 21–23). The cccDNA molecules serve as the transcriptional template for the production of viral RNAs that encode viral structural and non-structural proteins. Reverse transcription of the viral pregenomic RNA and second-strand DNA synthesis occur in the cytoplasm within viral capsids formed by the HBV core protein. Because lamivudine, a nucleoside analogue, inhibits reverse transcription of the pregenomic RNA, it directly suppresses the production of HBV virion. Thus, serum HBV DNA levels decrease rapidly after the initiation of lamivudine administration. On the other hand, the production of viral proteins is not suppressed by lamivudine because the production process does not include reverse transcription. Furthermore, it has been reported that the amount of cccDNA, which serves as a template for mRNA, decreases quite slowly after starting the administration of nucleoside analogues (24–26). Thus, it is reasonable that serum HBVcrAg levels decrease much more slowly than HBV DNA levels after the initiation of lamivudine therapy.

Significant markers that can predict the presence or absence of lamivudine resistance are clinically valuable because the emergence of this resistance and the subsequent recurrence of hepatitis are fundamental problems in lamivudine therapy. Serum markers that reflect the activity of HBV replication have been reported to be associated with the occurrence of lamivudine resistance (11, 12, 27, 28). However, neither the pretreatment existence of HBe antigen nor pretreatment levels of HBV DNA or HBVcrAg were found to be significant markers in the present study. These results may reflect a weak association between the pretreatment activity of HBV replication and the occurrence of lamivudine resistance (13, 29). Changes in HBV DNA and HBVcrAg levels after starting lamivudine administration clearly differed between patients with and without lamivudine resistance. Thus, HBV DNA and HBVcrAg levels at 6 months after starting lamivudine administration were analyzed to determine whether these levels might serve as predictive markers; both were found to be significantly lower in patients without lamivudine resistance at the tested point in time. Furthermore, patients who showed higher levels of HBV DNA and HBVcrAg at 6 months after the initiation of treatment were significantly more likely to develop lamivudine resistance than those who showed lower levels.

We believe that the measurement of HBV DNA levels is useful to identify patients who are at high risk for lamivudine resistance because as many as 70% of patients who were positive for HBV DNA at 6 months after starting lamivudine

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.

Acknowledgements

This research was supported in part by a research grant on hepatitis from the Ministry of Health, Labour and Welfare of Japan.

References

1. Doong S L, Tsai C H, Schinazi R F, Liotta D C, Cheng Y C. Inhibition of the replication of hepatitis B virus in vitro by 2′, 3′-dideoxy-3′-thiacytidine and related analogues. Proc Natl Acad Sci USA 1991; 88: 8495–9.
2. Benhamou Y, Dohin E, Lunel-Fabiani F, Poynard T, Huraox J M, Katlama C. et al. Efficacy of lamivudine on replication of hepatitis B virus in HIV-infected patients. Lancet 1995; 345: 396–7.
3. Dienstag J L, Goldin R D, Heathcote E J, Hann H W, Woessner M, Stephenson S L, et al. Histological outcome during long-term lamivudine therapy. Gastroenterology 2003; 124: 105–17.
4. Dienstag J L, Perrillo R P, Schiff E R, Bartholomew M, Vicary C, Rubin M. A preliminary trial of lamivudine for chronic hepatitis B infection. N Engl J Med 1995; 333: 1657–61.
5. Lai C L, Chien R N, Leung N W, Chang T T, Guan R, Tai D I, et al. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med 1998; 339: 61–8.
6. Liaw Y F, Sung J J, Chow W C, et al. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med 2004; 351: 1521–31.
7. Matsumoto A, Tanaka E, Rokuhara A, et al. Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B: a multicenter retrospective study of 2,795 patients. Hepatol Res 2005; 32: 173–84.
8. Ling R, Mutimer D, Ahmed M, et al. Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. Hepatology 1996; 24: 711–3.
9. Lok A S, Lai C L, Leung N, Yao G B, Cui Z Y, Schiff E R, et al. Long-term safety of lamivudine treatment in patients with chronic hepatitis B. Gastroenterology 2003; 125: 1714–22.
10. Tipples G A, Ma M M, Fischer K P, Bain V G, Kneteman N M, Tyrrell D L. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine in vivo. Hepatology 1996; 24: 714–7.
11. Liaw Y F, Chien R N, Yeh C T, Tsai S L, Chu C M. Acute exacerbation and hepatitis B virus clearance after emergence of YMDD motif mutation during lamivudine therapy. Hepatology 1999; 30: 567–72.
12. Suzuki F, Tsubota A, Arase Y, Suzuki Y S, Akuta N, Hosaka T, et al. Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. Intervirology 2003; 46: 182–9.
13. Zollner B, Schafer P, Feucht H H, Schroter M, Petersen J, Laufs R. Correlation of hepatitis B virus load with loss of e antigen and emerging drug-resistant variants during lamivudine therapy. J Med Virol 2001; 65: 659–63.
14. Kimura T, Rokuhara A, Sakamoto Y, Yagi S, Tanaka E, Klyosawa K, et al. Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. J Clin Microbiol 2002; 40: 439–45.
15. Rokuhara A, Tanaka E, Matsumoto A, Kimura T, Yamaura T, Orii K, et al. Clinical evaluation of a new enzyme immunoassay for hepatitis B virus core-related antigen; a marker distinct from viral DNA for monitoring lamivudine treatment. J Viral Hepatol 2003; 10: 324–30.
16. Bruss V, Gerlich W H. Formation of transmembraneous hepatitis B e-antigen by cotranslational in vitro processing of the viral precore protein. Virology 1988; 163: 268–75.
17. Garcia P D, Ou J H, Rutter W J, Walter P. Targeting of the hepatitis B virus precore protein to the endoplasmic reticulum membrane: after signal peptide cleavage translocation can be aborted and the product released into the cytoplasm. J Cell Biol 1988; 106: 1093–104.
18. Lee W M. Hepatitis B virus infection. N Engl J Med 1997; 337: 1733–45.
19. Mizokami M, Nakano T, Orito E, Tanaka Y, Sakugawa H, Mukaide M, et al. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. FEBS Lett 1999; 450: 66–71.
20. Kobayashi S, Shimada K, Suzuki H, Tanikawa K, Sata M. Development of a new method for detecting a mutation in the gene encoding hepatitis B virus reverse transcriptase active site (YMDD motif). Hepatol Res 2000; 17: 31–42.
21. Mason W S, Halpern M S, England J M, Seal G, Egan J, Coates L, et al. Experimental transmission of duck hepatitis B virus. Virology 1983; 131: 375–84.
22. Summers J, Smith P M, Horwich A L. Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification. J Virol 1990; 64: 2819–24.
23. Tuttleman J S, Pourcel C, Summers J. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. Cell 1986; 47: 451–60.

24. MORALEDA G. SAPUTELLI J. ALDRICH C E. AVERETT D. CONDREAY L, MASON W S. Lack of effect of antiviral therapy in nondividing hepatocyte cultures on the closed circular DNA of woodchuck hepatitis virus. J Virol 1997; 71: 9392–9.
25. WERLE-LAPOSTOLLE B. BOWDEN S, LOCARNINI S, WORSTHORN K, PETERSEN J, LAU G. et al. Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. Gastroenterology 2004; 126: 1750–8.
26. ZHU Y. YAMAMOTO T, CULLEN J. et al. Kinetics of hepadnavirus loss from the liver during inhibition of viral DNA synthesis. J Virol 2001: 75: 311–22.
27. LAU D T, KHOKHAR M F. DOO E. GHANY M G, HERION D, PARK Y. et al. Long-term therapy of chronic hepatitis B with lamivudine. Hepatology 2000; 32: 828–34.
28. YUEN M F. SABLON E, HUI C K. YUAN H J, DECRAEMER H, LAI C L. Factors associated with hepatitis B virus DNA breakthrough in patients receiving pro longed lamivudine therapy. Hepatology 2001; 34: 785–91.
29. MIHM U. SARRAZIN C. HERRMANN E, TEUBER G, VON WAGNER M, KRONEA BERGER B, et al.Response predictors and results of a long-term treatment with lamivudine in patients with chronic hepatitis B. Z Gastroenterol 2003; 41: 249–54.

PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

ELSEVIER

World J Gastroenterol 2005;11(16):2398-2401.
World Journal of Gastroenterology ISSN 1007-9327
© 2005 The WJG Press and Elsevier Inc. All rights reserved.

Expression of β-catenin in hepatocellular carcinoma

Liem Thanh Tien, Masahiro Ito, Mikiko Nakao, Daisuke Niino, Meirmanov Serik, Masahiro Nakashima, Chun-Yang Wen, Hiroshi Yatsuhashi, Hiromi Ishibashi

Liem Thanh Tien, Masahiro Ito, Mikiko Nakao, Department of Pathology, National Nagasaki Medical Center, 2-1001-1 Kubara, Omura, Nagasaki 856-8562, Japan
Daisuke Niino, Meirmanov Serik, Chun Yang Wen, Department of Molecular Pathology, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Science, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan
Masahiro Nakashima, Tissue and Histopathology Section Division of Scientific Data Registry, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Science, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan
Chun-Yang Wen, Department of Digestive Disease, Nanjing Drum Tower Hospital, Medical School of Nanjing University, Nanjing 210008, Jiangsu Province, China
Hiroshi Yatsuhashi, Hiromi Ishibashi, Clinical Research Center, National Nagasaki Medical Center, 2-1001-1 Kubara, Omura, Nagasaki 856-8562, Japan
Correspondence to: Masahiro Ito, MD, Department of Pathology, National Nagasaki Medical Center, 2-1001-1 Kubara, Omura, Nagasaki 856-8562, Japan. itohm@nmc.hosp.go.jp
Telephone: +81-957-52-3128 Fax: +81-957-54-0292
Received: 2004-06-08 Accepted: 2004-08-12

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.

© 2005 The WJG Press and Elsevier Inc. All rights reserved.

Key words: Hepatocellular carcinoma; β-Catenin; Immunohistochemistry

Tien TL, Ito M, Nakao M, Niino D, Serik M, Nakashima M, Wen CY, Yatsuhashi H, Ishibashi H. Expression of β-catenin in hepatocellular carcinoma. *World J Gastroenterol* 2005; 11(16): 2398-2401
http://www.wjgnet.com/1007-9327/11/2398.asp

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

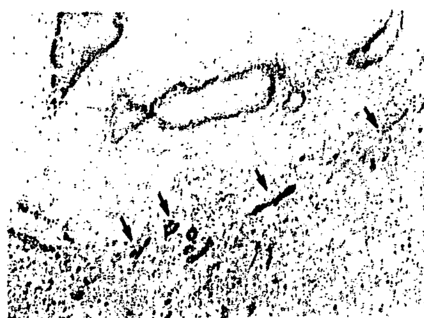

Figure 1 Constitutive expression of β-catenin in normal bile ducts. Membranous expression is conspicuous in normal bile ducts and newly formed bile ducts (arrow heads), but no expression is observed in normal hepatocytes.

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

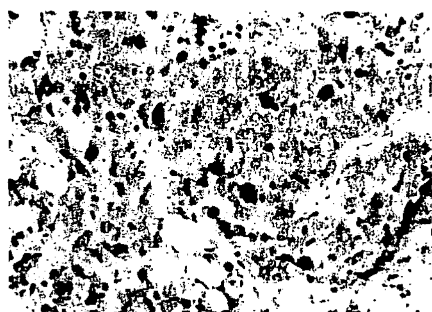

Figure 3 Nuclear expression. Nuclear expression is encountered in the poorly-differentiated HCC (arrows). Most cells co-expressed β-catenin in the cytoplasm.

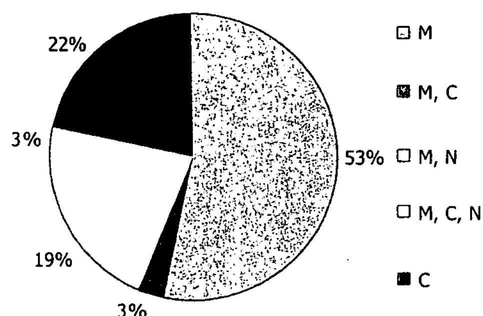

Figure 4 Localization of β-catenin expression in cell level. M: membranous, M+C: membranous and cytoplasmic, M+N: membranous and nuclear, M+N+C: membranous, cytoplasmic, and nuclear, C: cytoplasmic.

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.

REFERENCES

1 **Calvisi DF**, Factor VM, Ladu S, Conner EA, Thorgeirsson SS. Disruption of beta-catenin pathway or genomic instability define two distinct categories of liver cancer in transgenic mice. *Gastroenterology* 2004; **126**: 1374-1386

2 **Ozturk M**. Genetic aspects of hepatocellular carcinogenesis. *Semin Liver Dis* 1999; **19**: 235-242

3 **Prange W**, Breuhahn K, Fischer F, Zilkens C, Pietsch T, Petmecky K, Eilers R, Dienes HP, Schirmacher P. Beta-catenin accumulation in the progression of human hepatocarcinogenesis correlates with loss of E-cadherin and accumulation of p53, but not with expression of conventional WNT-1 target genes. *J Pathol* 2003; **201**: 250-259

4 **Torbenson M**, Kannangai R, Abraham S, Sahin F, Choti M, Wang J. Concurrent evaluation of p53, beta-catenin, and alpha-fetoprotein expression in human hepatocellular carcinoma. *Am J Clin Pathol* 2004; **122**: 377-382

5 **Wei Y**, Van Nhieu JT, Prigent S, Srivatanakul P, Tiollais P, Buendia MA. Altered expression of E-cadherin in hepatocellular carcinoma: correlations with genetic alterations, beta-catenin expression, and clinical features. *Hepatology* 2002; **36**: 692-701

6 **Cui J**, Zhou X, Liu Y, Tang Z, Romeih M. Wnt signaling in hepatocellular carcinoma: analysis of mutation and expression of beta-catenin, T-cell factor-4 and glycogen synthase kinase 3-beta genes. *J Gastroenterol Hepatol* 2003; **18**: 280-287

7 **Suzuki T**, Yano H, Nakashima Y, Nakashima O, Kojiro M. β-Catenin expression in hepatocellular carcinoma: A possible participation of β-catenin in the dedifferentiation process. *J Gastroenterol Hepatol* 2002; **17**: 994-1000

8 **Harada N**, Oshima H, Katoh M, Tamai Y, Oshima M, Taketo MM. Hepatocarcinogenesis in mice with beta-catenin and Ha-ras gene mutations. *Cancer Res* 2004; **64**: 48-54

9 **Inagawa S**, Itabashi M, Adachi S, Kawamoto T, Hori M, Shimazaki J, Yoshimi F, Fukao K. Expression and prognostic roles of β-Catenin in hepatocellular carcinoma. *Clin Cancer Res* 2002; **8**: 450-456

10 **Hsu HC**, Jeng YM, Mao TL, Chu JS, Lai PL, Peng SY. β-catenin mutations are associated with a subset of low-stage hepatocellular carcinoma negative for hepatitis B virus and with favorable prognosis. *Am J Pathol* 2000; **15**: 763-770

11 **Buendia MA**. Genetics of hepatocellular carcinoma. *Semin Cancer Biol* 2000; **10**: 185-200

12 **Nhieu JTV**, Renard CA, Wei Y, Cherqui D, Zafrani ES, Buendia MA. Nuclear accumulation of mutated β-catenin in hepatocellular carcinoma is associated with increased cell proliferation. *Am J Pathol* 1999; **155**: 703-710

13 **Huang H**, Fujii H, Sankila A, Mahler-Araujo BM, Matsuda M, Cathomas G, Ohgaki H. Beta-catenin mutations are frequent in human hepatocellular carcinomas associated with hepatitis C virus infection. *Am J Pathol* 1999; **155**: 1795-1801

14 **Wong CM**, Fan ST, Ng IO. Beta-Catenin mutation and overexpression in hepatocellular carcinoma: clinicopathologic and prognostic significance. *Cancer* 2001; **92**: 136-145

15 **Kondo Y**, Kanai Y, Sakamoto M, Genda T, Mizokami M, Ueda R, Hirohashi S. Beta-catenin accumulation and mutation of exon 3 of the beta-catenin gene in hepatocellular carcinoma. *Jpn J Cancer Res* 1999; **90**: 1301-1309

16 **Fujito T**, Sasaki Y, Iwao K, Miyoshi Y, Yamada T, Ohigashi H, Ishikawa O, Imaoka S. Prognostic significance of beta-catenin nuclear expression in hepatocellular carcinoma. *Hepatogastroenterology* 2004; **51**: 921-924

17 **Edamoto Y**, Hara A, Biernat W, Terracciano L, Cathomas G, Riehle HM, Matsuda M, Fujii H, Scoazec JY, Ohgaki H. Alterations of RB1, p53 and Wnt pathways in hepatocellular carcinomas associated with hepatitis C, hepatitis B and alcoholic liver cirrhosis. *Int J Cancer* 2003; **106**: 334-341

18 **Levy L**, Renard CA, Wei Y, Buendia MA. Genetic alterations and oncogenic pathways in hepatocellular carcinoma. *Ann N Y Acad Sci* 2002; **963**: 21-36

19 **Shtutman M**, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A. The cyclin D1 gene is a target of the β-catenin/ LEF-1 pathway. *Proc Natl Acad Sci USA* 1999; **96**: 5222-5227

20 **Shang XZ**, Zhu H, Lin K, Tu Z, Chen J, Nelson DR, Liu C. Stabilized beta-catenin promotes hepatocyte proliferation and inhibits TNFalpha-induced apoptosis. *Lab Invest* 2004; **84**: 332-341

21 **Monga SP**, Monga HK, Tan X, Mule K, Pediaditakis P, Michalopoulos GK. Beta-catenin antisense studies in embryonic liver cultures: role in proliferation, apoptosis, and lineage specification. *Gastroentrerology* 2003; **124**: 202-216

22 **Delpuech O**, Trabut JB, Carnot F, Feuillard J, Brechot C, Kremsdorf D. Identification, using cDNA macroarray analysis, of distinct gene expression profiles associated with pathological and virological features of hepatocellular carcinoma. *Oncogene* 2002; **21**: 2926-2937

Science Editor Guo SY **Language Editor** Elsevier HK

Original Paper

Intervirology

Intervirology 2005;48:112–119
DOI: 10.1159/000081737

Received: March 15, 2004
Accepted: May 5, 2004

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

Kojiro Michitaka[a] Norio Horiike[b] Yan Chen[b] Hiroshi Yatsuhashi[g] Michitami Yano[f] Naohiko Kojima[c] Keiji Ohkubo[c] Yosikazu Tanaka[d] Kazuhisa Yamamoto[e] Naofumi Ohno[f] Morikazu Onji[b]

[a]Endoscopy Center and [b]Third Department of Internal Medicine, Ehime University School of Medicine, [c]Internal Medicine, Ohzu Central Hospital, [d]Internal Medicine, Ehime Prefectural Central Hospital, [e]Department of Gastroenterology, Matsuyama Shimin Hospital, and [f]Ohno Naika, Ehime, and [g]Clinical Research Center, National Nagasaki Medical Center, Nagasaki, Japan

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.

Copyright © 2005 S. Karger AG, Basel

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

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2005 S. Karger AG, Basel
0300–5526/05/0483–0112$22.00/0

Accessible online at:
www.karger.com/int

Morikazu Onji, MD
Third Department of Internal Medicine, Ehime University School of Medicine
Shigenobu-cho, Onsen-gun
Ehime-ken, 791-0295 (Japan)
Tel. +81 89 960 5308, Fax +81 89 960 5310, E-Mail onjimori@m.ehime-u.ac.jp

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 (BigDye 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.