ished by gonadectomy of male rats (24). In our analysis of male patients, their ALT-CPR correlation curve was similar to that of postmenopausal women. However, the correlation was weak, and was not reliable because the population with high insulin resistance was very small. This low population was mainly due to the exclusion of a large number of patients who habitually drank alcohol. A study involving a larger number of patients would be required to confirm the influence of androgens. In conclusion, our results strongly indicate that sex steroids are key factors of progression of NAFLD and NASH. If estrogen is an aggravating factor, our findings might lead to understanding of two unresolved clinical problems, the mechanism of acute fatty liver in pregnancy and the rapid progression of alcoholic hepatitis in females. Further studies focusing on the roles of sex steroids in NFALD and NASH are needed, both clinical studies and animal experiments using their models.

## REFERENCES


1 **Mehta K, Van Thiel DH, Shah N, Mobarhan SL:** Nonalcoholic fatty liver disease: pathogenesis and the role of antioxidants. Nutr Rev 2002; 60:289-293.
2 **Harrison SA, Di Bisceglie AM:** Advances in the understanding and treatment of nonalcoholic fatty liver disease. Drugs 2003; 63:2379-2394.
3 **Day CP:** Pathogenesis of steatohepatitis. Best Pract Res Clin Gastroenterol 2002; 16:663-678.
4 **Marchesini G, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, Manini R, et al:** Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. Hepatology 2003; 37:917-923.
5 **Deschamps D, De Beco V, Fisch C, Fromentry B, Guillouzo A, Pessayre D:** Inhibition by perhexiline of oxidative phosphorylation and the β-oxidation of fatty acids: possible role in pseudoalcoholic liver disease. Hepatology 1994; 19:948-961.
6 **Pérez-Carreras M, Del Hoyo P, Martín MA, Rubio JC, Martín A, Castellano G, et al:** Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. Hepatology 2003; 38:999-1007.
7 **Chalasarni N, Gorski C, Asghar MS, Asghar A, Foresman B, Hall SD, Crabb W:** Hepatic cytochrome P450 2E1 activity in nondiabetic patients with nonalcoholic steatohepatitis. Hepatology 2003; 37:544-550.
8 **Lieber CS:** CYP2E1: from ASH to NASH. Hepatol Res 2004; 28:1-11.
9 **Harrison SA, Torgerson S, Hayashi PH:** The natural history of nonalcoholic fatty liver disease: a clinical histopathological study. Am J Gastroenterol 2003; 98:2042-2047.
10 **Angulo P, Keach JC, Batts KP, Lindor KD:** Independent predictors of liver fibrosis in patients with nonalcoholic steatohepatitis. Hepatology 1999; 30:1356-1362.
11 **Kashireddy PR, Rao MS:** Sex differences in choline-deficient diet-induced steatohepatitis in mice. Exp Biol Med (Maywood) 2004; 229:158-162.
12 **Fujimoto N, Kohta R, Kitamura S, Honda H:** Estrogenic activity of an antioxidant, nordihydroguaiaretic acid (NDGA). Life Sci 2004; 74:1417-1425.
13 **Perez-Carreras M, Del Hoyo P, Martin MA, Rubio JC, Martin A, Castellano G, Colina F, et al:** Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. Hepatology 2003; 38:999-1007.
14 **Luyckx FH, Lefebvre PJ, Scheen AJ:** Non-alcoholic steatohepatitis: association with obesity and insulin resistance, and influence of weight loss. Diabetes Metab 2000; 26:98-106.
15 **Xu JW, Gong J, Chang XM, Luo JY, Dong L, Hao ZM, Jia A, et al:** Estrogen reduces CCL4- induced liver fibrosis in rats. World J Gastroenterol 2002; 8:883-887.
16 **Starkel P, Sempoux C, Leclercq I, Herin M, Deby C, Desager JP, Horsmans Y:** Oxidative stress, KLF6 and transforming growth factor-beta up-regulation differentiate non-alcoholic steatohepatitis progressing to fibrosis from uncomplicated steatosis in rats. J Hepatol 2003; 39:538-546.
17 **Shimizu I:** Impact of oestrogens on the progression of liver disease. Liver Int 2003; 23:63-69.
18 **Robertson G, Leclercq I, Farrell GC:** Nonalcoholic steatosis and steatohepatitis. II. Cytochrome P-450 enzymes and oxidative stress. Am J Physiol Gastrointest Liver Physiol 2001; 281:G1135-1139.
19 **Stork S, von Schacky C, Angerer P:** The effect of 17beta-estradiol on endothelial and inflammatory markers in postmenopausal women: a randomized, controlled trial. Atherosclerosis 2002; 165:301-307.
20 **O'Sullivan AJ, Martin A, Brown MA:** Efficient fat storage in premenoposal women and in earuly pregnancy: a role for estrogen. J Clin Endocrinol Metab 2001; 86:4951-4956.
21 **O'Sullivan AJ, Crampton LJ, Fruend J, Ho KKY:** The route of estrogen replacement therapy confers divergent effects on substrate oxidation and body composition in postmenopausal women. J Clin Invest 1998; 102:1035-1040.
22 **Kishimoto R, Ogishi Y, Ueda M, Matsusaki M, Amano K, Goda K, Park SS:** Gender-related difference in mouse hepatic ethanol metabolism. J Nutr Sci Vitaminol 2002; 48:216-224.
23 **Jarvelainen HA, Lukkari TA, Heinaro S, Sipple H, Lindros KO:** The antiestrogen toremifene protects against alcoholic liver injury in female rats. J Hepatol 2001; 35:46-52.
24 **Jalouli M, Carlsson L, Ameen C, Linden D, Ljungberg A, Michalik L, et al:** Sex difference in hepatic peroxisome proliferator-activated receptor alpha expression: influence of pituitary and gonadal hormones. Endocrinology 2003; 144:101-109.

Liver International ISSN 1478-3223

BASIC STUDIES

# Decreased portal flow volume increases the area of necrosis caused by radio frequency ablation in pigs

Tsuyoshi Yoshimoto, Kazuhiro Kotoh, Yuki Horikawa, Motoyuki Kohjima, Shusuke Morizono, Shinsaku Yamashita, Munechika Enjoji and Makoto Nakamuta

Department of Medicine and Bioregulatory Science, Graduate School of Medical Science, Kyushu University, Fukuoka, Japan

**Keywords**
complication – hepatocellular carcinoma – over-ablation – RFA

**Correspondence**
Kazuhiro Kotoh, Department of Medicine and Bioregulatory Science, Graduate School of Medical Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
Tel: +81 92 642 5282
Fax: +81 92 642 5287
e-mail: kotoh-k@intmed3.med.kyushu-u.ac.jp

Received 15 September 2006
accepted 17 November 2006

DOI:10.1111/j.1478-3231.2007.01454.x

## Abstract

*Background/aims:* Although radio frequency ablation (RFA) has been widely accepted as an effective treatment for hepatocellular carcinoma (HCC), severe complications are not uncommon. Major complications seem to occur as a result of over-ablation beyond the intended area. As most patients with HCC have underlying cirrhosis, we speculated that decreased portal flow might cause the necrosis associated with RFA. To confirm this hypothesis, we examined the area of necrosis resulting from RFA under varying conditions of portal flow in a porcine model. *Methods:* RFA was performed using ultrasonographic guidance in anesthetized pigs. During the RFA procedure, portal flow was regulated by a balloon catheter, which was set in a portal trunk. The necrosis area was measured after sacrifice and was compared with the hyperechoic area that appeared during ablation. In another session, RFA was performed close to the hepatic vein and endothelial damage was examined. *Results:* The necrosis area caused by RFA was significantly larger when the portal flow volume was decreased by 50% or more. The hyperechoic lesion was always larger than the area of pathological necrosis regardless of portal flow volume. Under conditions of decreased portal flow, the vessel endothelium near the ablated area was more readily damaged. *Conclusion:* Decreased portal flow volume resulted in enlargement of the area of necrosis caused by RFA. Our results indicate that over-ablation could easily occur in patients with advanced cirrhosis, and that this could lead to major complications. Ultrasonographic guidance may be helpful for avoiding over-ablation.

The efficacy of radio frequency ablation (RFA) for the treatment of hepatocellular carcinoma (HCC) has been widely recognized (1–5). Recent studies comparing RFA with earlier ablation methods such as ethanol injection therapy and microwave coagulation therapy showed that RFA resulted in wider and more complete necrosis (3, 6, 7). However, several studies have also shown that RFA can be associated with serious complications such as gastrointestinal perforation, hepatic abscess, intraperitoneal hemorrhage, portal thrombus, liver infarction, and bile peritonitis (4, 8–12). Despite the prevalence of such complications, the background of patients most likely to experience complications related to RFA has not been investigated.

Most of the reported complications seem to have occurred when the ablated area spread beyond the intended margin, which caused heat damage in vessels, bile ducts, and contiguous viscera. Because the RFA procedure has been generally performed using two-dimensional ultrasonographic imaging, it is conceivable that vessels or viscera located apart from guiding-image plane could be unexpectedly injured. However, not only is it difficult to visualize three-dimensional structure but the actual ablation area achieved with RFA may not always be equal to that which was intended. In our limited experience, we have found that the area of ablation confirmed by computed tomography after RFA was not equal among patients despite treatment with the same protocol and device. To our knowledge, such unevenness in the ablation area among patients has not been widely addressed. However, it is important to determine why unevenness of the ablated area occurs, as it could contribute to complications associated with RFA.

To explain why the ablated area is often uneven, we speculated that the portal flow volume, which is decreased to varying degrees among patients with liver cirrhosis, would influence the ablation size. It is well known that the most of patients with HCC have liver cirrhosis owing to hepatitis B or C virus. It has

previously been shown in a porcine model that RFA during the Pringle maneuver spreads the ablated area owing to a 'heat sink effect' (13, 14). However, most cirrhotic patients have at least some residual portal flow volume (15, 16), while the Pringle maneuver results in complete occlusion of portal flow. In discussing the influence of portal flow volume on RFA in the patients with liver cirrhosis we should confirm the effects under various degree of portal flow volume.

To determine the influence of portal flow volume on RFA in patients with liver cirrhosis we used a porcine model in which the portal flow volume was varied using a balloon catheter set in the portal vein. We compared the histological ablation area and the expected ablation area based on ultrasonographic imaging, as differences between these two parameters may contribute to complications associated with RFA.

## Methods

### Animals

Five healthy female pigs (58–61 kg) were anaesthetized with ketamine 10 mg/kg, clonidine 5 μg/kg, and atropine 0.02 mg/kg. Anaesthesia was maintained by ventilation with a mixture of oxygen and nitrous oxide. The RFA procedure was performed following midline laparotomy and the animals were subsequently sacrificed.

### Regulation of portal flow volume and RFA

In order to regulate the portal flow volume, a balloon catheter (Occlusion Balloon Catheter, OBW/20/8/100, Boston Scientific Japan K.K., Tokyo, Japan) was set in the main trunk of the portal vein and X-ray imaging was used to confirm that the tip of the catheter was located within the main trunk. The portal flow volume was measured at the first right branch of the portal vein by expanding the balloon to various degrees before puncture with the electrode needle.

The electrode needle was a LeVeen™ multipolar array needle (20 mm diameter type; Boston Scientific Corporation, Natick, MA, USA) used in combination with an RF 2000 generator™ (Radio Therapeutics Corporation, Sunnyvale, CA, USA) according to the manufacturers protocol. In short, the tines were fully expanded after the needle was inserted to the target position and RF energy was then applied to the tissue using an initial power setting of 30 W, which was subsequently increased in increments of 10 W/min to a maximum power of 75 W. The power setting was then maintained at 75 W until power 'roll-off' occurred; tissue impedance (an increase in tissue resistance caused by decreased conductivity of electrical current by protein denaturation and loss of intracellular fluids) rose to over 200 ω, at which time the power passively decreased to $< 10$ W. For the ablations, all electrode needle punctures were performed under ultrasonographic guidance confirming that visible vessels were at least 20 mm away from the expanded electrode.

After ablation, the hyperechoic areas on ultrasonographic images were evaluated. The maximum and minimum dimensions (length and width) of the hyperechoic lesion were determined and the area was calculated as follows:

$$\text{Area of hyperechoic lesion} = ((\text{maximum distance} + \text{minimum distance})/4)^2 \times \pi.$$

We also performed RFA close to the hepatic vein to observe the influence of portal flow volume on blood temperature in the hepatic vein and on endothelial damage. For these procedures, the tip of the electrode was placed 10 mm from hepatic vein and a thin thermometer (Digital Thermometer; SK-250WP, Sato Co., Tokyo, Japan) was set in the vein during the ablation.

Data shown in the figures are expressed as mean ± SD. Dunnett's *post hoc* test was used to analyse differences in the ablated areas from the RFA group and the non-occlusion control group.

### Histopathology

To determine the area of necrosis caused by RFA, the ablated lesion was removed *en bloc* and sectioned tangential to the long axis of the probe shaft. For each specimen, the maximum and minimum distances of the region of necrosis area were measured, and the area of necrosis was calculated as follows:

$$\text{Pathological necrosis area} = ((\text{maximum distance} + \text{minimum distance})/4)^2 \times \pi.$$

Portal vein specimens including liver parenchyma were taken from near the ablated area, cut into blocks, and frozen in liquid nitrogen. Cryostat sections (8 μm) were fixed with 4% (w/v)-buffered formaldehyde, rinsed with distilled water, and mounted in glycerol jelly. Sections were incubated with a solution containing the substrate NADH (Sigma Diagnostics, St. Louis, MO, USA) and the indicator nitro-blue tetrazolium (Sigma Diagnostics) for 30 min at room temperature. Cell viability was judged by the detection of reduced nicotinamide adenine dinucleotide diaphorase (NADHd).

## Results

When the balloon in the portal vein trunk was expanded, no change of blood pressure, heart rate, or respiration was observed. The RFA procedure and the puncture of the hepatic vein did not affect vital signs.

As shown in Fig. 1A, the pathological necrosis area negatively correlated with the portal flow volume. The areas of necrosis under conditions of 25% occlusion did not differ significantly from the control group (0% occlusion), whereas the area was significantly increased by occlusion of 50% or more. We also examined the effect of RFA just after sacrifice to determine the effect on necrosis area when both portal vein and hepatic artery flow was interrupted. The necrosis area caused by RFA after sacrifice was almost equal to that under conditions of 100% portal vein occlusion.

The hyperechoic area produced by RFA gradually increased as a result of portal vein occlusion and was consistent with pathological findings. We aimed to separately examine the US-image change and the pathological change. After that, we confirmed the correlation between them. (Fig. 1B). The pathological necrosis area was always larger than the hyperechoic area determined by ultrasonographic imaging regardless of the degree of portal vein occlusion (Fig. 1C).

In the series of ablations conducted close to the hepatic vein, the increase of intravenous temperature was significant with 100% portal vein occlusion but not with 50% occlusion (Fig. 2). NADHd staining of hepatic vein specimens revealed massive loss of endothelial cells in all of the samples from the group that received 100% portal vein occlusion. Endothelial cell viability was decreased by expansion of the balloon in the portal vein (Fig. 3).

## Discussion

Although the effect of RFA for HCC has been confirmed in past studies, reports of complications were not infrequent. In recent analyses of large numbers of patients with HCC, the rate of major complications was 4.0–5.7% (4, 8, 9, 12). Despite the relatively high frequency of complications, the backgrounds of patients who are most likely to develop complications have rarely been discussed. Recently, Curley et al. (12) prospectively analysed the complications related to RFA for primary and metastatic liver tumors and showed that the prevalence was higher in cirrhotic than in non-cirrhotic patients. However, they also pointed out that there was only a slight difference in the rate of complications between these two groups of patients, and emphasized that the higher frequency of complications in cirrhotic patients was acceptable because most patients with advanced liver cirrhosis would not be candidates for liver resection. Although we consider their results to be merely suggestive, the correlation between the prevalence of complications and the degree of cirrhosis progression should be analysed in order to clarify the importance of cirrhosis as a predictive factor for the occurrence of complications.

On the basis of the earlier studies that showed increased ablated area under Pringle maneuver, it is natural to speculate that decreased portal flow volume might negatively influence the ablation size with RFA. Several prior studies have evaluated portal flow volume in cirrhotic patients using Doppler ultrasound. According to one study, the portal flow volume in Child's A patients was maintained at about 90% of healthy controls, whereas that in Child's B or C patients decreased to about 50–60% (16). Our results

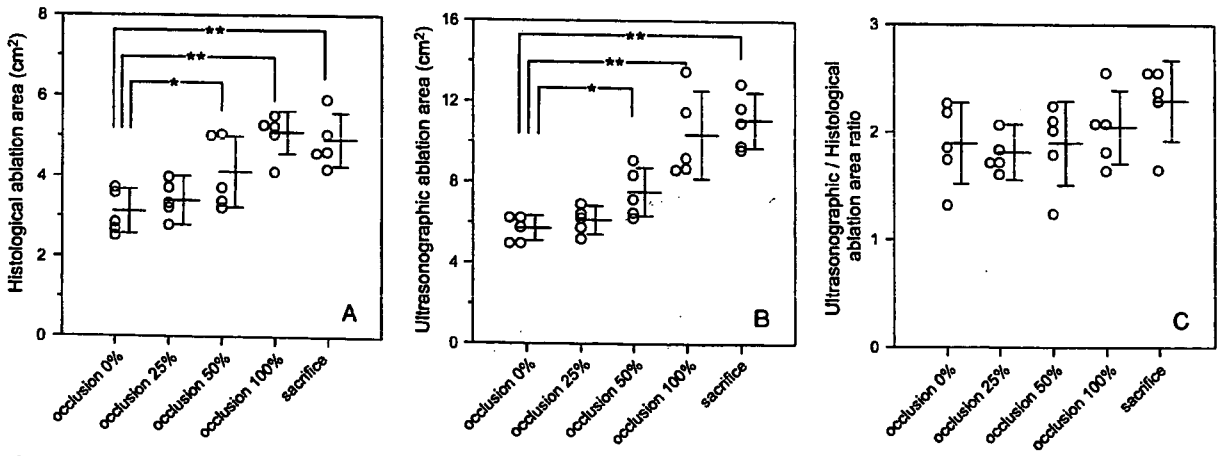


**Fig. 1.** Both the pathological necrosis area (A) and the hyperechoic area (B) caused by radio frequency ablation (RFA) negatively correlated with portal flow volume. The hyperechoic area was always larger than necrosis area regardless of portal flow volume (C).

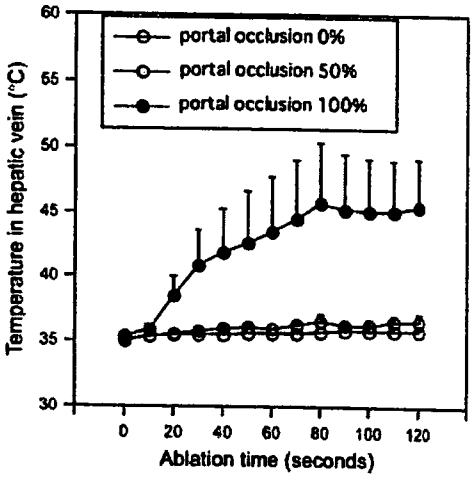


**Fig. 2.** Intravenous temperature during ablation close to the hepatic vein. The temperature significantly increased as a result of 100% occlusion of portal flow, while temperature did not change significantly with either 50% or 0% occlusion.

revealed that a decrease in portal flow volume of 50% or more caused a significant increase in the ablation size, indicating that unexpected spread of the ablation area might occur with RFA in advanced cirrhotic patients. Furthermore, we also observed an increase of intravenous temperature and endothelial damage associated with ablation close to the hepatic vein under conditions of decreased portal flow volume. Unintentional spread of the ablation area might cause adjacent visceral perforation and bile duct injury, and vessel endothelial damage might lead to thrombosis. Although such complications were commonly reported in prior clinical studies of RFA for liver tumor treatment, we noticed that the incidence of intestinal perforation has decreased in recent reports. We speculate that this decrease may be owing to improved technical skill of the operators who are more proficient in avoiding critical complications. If this assumption is true, then the negative influence of portal flow volume on ablation area could represent a greater risk for immature operators.

In recent studies, the percutaneous ultrasound-guided procedure has been used more often than has RFA with laparoscopy (5, 17, 18). With the ultrasound-guided technique, the hyperechoic area is regarded as the margin of necrosis. However, no prior studies have confirmed whether the hyperechoic image during ablation actually corresponds with histological necrosis. In order to confirm the usefulness of ultrasonographic imaging during RFA, we compared the histological necrosis area after RFA with the hyperechoic area achieved by thermal ablation. Our result

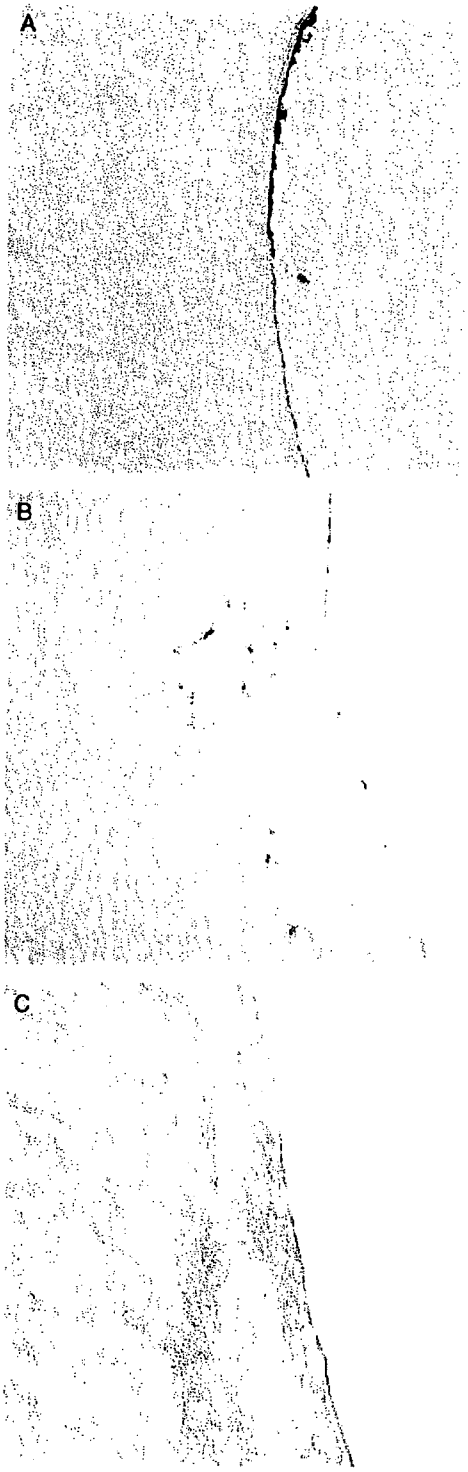


**Fig. 3.** Nicotinamide adenine dinucleotide diaphorase (NADHd) staining of sections of liver tissue obtained following ablation close to the hepatic vein under conditions of 0% (A), 50% (B), and 100% occlusion (C) of portal flow. The viability of venous endothelial cells declined in proportion to the decrease in portal flow volume.

showed that the area of histological necrosis was always smaller than the ultrasonographic image regardless of the portal flow volume. Hence, major complications can be avoided when the ablation procedure is performed using the spread of the hyperechoic image on ultrasonography as a guide.

Our results indicate that the necrosis area caused by RFA is influenced by portal flow volume. Considering that most of the patients with HCC have underlying cirrhosis, the unevenness of ablation size, even when using the same device and protocol, is likely owing to differences in portal flow volume. When using RFA in cirrhotic patients, especially those with Child's B and C, the resultant area of ablation may be larger than intended, which could lead to severe complications. Careful observation of the hyperechoic image that develops during ablation when using ultrasonographic guidance can be helpful for avoiding major complications related to RFA.

## References

1. Machi J, Bueno RS, Wong LL. Long-term follow-up outcome of patients undergoing radiofrequency ablation for unresectable hepatocellular carcinoma. *World J Surg* 2005; 29: 1364–73.
2. Poon RT, Ng KK, Lam CM, *et al.* Learning curve for radiofrequency ablation of liver tumors: prospective analysis of initial 100 patients in a tertiary institution. *Ann Surg* 2004; 239: 441–9.
3. Shiina S, Teratani T, Obi S, Hamamura K, Koike Y, Omata M. Nonsurgical treatment of hepatocellular carcinoma: from percutaneous ethanol injection therapy and percutaneous microwave coagulation therapy to radiofrequency ablation. *Oncology* 2002; 62(Suppl. 1): 64–8.
4. Tateishi R, Shiina S, Teratani T, *et al.* Percutaneous radiofrequency ablation for hepatocellular carcinoma. An analysis of 1000 cases. *Cancer* 2005; 103: 1201–9.
5. Raut CP, Izzo F, Marra P, *et al.* Significant long-term survival after radiofrequency ablation of unresectable hepatocellular carcinoma in patients with cirrhosis. *Ann Surg Oncol* 2005; 12: 616–28.
6. Luo BM, Wen YL, Yang HY, *et al.* Percutaneous ethanol injection, radiofrequency and their combination in treatment of hepatocellular carcinoma. *World J Gastroenterol* 2005; 11: 6277–80.
7. Omata M, Tateishi R, Yoshida H, Shiina S. Treatment of hepatocellular carcinoma by percutaneous tumor ablation methods: ethanol injection therapy and radiofrequency ablation. *Gastroenterology* 2004; 127: S159–66.
8. Livraghi T, Solbiati L, Meloni MF, Gazelle GS, Halpern EF, Goldberg SN. Treatment of focal liver tumors with percutaneous radio-frequency ablation: complications encountered in a multicenter study. *Radiology* 2003; 226: 441–51.
9. Chen MH, Yang W, Yan K, *et al.* Treatment efficacy of radiofrequency ablation of 338 patients with hepatic malignant tumor and the relevant complications. *World J Gastroenterol* 2005; 11: 6395–401.
10. Jansen MC, van Duijnhoven FH, van Hillegersberg R, *et al.* Adverse effects of radiofrequency ablation of liver tumours in the Netherlands. *Br J Surg* 2005; 92: 1248–54.
11. Moumouh A, Hannequin J, Chagneau C, *et al.* A tamponade leading to death after radiofrequency ablation of hepatocellular carcinoma. *Eur Radiol* 2005; 15: 234–7.
12. Curley SA, Marra P, Beaty K, *et al.* Early and late complications after radiofrequency ablation of malignant liver tumors in 608 patients. *Ann Surg* 2004; 239: 450–8.
13. Patterson EJ, Scudamore CH, Owen DA, Nagy AG, Buczkowski AK. Radiofrequency ablation of porcine liver *in vivo*: effects of blood flow and treatment time on lesion size. *Ann Surg* 1998; 227: 559–65.
14. Shen P, Fleming S, Westcott C, Challa V. Laparoscopic radiofrequency ablation of the liver in proximity to major vasculature: effect of the Pringle maneuver. *J Surg Oncol* 2003; 83: 36–41.
15. Annet L, Materne R, Danse E, Jamart J, Horsmans Y, Van Beers BE. Hepatic flow parameters measured with MR imaging and Doppler US: correlations with degree of cirrhosis and portal hypertension. *Radiology* 2003; 229: 409–14.
16. Kayacetin E, Efe D, Dogan C. Portal and splenic hemodynamics in cirrhotic patients: relationship between esophageal variceal bleeding and the severity of hepatic failure. *J Gastroenterol* 2004; 39: 661–7.
17. Lencioni R, Della Pina C, Bartolozzi C. Percutaneous image-guided radiofrequency ablation in the therapeutic management of hepatocellular carcinoma. *Abdom Imaging* 2005; 30: 401–8.
18. Pompili M, Mirante VG, Rondinara G, *et al.* Percutaneous ablation procedures in cirrhotic patients with hepatocellular carcinoma submitted to liver transplantation: assessment of efficacy at explant analysis and of safety for tumor recurrence. *Liver Transpl* 2005; 11: 1117–26.

ELSEVIER

Journal of Hepatology 45 (2006) 646–653

Journal of Hepatology

www.elsevier.com/locate/jhep

# Specific mutations in enhancer II/core promoter of hepatitis B virus subgenotypes C1/C2 increase the risk of hepatocellular carcinoma

Yasuhito Tanaka[1], Motokazu Mukaide[1,3], Etsuro Orito[2], Man-Fung Yuen[4], Kiyoaki Ito[2], Fuat Kurbanov[1], Fuminaka Sugauchi[2], Yasuhiro Asahina[5], Namiki Izumi[5], Michio Kato[6], Ching-Lung Lai[4], Ryuzo Ueda[2], Masashi Mizokami[1,*]

[1]*Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan*
[2]*Internal Medicine and Molecular Science, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan*
[3]*SRL Inc., Tokyo, 51 Komiya, Hachiouji, Tokyo 192-8535, Japan*
[4]*Department of Medicine, The University of Hong Kong, Queen Mary Hospital, Hong Kong*
[5]*Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan*
[6]*National Hospital Organization Osaka National Hospital, Osaka, Japan*

***Background/Aims*: Hepatitis B virus genotype C (HBV/C) has been classified into two geographically distinct subgenotypes; HBV/C1/Cs (Southeast Asia) and HBV/C2/Ce (East Asia).**

***Methods*: Viral differences in enhancer II/core promoter and precore regions between the subgenotypes and their association with hepatocellular carcinoma (HCC) were assessed in a matched cross-sectional control study of 118 carriers (from Hong Kong) with HBV/C1/Cs (48.0 years, 81% male, 40% HBeAg+, 44% HCC) and 210 HBV/C2/Ce (172 from Japan, 38 from Hong Kong) (50.2 years, 78% male, 30% HBeAg+, 46% HCC).**

***Results*: Univariate analyses showed that mutation V1753 was predictive for HCC among HBeAg-positive-C1/Cs-carriers ($P = 0.0055$), and T1653 among HBeAg-positive-C2/Ce-carriers ($P = 0.018$), and T1653 or V1753 or T1762/A1764 among HBeAg-negative-C2/Ce-carriers ($P < 0.05$). In the multivariate analysis on all HBV/C subjects, independent predictive factors for HCC were subgenotype C2/Ce (odds ratio, 4.21; 95% confidence interval, 1.07–16.23), T1653 (3.64; 1.93–6.86), V1753 (3.07; 1.66–5.65) and T1762/A1764 (2.58; 1.21–5.49) mutations, age (≥50 years), gender (male) and HBeAg (positive).**

***Conclusions*: Our data indicate that T1653 and/or V1753 mutations in addition to T1762/A1764 are differently associated with HCC in context of HBeAg status among HBV/C1/Cs and C2/Ce-carriers. HBV/C subgenotypes have specific mutation patterns, which is probably responsible for increased carcinogenesis of HBV/C2/Ce.**



***Keywords*: Hepatitis B virus; Hepatocellular carcinoma; Subgenotype C; Enhancer II; Core promoter; Precore genome**

## 1. Introduction

HBV genotypes have a distinct geographical distribution and correlate with severity of liver disease [1,2]. Genotypes B and C are prevalent in Asia, and genotype C causes more serious liver disease than genotype B [3,4]. There are two subtypes (subgenotypes) of genotype B in distinct geographical distributions, designated Ba ("a" standing for Asia) and Bj ("j"

*Received 5 April 2006; received in revised form 23 May 2006; accepted. 26 June 2006; available online 10 August 2006*

* Corresponding author. Tel.: +81 52 853 8292; fax: +81 52 842 0021.

*E-mail address:* mizokami@med.nagoya-cu.ac.jp (M. Mizokami).

*Abbreviations:* HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; ALT, alanine aminotransferase; ELISA, enzyme-linked immunosorbent assay; RFLP, restriction fragment length polymorphism; RTD-PCR, real-time detection polymerase chain reaction.

0168-8278/$32.00 
doi:10.1016/j.jhep.2006.06.018

for Japan) provisionally [5], and clinical differences between patients infected with HBV/Ba and HBV/Bj are coming to the fore [6,7]. Recently, a phylogenetic analysis of the pre-S1/pre-S2 genes revealed two major groups within genotype C: one for strains from Southeast Asia including Vietnam, Myanmar, Thailand and Hong Kong (named HBV/C1) and the other for strains from (Far) East Asia including Japan and China (named HBV/C2). This finding was confirmed by phylogenetic analyses based on the complete sequences of 32 HBV/C strains [8], and by recent independent studies in Hong Kong [9] and Japan [10]. The latter papers designated the 2 subgenotypes as HBV/Cs in Southeast Asia and HBV/Ce in the (Far) East Asia that have not only different epidemiological distributions but also different virological findings in precore regions [9,10].

Mutations in the basic core promoter (BCP) region at nucleotides (nt) 1762/1764 (T1762/A1764) and mutation in the precore region at nt 1896 (A1896) are associated with HBe antigen seroconversion (SC) and viral replication. It is noteworthy that the both BCP and precore stop-codon mutations are often found in patients with advanced liver disease such as hepatocellular carcinoma (HCC) [11–14]. Beyond these mutations, the C to T mutation in the upstream regulatory sequence (C1653T) is associated with fulminant hepatitis [15] and located in the alpha box, which is a strong activating element of both enhancer II and core promoter [16]. Takahashi et al. [17,18] reported that C-to-T1653 and T-to-V(not T)1753 mutants were more closely associated with the progression of liver disease from chronic hepatitis to cirrhosis and/or HCC in HBeAg-positive patients, compared with the BCP double mutation. Our recent case-control study supports that the addition of T1653 mutation in enhancer II to the BCP mutation increases the risk of HCC in anti-HBe-positive patients with HBV/C [19].

To evaluate clinical and virological significances between HBV/C1/Cs and HBV/C2/Ce, in the present study, we performed a multi-center cross-sectional matched control study among HBV/C carriers [inactive carriers (IC), chronic hepatitis (CH), HCC] and determined the specific HBV mutations associated with disease progression.

## 2. Materials and methods

### 2.1. Serum samples

A total of 328 sera were obtained from chronic HBV/C carriers who visited Nagoya City University Hospital, Musashino Red Cross Hospital, Osaka National Hospital in Japan and Queen Mary Hospital in Hong Kong. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the Ethics Committees of the institutions, and an informed consent was obtained from each carrier.

### 2.2. Serological assays for HBV markers

HBeAg and anti-HBe were detected by Chemiluminescent enzyme immunoassay (CLEIA) (Lumipulse f, FUJIREBIO INC., Tokyo, Japan). HBV Genotypes were determined by enzyme-linked immunosorbent assay with monoclonal antibodies directed to distinct epitopes on the preS2-region [20,21], with use of commercial kits (HBV GENOTYPE EIA; Institute of Immunology Co., Ltd., Tokyo, Japan).

### 2.3. PCR-RFLP for distinguishing between subgenotypes C1/Cs and C2/Ce of HBV genotype C

Nucleic acids were extracted from 100 μL of serum using QIAamp DNA Blood Mini Kit (Qiagen Inc., Hilden, Germany). A novel method for specific determination of HBV/C consisted of two PCR cycles with hemi-nested primers followed by RFLP with the restriction site specific for HBV/C1/Cs or C2/Ce [10]. The first-round PCR was performed with a sense primer (HBV964F) and an antisense primer (HBV1272R) within non-overlapping polymerase region. The second-round PCR was performed with a sense primer (HBV970F2) and an antisense primer (HBV1272R). To determine HBV/C1/Cs, a portion (5 μL) of the amplification product of 309 base pairs (bp) in size was digested with 5 U of *Ase*I at 37 °C and *Bst*EII at 60 °C for 1 h each. For HBV/C2/Ce digestion, *Nci*I was used at 37 °C for 2 h. Digests with these enzymes were run on electrophoresis in 3.0% (wt/vol) agarose gel, stained with ethidium bromide and examined for their sizes under the ultraviolet light.

### 2.4. Detection and quantification of serum HBV DNA

HBV DNA sequences spanning the S gene were amplified with real-time detection polymerase chain reaction (RTD-PCR) according to the method of Abe et al. [22] with a forward primer (HBSF2), a reverse primer (HBSR2), and Taq Man probe (HBSP2′) with an additional G at the 3′-end of the original HBSP2 [23]. The detection limit of this method was 100 copies/mL.

### 2.5. Amplification and sequencing of the core promoter as well as the precore region plus core gene

To confirm the results by PCR-RFLP, HBV DNA sequences bearing the core promoter and precore/core regions were amplified by PCR with hemi-nested primers by the method described previously [24], with slight modifications [23]. PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, CA) in the ABI 3100 DNA automated sequencer. The sequences covered enhancer II/core promoter (Fig. 1A) and precore genes (Fig. 1B), which could be associated with HBeAg production, viral replication and disease progression.

### 2.6. A cross-sectional control study for clinical and virological differences between HBV/C1/Cs and C2/Ce

The clinical diagnosis was established after serum biochemistry tests ultasonography, computerized tomography (CT), the magnetic resonance imaging (MRI), and the liver biopsy. To compare the clinical differences between HBV/C1/Cs ($n = 118$) and C2/Ce ($n = 210$), age-, sex-, HBeAg status-matched HBV carriers were enrolled (Table 1). The carriers were also matched according to the severity of liver disease in each group. The HBsAg-positive individuals with normal alanine aminotransferase (ALT) levels over 2 years (examined at least four times at 3-month intervals), and without the presence of portal hypertension were defined as IC. Individuals with persistent elevation of ALT levels (>1.5× upper limit of normal) [35 U/L] over

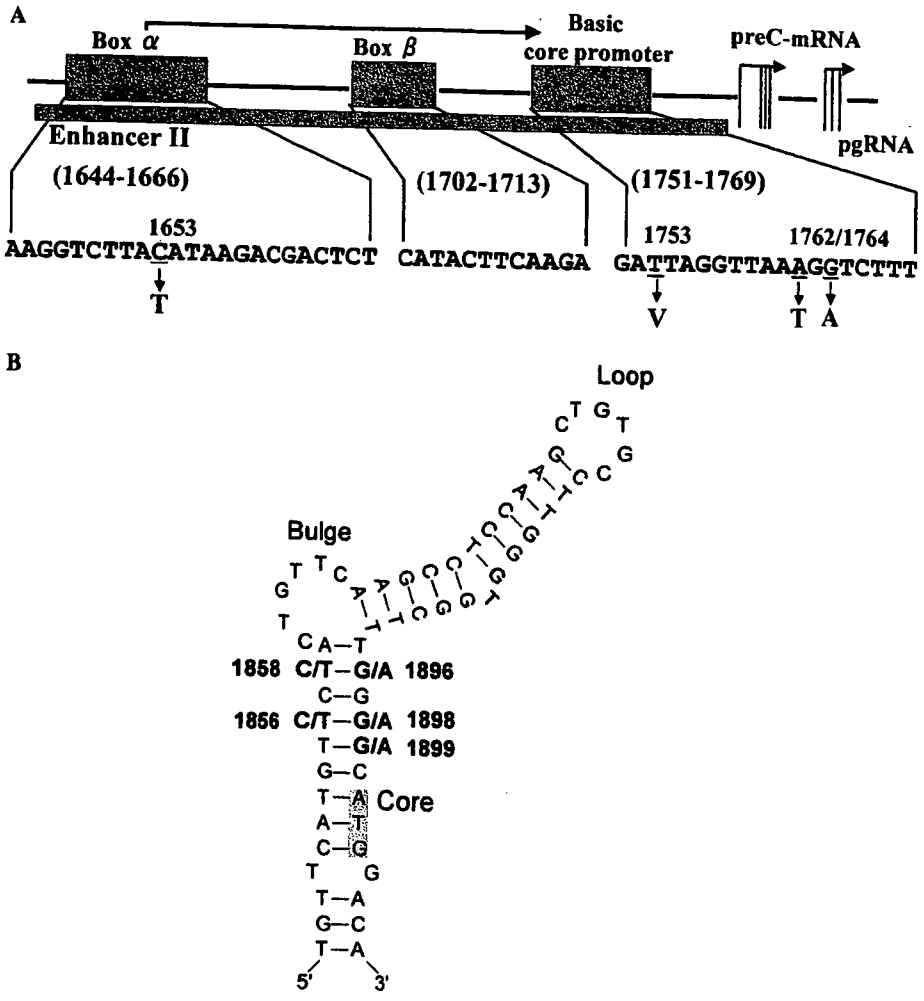


**Fig. 1. (A) C1653T, T1753V (not T), A1762T/G1764A mutations in the enhancer II/core promoter region, and (B) ε loop structure of the encapsidation signal in precore genome. pgRNA, pregenomic RNA.**

a 6-month period (at least three readings at 2-month intervals) without decrease of platelet count, albumin and hypersplenism (splenomegaly on ultrasonography) were defined as CH. Patients with established hepatocellular carcinoma according to the clinical biochemical investigation [Alpha-fetoprotein (AFP) and/or serum protein induced by vitamin K absence named antagonist II (PIVKA-II)], CT and/or MRI, and liver biopsy were included in HCC group. Patients with hepatitis C virus or human immunodeficiency virus co-infection were excluded, and none had received antiviral treatment during the follow-up period.

### 2.7. Statistical evaluation

Statistical analyses were performed using chi squared test and Fisher's exact test for categorical variables. Mann–Whitney *U* test was used for continuous variables where appropriate. The univariate general linear modeling (GLM) was used to test the effects of specific HBV mutations on the HCC and non-HCC groups of the HBV-carriers in the context of their HBeAg status. Multivariate analyses with logistic regression were used to determine the independent factors associated with

**Table 1**
**Demographic and clinical characteristics of patients with HBV subgenotype C1/Cs and C2/Ce who were matched for age, sex, HBeAg status and clinical states**

| Characteristics | C1/Cs ($n = 118$) | C2/Ce ($n = 210$) | *P* value |
|---|---|---|---|
| Country (Japan/Hong Kong) | 0/118 | 172/38 | <0.0001 |
| Age, mean ± SD | 48.0 ± 13.7 | 50.2 ± 10.7 | Matched |
| Sex, male (%) | 96 (81%) | 163 (78%) | Matched |
| HBeAg positive (%) | 47 (40%) | 62 (30%) | Matched |
| HBV DNA (log copies/mL), mean ± SD | 5.7 ± 1.7 | 5.4 ± 1.4 | NS |
| ALT (U/L), median (range) | 51 (4–1154) | 46 (8–773) | NS |
| IC/CH/HCC* | 19/47/52 | 29/85/96 | Matched |
| HCC (%) | 52 (44%) | 96 (46%) | Matched |

* IC, inactive carriers; CH, chronic hepatitis; HCC, hepatocellular carcinoma; NS, not significant.

HCC. Differences were considered significant for $P$ values less than .05. All statistical analyses were performed using the Statistical Program for Social Sciences (SPSS 12.0 for windows, SPSS Inc., Chicago, IL).

## 3. Results

For comparative analyses, HBV/C1/Cs ($n = 118$) and C2/Ce ($n = 211$) carriers' groups were matched in respect to age, sex, HBeAg status, and clinical states. Table 1 shows comparative summary for HBV/C1/Cs and C2/Ce groups according to the origin country of the enrolled carriers, age, sex, HBe Ag status, HBV DNA levels, ALT levels and clinical states (IC/CH/HCC); no significant differences demonstrated with exception for the original country. HBV/C1/Cs is found in Hong Kong, whereas HBV/C2/Ce is predominant in Japan.

When HCC patients in HBV/C1/Cs ($n = 52$) and C2/Ce ($n = 96$) groups were compared, no significant difference was observed in mean age, sex, HBeAg positivity, HBV DNA, and ALT levels (Table 2). However, the frequency of C-to-T1653 mutation in the box alpha (Fig. 1A) was significantly higher in HBV/C2 (C1/Cs = 23%, C2/Ce = 48%, $P = 0.0055$), whereas T-to-V1753 mutation was significantly prevalent in HBV/C1/Cs (C1/Cs = 75%, C2/Ce=39%, $P < 0.0001$). The prevalence of T1762/A1764 was high in both of these groups with no significant difference (Table 2). In the precore region including encapsidation signal (ε) (Fig. 1B), the precore stop mutation (A1896), accompanied by a C-to-T substitution at nt 1858 forming a base pairing within ε structure, was frequently found in HBV/C2/Ce strains (40/96, 42%), whereas another precore mutation (A1898), accompanied by a C-to-T substitution at nt 1856, was found only in HBV/C1/Cs strains (18/52, 35%) (Table 2). A1899 mutation was prevalent in HBV/C2/Ce (C1/Cs, 12% vs. C2/Ce, 27%) as well as A1896 mutation.

As the above mutations in the enhancer II/core promoter and precore regions could be associated with subgenotypes as well as HBeAg status, we examined the mutations predictive for HCC among all HBV/C1/Cs and C2/Ce patients in the context of HBeAg status. The prevalence of HBV mutations such as C1653T, T1753V, A1762T/G1764A and G1896A was compared among HBeAg-positive, and -negative patients with and without HCC within the C1/Cs and C2/Ce groups (Table 3). As summarized in Table 3, V1753 was frequently found among HCC patients infected with C1/Cs, when compared to those without HCC. Interestingly, the difference was greater in HBeAg-positive group ($P = 0.0055$), whereas in HBeAg-negative group the trend was only remaining ($P = 0.051$). When C2/Ce infected patients with and without HCC were compared, T1653 was frequently found among HCC patients in both HBeAg-positive and -negative groups ($P = 0.018$, 0.012, respectively), and V1753 or T1762/A1764 was also frequent in HBeAg-negative group ($P = 0.046$, 0.024, respectively). The univariate GLM confirmed the above results; V1753 mutation was predictive for HCC in the HBeAg-positive-C1/Cs ($P = 0.0092$), T1653 mutation in the HBeAg-positive-C2/Ce ($P = 0.0056$), both T1653 ($P = 0.0046$) and V1753 mutations ($P = 0.016$) in the HBeAg-negative-C2/Ce group. On the other hand, A1896 mutation was negatively correlated with HCC ($P = 0.0015$).

The factors possible attributable for HCC; age, sex, HBeAg positivity, HBV DNA level, ALT (two groups divided by median values), subgenotypes and mutations; T1653, V1753, T1762/A1764, T1856, T1858, A1896, A1898 and A1899 were tested in multiple logistic regression analysis for all 328 HBV-carriers (Table 4). Age

**Table 2**
**Clinical and virologic characteristics of HCC patients infected with HBV subgenotype C1/Cs and C2/Ce**

| Characteristics | C1/Cs ($n = 52$) | C2/Ce ($n = 96$) | $P$ value |
|---|---|---|---|
| Age, mean ± SD | 54.2 ± 11.9 | 53.7 ± 9.0 | NS |
| Sex, male (%) | 43 (83%) | 81 (84%) | NS |
| HBeAg-positive (%) | 24 (46%) | 36 (38%) | NS |
| HBV DNA (log copies/mL), mean ± SD | 5.5 ± 1.6 | 5.2 ± 1.4 | NS |
| ALT (U/L), median (range) | 50 (4–1154) | 53 (16–473) | NS |
| Mutation in the box alpha | | | |
| T1653 | 12 (23%) | 46 (48%) | 0.0055 |
| Mutations in the core promoter | | | |
| V(not T)1753 | 39 (75%) | 37 (39%) | <0.0001 |
| T1762/A1764 | 47 (90%) | 88 (92%) | NS |
| Mutation in the precore region | | | |
| T1856 | 21 (40%) | 0 | <0.0001 |
| T1858 | 2 (4%) | 96 (100%) | <0.0001 |
| A1896 | 2 (4%) | 40 (42%) | <0.0001 |
| A1898 | 18 (35%) | 0 | <0.0001 |
| A1899 | 6 (12%) | 26 (27%) | 0.05 |

NS, not significant.

**Table 3**
**HBV/C1 and C2 mutations among patients with and without HCC in context of HBeAg**

| No. | T1653 | V1753 | 1762/A1764 | A1896 |
|---|---|---|---|---|
| *C1/CS* | | | | |
| HBeAg (+) | | | | |
| Non-HCC ($n = 23$) | 2 (8.7%) | 7 (30.4%) | 16 (69.6%) | 4 (17.4%) |
| HCC ($n = 24$) | 6 (25.0%) | **18 (75.0%)*** | 22 (91.7%) | 1 (4.2%) |
| HBeAg (−) | | | | |
| Non-HCC ($n = 43$) | 8 (18.6%) | 21 (48.8%) | 33 (76.7%) | 10 (23.2%) |
| HCC ($n = 28$) | 6 (21%) | 21 (75%) | 25 (89%) | 1 (4%) |
| *C2/Ce* | | | | |
| HBeAg (+) | | | | |
| Non-HCC ($n = 26$) | 1 (3.8%) | 7 (26.9%) | 17 (65.4%) | 6 (23.1%) |
| HCC ($n = 36$) | **10 (27.8%)** | 15 (41.7%) | 31 (86.1%) | 9 (25.0%) |
| HBeAg (−) | | | | |
| Non-HCC ($n = 88$) | 33 (37.5%) | 18 (20.5%) | 71 (80.7%) | 57 (64.8%) |
| HCC ($n = 60$) | **36 (60.0%)*** | **22 (36.7%)*** | **57 (95.0%)*** | 31 (51.7%) |

* Non-HCC vs. HCC, $P < 0.05$ (Yates corrected chi-square). Significant data are shown in bold.

($\geqslant 50$) [odds ratio (95% CI): 2.90 (1.72–4.89), $P < 0.0001$], sex (male) [2.29 (1.18–4.43), $P = 0.014$], HBeAg (positive) [2.39 (1.34–4.28), $P = 0.003$] and subgenotype (C2/Ce) [4.21 (1.07–16.23), $P = 0.039$] were significantly associated with the development of HCC. HBV mutations found in strong association with HCC were T1653 [3.64 (1.93–6.86)], V1753 [3.07 (1.66–5.65)], and T1762/A1764 [2.58 (1.21–5.49)]. A1896 stop-codon mutation was negatively correlated with HCC [0.31 (0.16–0.62), $P = .001$] in this population (Table 4).

## 4. Discussion

In this study, we focused on HBV/C, which is prevalent in Asia and possibly contribute to progressive liver disease and poor clinical outcomes in HBV-carriers [3,25]. Previous reports containing epidemiological and phylogenetic analyses of the HBV/C complete genome determined at least 4 subgenotypes (C1–4) with different geographic distribution [10,26]. HBV/C1 was found only in Southeast Asia, and HBV/C2 was found in Far East Asia; while remaining two were rarely found in most Asian countries, and probably represent isolated local epidemics; HBV/C3 found in Pacific countries and HBV/C4 strains were isolated from Aborigines in Northeast Australia [24]. In the present study, we examined the clinical and virological differences between C1/Cs and C2/Ce.

The multivariate analysis in this study showed that the following factors were predictive for HCC; subgenotype C2/Ce, and mutations in the enhancer II/core promoter; T1653, V1753 and T1762/A1764. In agreement with the previous reports [27–29], the elder age ($\geqslant 50$), male sex and HBeAg positive were also independent risk factors for HCC. The T1653 and V1753 mutations had been first reported by Takahashi et al. [17]; these specific mutations were prevalent among Japanese HCC patients. Our recent age-, sex-matched case-control study also confirmed that the T1653 mutation in the box alpha in addition to the T1762/A1764 double mutation increases the risk of HCC in anti-HBe-positive patients with HBV/C [19]. The T1762/A1764 mutation had been highly frequent in the elder HBV/C carriers ($\geqslant 50$) regardless of the clinical states [19]; however, these results do not contradict that T1762/A1764 is associated with hepatocarcinogenesis, because poor prognosis of HBV/C compared to HBV/B (Ba and Bj) correlated with high prevalence of T1762/A1764 [3,7,11]. A prospective cohort of 1638 high-risk individuals in Qidong (China) showed that the T1762/A1764 mutation was detected in 8 of the 15 HCC cases (53.3%) before cancer [30], suggesting that the T1762/A1764 double mutation would indicate a high potential risk for hepatocarcinogenesis. Hence, T1653 and/or V1753 mutations in addition to T1762/A1764 are strongly associated with HCC development.

Buckwold et al. reported that the emerging T1762/A1764 dramatically decreases the affinity with the liver-enriched transcription factors resulting in the reduction of the HBeAg expression [31]. Thereafter, Li et al. reported that this mutation not only affects the nuclear receptor binding site but also creates a new HNF1 transcription factor binding site [32]. In the previous study, it was demonstrated that the box alpha elements (1646–1668) individually stimulate the promoter activity for more than 100-fold [16]. The T1653 mutation converts the alpha box binding site for C/EBP and related factors into the perfect palindromic sequence 1648-TCTTATATAAGA, which might enhance binding affinity and enhancer II/core promoter activity. Hence, the T1653 mutation could influence the HBeAg production and viral replication through the BCP activity. Although a number of studies have reported the role of the BCP mutations in the viral features, the exact mechanisms of HCC development still remains unclear,

**Table 4**
**Variables with independent predictive value for HCC in the multivariate analysis**

| Factor | Total ($n = 328$) | |
|---|---|---|
| | Odds ratio (95% CI) | $P$ value |
| Age* | | |
| <50 | 1 | |
| ⩾50 | 2.90 (1.72–4.89) | $P < 0.0001$ |
| Sex | | |
| Female | 1 | |
| Male | 2.29 (1.18–4.43) | $P = 0.014$ |
| HBeAg | | |
| Negative | 1 | |
| Positive | 2.39 (1.34–4.28) | $P = 0.003$ |
| HBV DNA (log copies/ml)* | | |
| <5.5 | 1 | |
| ⩾5.5 | 0.74 (0.25–2.86) | NS |
| ALT (U/L)* | | |
| <50 | 1 | |
| ⩾50 | 1.75 (0.56–6.63) | NS |
| HBV subgenotypes | | |
| C1/Cs | 1 | |
| C2/Ce | 4.21 (1.07–16.23) | $P = 0.039$ |
| T1653 mutation | | |
| Absence | 1 | |
| Presence | 3.64 (1.93–6.86) | $P < 0.0001$ |
| V(not T)1753 mutation | | |
| Absence | 1 | |
| Presence | 3.07 (1.66–5.65) | $P < 0.0001$ |
| T1762/A1764 mutation | | |
| Absence | 1 | |
| Presence | 2.58 (1.21–5.49) | $P = 0.014$ |
| T1856 mutation | | |
| Absence | 1 | |
| Presence | 0.41 (0.10–1.69) | NS |
| T1858 mutation | | |
| Absence | 1 | |
| Presence | 0.32 (0.07–1.43) | NS |
| A1896 mutation | | |
| Absence | 1 | |
| Presence | 0.31 (0.16–0.62) | $P = 0.001$ |
| A1898 mutation | | |
| Absence | 1 | |
| Presence | 3.31 (0.72–15.35) | NS |
| A1899 mutation | | |
| Absence | 1 | |
| Presence | 1.14 (0.57–2.29) | NS |

* Two groups were divided by each median value. NS, not significant.

particularly in respect to reflection of the mutations on the X protein. The T1653 mutation responsible for an amino acid change from histidine to tyrosine at aa 94 of the X protein, so this alteration of X protein might be somehow associated with hepatocarcionogenesis. Similarly, the changes of amino acids from isoleucine to asparagine/serine/threonine by V1753 mutation may also affect the function of X protein.

Many previous studies on the HBV encapsidation sequence focused on the configuration of nucleotide 1858 [33,34]. Of note, all HBV/C2/Ce strains possessed T1858 and most HBV/C1/Cs had C1858. A previous study carried among multi-ethnic carriers in Hawaii indicated no significant difference in clinical characteristics between C1858 and T1858 variants [35]. Although the polymorphism of C or T at nucleotide 1858 affects the development of the precore stop-codon mutation, it does not seem to influence disease activity [4,11,36,37]. A recent report showed that HBV carriers bearing TCC at nucleotides 1856–1858 had higher HBeAg positivity and ALT levels than those with CCT; but similar prevalence of liver cirrhosis was observed between them [37]. The precore stop-codon mutation (A1896), accompanied by a C-to-T substitution at nt 1858 forming a base pair with it, was found mainly in HBV/C2/Ce, and another precore mutation (A1898), accompanied by a C-to-T mutation at nt 1856, was found only in HBV/C1/Cs strains. These mutations could stabilize the ε loop structure and the former HBeAg-negative mutants bearing a TAG stop-codon mutation at codon 28 (A1896) uniformly replicate at least 20-fold better than mutants bearing a TGA stop-codon at the same amino acid position enhance viral replication [38]. Although the stringent selection for a highly efficient RNA encapsidation element may play a crucial role in the natural occurrence of these two closely linked precore mutations, the multivariable analysis in this study showed that A1896 stop-codon mutation was negatively correlated with HCC development. The result remains controversial [36,39,40] and the mechanism of HCC development in association with A1896 mutation remains unclear.

HBeAg positivity was one of independent predictive factor for HCC in this study, which was consistent with a previous prospective study by Yang et al. [41]. The biologic function of HBeAg remains controversial. HBeAg is not required for viral replication; but it appears to be necessary for the establishment of chronic infection in animal models [42]. The most common mutation in the precore sequence that abrogates the synthesis of HBeAg is a stop-codon mutation (G1896A). As all HBV/C2/Ce strains possessed T1858 and most C1/Cs had C1858; the C1/Cs with C1858 might be responsible for a delayed seroconversion for the loss of HBeAg in the carriers of C1/Cs [37]. The mechanisms of HBeAg seroconversion and its association with HCC development remain unclear. Assuming that different mechanism may exist leading to carcinogenesis in context of HBeAg status of patients, in the present cross-sectional control study, we examined patients divided into four subgroups in respect to subgenotypes/HBeAg status. In univariate analysis the V1753 mutation was confirmed as predictor for HCC in the HBeAg-positive-C1/Cs, and T1653 mutation in the HBeAg-positive-C2/Ce. Interestingly, in the HBeAg-negative-C2/Ce group, T1653 or V1753 or T1762/A1764 mutations

appeared to be significantly associated with HCC development, which supported the previous reports [17–19]. These data might indicate that different HBV mutation patterns might be predictive for HCC in HBV/C1/Cs and C2/Ce-infected carriers in the context of HBeAg status.

In this cross-sectional study, however, HBV DNA level was retracted from predictive factors for HCC. One of the reasons is that HBV DNA data were available only at the time of diagnosis of HCC, when it has already decreased. A recent prospective study in Taiwan indicated that high HBV DNA levels at baseline and genotype C were independent predicting factors for HCC, but the mean viral load at the time of diagnosis of HCC was significantly lower than that at baseline [29]. Our recent cross-sectional case-control study [19] also showed that HBV DNA level was retracted from predicting factor for HCC.

In conclusion, the present multi-center cross-sectional control study indicated that subgenotype C2/Ce, T1653, V1753 and T1762/A1764 mutations in the enhancer II/core promoter are independent factors strongly associated with HCC development as well as the elder age, male sex and HBeAg positivity. The mutation patterns are associated with subgenotypes and HBeAg, suggesting clinical importance of the HBV/C subgenotyping and detection of the mutation pattern for the prediction of HCC. Further prospective studies in countries where HBV genotype C is endemic are required to confirm whether the accumulation of these mutations during the follow-up causes clinical and virological differences between HBV-infected carriers with HBV/C1/Cs and C2/Ce subgenotypes.

## Acknowledgments

The work was supported by a Grant-in-Aid from the Ministry of Health, Labour and Welfare of Japan (H16-kanen-3), Grants-in-Aid for Young Scientists (A) from the Ministry of Education, Culture, Science, and Sports of Japan (16689016), Uehara Memorial Foundation and Toyoaki Foundation.

## References

[1] Kidd-Ljunggren K, Miyakawa Y, Kidd AH. Genetic variability in hepatitis B viruses. J Gen Virol 2002;83:1267–1280.
[2] Kidd-Ljunggren K, Myhre E, Blackberg J. Clinical and serological variation between patients infected with different Hepatitis B virus genotypes. J Clin Microbiol 2004;42:5837–5841.
[3] Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, et al. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. Hepatology 2001;33:218–223.
[4] Chan HL, Hui AY, Wong ML, Tse AM, Hung LC, Wong VW, et al. Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. Gut 2004;53:1494–1498.
[5] Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, et al. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. J Virol 2002;76:5985–5992.
[6] Akuta N, Suzuki F, Kobayashi M, Tsubota A, Suzuki Y, Hosaka T, et al. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. J Hepatol 2003;38:315–321.
[7] Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, et al. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. Gastroenterology 2003;124:925–932.
[8] Huy TT, Ushijima H, Quang VX, Win KM, Luengrojanakul P, Kikuchi K, et al. Genotype C of hepatitis B virus can be classified into at least two subgroups. J Gen Virol 2004;85:283–292.
[9] Chan HL, Tsui SK, Tse CH, Ng EY, Au TC, Yuen L, et al. Epidemiological and virological characteristics of 2 subgroups of hepatitis B virus genotype C. J Infect Dis 2005;191:2022–2032.
[10] Tanaka Y, Orito E, Yuen MF, Mukaide M, Sugauchi F, Ito K, et al. Two subtypes (subgenotypes) of hepatitis B virus genotype C: A novel subtyping assay based on restriction fragment length polymorphism. Hepatol Res 2005.
[11] Kao JH, Chen PJ, Lai MY, Chen DS. Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. Gastroenterology 2003;124:327–334.
[12] Baptista M, Kramvis A, Kew MC. High prevalence of 1762(T) 1764(A) mutations in the basic core promoter of hepatitis B virus isolated from black Africans with hepatocellular carcinoma compared with asymptomatic carriers. Hepatology 1999;29:946–953.
[13] Blackberg J, Kidd-Ljunggren K. Mutations within the hepatitis B virus genome among chronic hepatitis B patients with hepatocellular carcinoma. J Med Virol 2003;71:18–23.
[14] Laskus T, Radkowski M, Nowicki M, Wang LF, Vargas H, Rakela J. Association between hepatitis B virus core promoter rearrangements and hepatocellular carcinoma. Biochem Biophys Res Commun 1998;244:812–814.
[15] Ogata N, Miller RH, Ishak KG, Purcell RH. The complete nucleotide sequence of a pre-core mutant of hepatitis B virus implicated in fulminant hepatitis and its biological characterization in chimpanzees. Virology 1993;194:263–276.
[16] Yuh CH, Chang YL, Ting LP. Transcriptional regulation of precore and pregenomic RNAs of hepatitis B virus. J Virol 1992;66:4073–4084.
[17] Takahashi K, Akahane Y, Hino K, Ohta Y, Mishiro S. Hepatitis B virus genomic sequence in the circulation of hepatocellular carcinoma patients: comparative analysis of 40 full-length isolates. Arch Virol 1998;143:2313–2326.
[18] Takahashi K, Ohta Y, Kanai K, Akahane Y, Iwasa Y, Hino K, et al. Clinical implications of mutations C-to-T1653 and T-to-C/A/G1753 of hepatitis B virus genotype C genome in chronic liver disease. Arch Virol 1999;144:1299–1308.
[19] Ito K, Tanaka Y, Orito E, Sugiyama M, Fujiwara K, Sugauchi F, et al. T1653 mutation in the box alpha increases the risk of hepatocellular carcinoma in patients with chronic hepatitis B virus genotype C infection. Clin Infect Dis 2006;42:1–7.
[20] Usuda S, Okamoto H, Tanaka T, Kidd-Ljunggren K, Holland PV, Miyakawa Y, et al. Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. J Virol Methods 2000;87:81–89.
[21] Usuda S, Okamoto H, Iwanari H, Baba K, Tsuda F, Miyakawa Y, et al. Serological detection of hepatitis B virus genotypes by

ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. J Virol Methods 1999;80:97–112.
[22] Abe A, Inoue K, Tanaka T, Kato J, Kajiyama N, Kawaguchi R, et al. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. J Clin Microbiol 1999;37:2899–2903.
[23] Tanaka Y, Hasegawa I, Kato T, Orito E, Hirashima N, Acharya SK, et al. A case-control study for differences among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. Hepatology 2004;40:747–755.
[24] Sugauchi F, Mizokami M, Orito E, Ohno T, Kato H, Suzuki S, et al. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. J Gen Virol 2001;82:883–892.
[25] Kao JH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. Gastroenterology 2000;118:554–559.
[26] Norder H, Courouce AM, Coursaget P, Echevarria JM, Lee SD, Mushahwar IK, et al. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. Intervirology 2004;47:289–309.
[27] Sumi H, Yokosuka O, Seki N, Arai M, Imazeki F, Kurihara T, et al. Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. Hepatology 2003;37:19–26.
[28] Orito E, Sugauchi F, Tanaka Y, Ichida T, Sata M, Tanaka E, et al. Differences of hepatocellular carcinoma patients with hepatitis B virus genotypes of Ba, Bj or C in Japan. Intervirology 2005;48:239–245.
[29] Yu MW, Yeh SH, Chen PJ, Liaw YF, Lin CL, Liu CJ, et al. Hepatitis B virus genotype and DNA level and hepatocellular carcinoma: a prospective study in men. J Natl Cancer Inst 2005;97:265–272.
[30] Kuang SY, Jackson PE, Wang JB, Lu PX, Munoz A, Qian GS, et al. Specific mutations of hepatitis B virus in plasma predict liver cancer development. Proc Natl Acad Sci USA 2004;101:3575–3580.
[31] Buckwold VE, Xu Z, Chen M, Yen TS, Ou JH. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J Virol 1996;70:5845–5851.
[32] Li J, Buckwold VE, Hon MW, Ou JH. Mechanism of suppression of hepatitis B virus precore RNA transcription by a frequent double mutation. J Virol 1999;73:1239–1244.
[33] Lindh M, Andersson AS, Gusdal A. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus–large-scale analysis using a new genotyping method. J Infect Dis 1997;175:1285–1293.
[34] Rodriguez-Frias F, Buti M, Jardi R, Cotrina M, Viladomiu L, Esteban R, et al. Hepatitis B virus infection: precore mutants and its relation to viral genotypes and core mutations. Hepatology 1995;22:1641–1647.
[35] Sakurai M, Sugauchi F, Tsai N, Suzuki S, Hasegawa I, Fujiwara K, et al. Genotype and phylogenetic characterization of hepatitis B virus among multi-ethnic cohort in Hawaii. World J Gastroenterol 2004;10:2218–2222.
[36] Lindh M, Horal P, Dhillon AP, Furuta Y, Norkrans G. Hepatitis B virus carriers without precore mutations in hepatitis B e antigen-negative stage show more severe liver damage. Hepatology 1996;24:494–501.
[37] Chan HL, Tse CH, Ng EY, Leung KS, Lee KH, Tsui SK, et al. Phylogenetic, virological, and clinical characteristics of genotype C hepatitis B virus with TCC at codon 15 of the precore region. J Clin Microbiol 2006;44:681–687.
[38] Yuan TT, Faruqi A, Shih JW, Shih C. The mechanism of natural occurrence of two closely linked HBV precore predominant mutations. Virology 1995;211:144–156.
[39] Kobayashi M, Arase Y, Ikeda K, Tsubota A, Suzuki Y, Saitoh S, et al. Precore wild-type hepatitis B virus with G1896 in the resolution of persistent hepatitis B virus infection. Intervirology 2003;46:157–163.
[40] Asahina Y, Izumi N, Uchihara M, Noguchi O, Nishimura Y, Inoue K, et al. Core promoter/pre-core mutations are associated with lamivudine-induced HBeAg loss in chronic hepatitis B with genotype C. J Hepatol 2003;39:1063–1069.
[41] Yang HI, Lu SN, Liaw YF, You SL, Sun CA, Wang LY, et al. Hepatitis B e antigen and the risk of hepatocellular carcinoma. N Engl J Med 2002;347:168–174.
[42] Chen HS, Kew MC, Hornbuckle WE, Tennant BC, Cote PJ, Gerin JL, et al. The precore gene of the woodchuck hepatitis virus genome is not essential for viral replication in the natural host. J Virol 1992;66:5682–5684.

*Hepatology Research* 2008; 38: 310–314 doi: 10.1111/j.1872-034X.2007.00263.x

***Short Communication***

# Prevalence of hepatitis B virus infection in Japanese patients with HIV

Kazuhiko Koike,[1] Yoshimi Kikuchi,[2] Michio Kato,[3] Junki Takamatsu,[4] Yoshizumi Shintani,[1] Takeya Tsutsumi,[1] Hajime Fujie,[1] Hideyuki Miyoshi,[1] Kyoji Moriya[1] and Hiroshi Yotsuyanagi[1]

*[1]Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, [2]AIDS Clinical Center, International Medical Center of Japan, Tokyo, [3]Department of Gastroenterology, Osaka National Hospital, Osaka and [4]Department of Transfusion Medicine, Nagoya University Hospital, Nagoya, Japan*

Patients with HIV infection are frequently infected with hepatitis viruses, which are presently the major cause of mortality in HIV-infected patients after the widespread use of highly active antiretrovirus therapy. We previously reported that approximately 20% of HIV-positive Japanese patients were also infected with hepatitis C virus (HCV). Hepatitis B virus (HBV) infection may also be an impediment to a good course of treatment for HIV-infected patients, because of recurrent liver injuries and a common effectiveness of some anti-HIV drugs on HBV replication. However, the status of co-infection with HIV and HBV in Japan is unclear. We conducted a nationwide survey to determine the prevalence of HIV–HBV co-infection by distributing a questionnaire to the hospitals belonging to the HIV/AIDS Network of Japan. Among the 5998 patients reported to be HIV positive, 377 (6.4%) were positive for the hepatitis B surface antigen. Homosexual men accounted for two-thirds (70.8%) of the HIV–HBV co-infected patients, distinct from HIV–HCV co-infection in Japan in which most of the HIV–HCV co-infected patients were recipients of blood products. One-third of HIV–HBV co-infected patients had elevated serum alanine aminotransferase levels at least once during the 1-year observation period. In conclusion, some HIV-infected Japanese patients also have HBV infection and liver disease. A detailed analysis of the progression and activity of liver disease in co-infected patients is needed.

**Key words:** co-infection, hepatitis B, HIV, liver disease.

## INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a major public health problem worldwide, along with hepatitis C virus (HCV) and HIV infections. In the USA, the estimated prevalence of HBV is less than 1%, but approximately 1 million people are persistently infected.[1] The prevalence of HIV in the USA is also <1%, and the virus is estimated to have infected approximately 800 000 people.[2] Because of the common transmission routes, that is, parenteral transmission routes, many people with HIV infection are also infected with HBV. Among the HIV-positive people in the USA, the prevalence of HBV co-infection is 6–14%.[1,2] Before the introduction of highly active antiretroviral therapy (HAART) in 1996, most patients with HIV infection died of HIV-associated opportunistic infections, such as *Pneumocystis jiroveci* pneumonia and cytomegaloviral infection. Since the widespread use of HAART, the mortality associated with HIV infection has declined. However, the reduction in mortality due to opportunistic infection, has left patients co-infected with HIV and hepatitis viruses faced with the menace of progressive liver diseases due to HBV infection,[3,4] in addition to HCV infection.[5]

HBV co-infection or superinfection of HIV-infected patients leads to several problematic situations. First, HBV infection tends to develop into persistent infection in HIV-infected patients,[1,6,7] which is a rare event in healthy adults, although it substantially depends on the genotype of HBV.[8] It results in the acceleration of the development of cirrhosis and eventually hepatocellular carcinoma. Second, some nucleoside reverse transcriptase inhibitors (NRTI) used in HAART also have

*Correspondence: Professor Kazuhiko Koike, Department of Infectious Diseases, Internal Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Email: kkoike-tky@umin.ac.jp*

*Received 22 June 2007; revision 29 July 2007; accepted 31 July 2007.*

inhibitory effects on the replication of HBV.[9–12] A careless administration or discontinuation of NRTI on HIV–HBV co-infected patients may cause reactivation and/or aggravation of hepatitis B. In addition, the administration of anti-HBV drugs in HIV–HBV co-infection may lead to the development of drug resistance.[11,12] Third, liver injury occurs more frequently in patients on HAART who are co-infected with HIV and HBV than those infected with HIV only.[9,10]

Importantly, co-infection with HIV and HCV increases the morbidity and mortality of HIV-infected patients in Japan,[13] where the prevalence of HIV infection is increasing linearly, and is exceptionally high among developed countries.[14] There are more than 14 000 HIV-positive people in Japan as of 2006, according to the AIDS National Survey in Japan,[14] and approximately 0.8 million chronic HBV carriers.[15] However, the prevalence of co-infection with HIV and HBV in Japan has not been clarified to date. Therefore, we conducted a nationwide study by distributing a postal mail-based questionnaire to the hospitals belonging to the HIV/AIDS Network of Japan.

## PATIENTS AND METHODS

IN THE QUESTIONNAIRE, the following information was obtained from the hospitals regarding the number of patients who visited the hospitals at least once between January and December in 2006: (i) the number of HIV-positive patients; (ii) the number of hepatitis B surface antigen (HBsAg)-positive patients among (i); (iii) the number of patients among (ii) who were determined at least once to have a serum alanine aminotransferase (ALT) level higher than 100 IU/L; (iv) the number of HIV-positive patients that contracted HIV from blood products; (v) the number of HBsAg-positive patients among (iv), (vi) the number of patients among (v) who were determined at least once to have a serum ALT level higher than 100 IU/L; (vii) the number of HIV-positive patients among homosexual men, (viii) the number of HBsAg-positive patients among (vii), (ix) the number of patients among (viii) who were determined at least once to have a serum ALT level higher than 100 IU/L; (x) the number of HIV-positive patients that contracted HIV through intravenous drug use (xi) the number of HBsAg-positive patients among (x), (xii) the number of patients among (xi) who had at least one determination of a serum ALT level more than 100 IU/L; (xiii) the number of HIV-positive patients whose transmission routes were classified as "others"; (xiv) the number of HBsAg-positive patients among (xiii); and (xv) the number of patients among (xiv) who were determined at least once to have a serum ALT level higher than 100 IU/L.

The questionnaire was sent to the 372 hospitals belonging to the HIV/AIDS Network of Japan by mail. Answers were mostly returned by mail and in some cases by fax. The list of the hospitals in the HIV/AIDS Network of Japan can be viewed at http://www.acc.go.jp/mLhw/mLhw_frame.htm.

## RESULTS

THE QUESTIONNAIRE WAS sent to all 372 hospitals that were on the list of the hospitals in the HIV/AIDS Network of Japan in January 2006. Two hundred and seven hospitals (55.6%) responded within the indicated period. In total, 5998 patients were reported to be HIV positive. The collection rate of 55.6% was higher than that (47.8%) for a questionnaire HIV–HCV co-infection study carried out in 2003.[15] It may appear rather low, particularly considering the number of reported HIV-positive people in 2006, which was approximately 14 000, according to the AIDS National Survey in Japan.[14] However, not all of the HIV-positive people were going to hospitals, and the answers to the questionnaire were obtained from most of the major hospitals in the HIV/AIDS Network in big cities around Japan. This suggests that not all, but a majority of HIV-positive Japanese patients were enrolled in the study.

Among the 5998 patients reported to be HIV positive, 377 (6.3%) patients were positive for HBsAg (Table 1). Of these 377 patients, 122 (32.4%) had elevated serum ALT levels at least one time during the 1-year observation period.

The HBV prevalence rates, when fractionated by the routes of transmission, were as follows: among the 508 HIV-positive patients who contracted HIV from blood products, such as unheated concentrated coagulation factors, only 30 (5.9%) were HBsAg positive, which shows a marked contrast to the prevalence of HCV in this cohort (Fig. 1).[16] Among the 23 intravenous drug users, three (13.0%) were HBsAg positive. Among the 3213 HIV-positive patients who were homosexual men, 267 (8.3%) were HBsAg positive. In the remaining 2254 patients who were HIV-positive and whose route of HIV transmission was classified as "others", most contracted HIV heterosexually. This number (2254) showed a substantial increase from the 1316 obtained in the questionnaire for the HIV–HCV co-infection study in 2003, while the total number of HIV-positive patients increased from 4877 to 5998.[16] Among these, 77 (3.4%)

**Table 1** Prevalence rates of hepatitis B virus infection among HIV-positive patients

| Routes of transmission | No. patients | HBsAg positive (% in HIV positive according to route) | ALT >100 IU/L (% in HBsAg positive according to route) |
|---|---|---|---|
| Blood products | 508 (5.9%) | 30 (40.0%) | 12 |
| Homosexual men | 3213 (8.3%) | 267 (32.2%) | 86 |
| Drug addicts | 23 (13.0%) | 3 (66.7%) | 2 |
| Others (heterosexual etc.) | 2254 (3.4%) | 77 (28.6%) | 22 |
| Total | 5998 | 377 (6.3%) | 122 (32.4%) |

ALT, serum alanine aminotransferase; HBsAg, hepatitis B surface antigen.

were HBsAg positive. In terms of the route of HIV infection, 267 (70.8%) of the 377 patients were homosexual men among the HIV–HBV co-infected patients. This shows a contrast to the status of HIV–HCV co-infection, in which the majority of HIV–HCV co-infected Japanese patients contracted both viruses from blood products.[16]

There were one or more HIV-positive patients in 154 (74.4%) of the 207 hospitals in the HIV/AIDS Network of Japan (Table 2). Twenty four (11.6%) of 207 hospitals had 20–49 HIV-positive patients, and 16 (7.7%) hospitals had 50 or more HIV-positive patients. There were one or more patients who were co-infected with HIV and HBV in 64 (30.9%) of the 207 hospitals. There were 10 or more HIV–HBV co-infected patients in nine (4.3%) hospitals, all of which had 50 or more HIV-positive patients (Table 2). HIV–HBV co-infected patients were concentrated in specific hospitals in big cities around Japan. In particular, in the Kanto area, HIV–HBV co-infected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area.

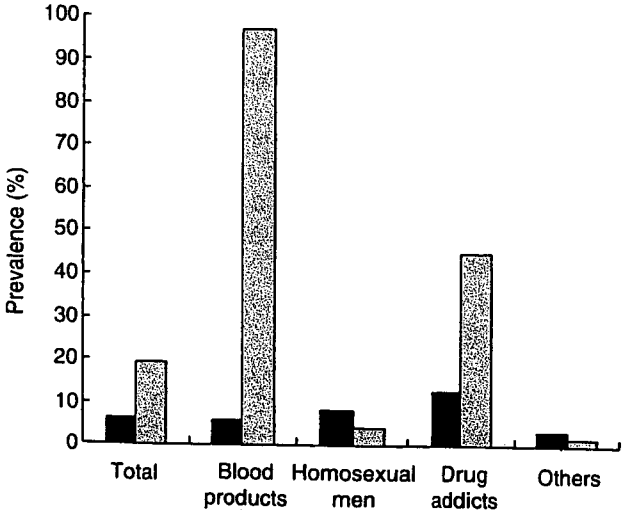


**Figure 1** Prevalence rates of persistent hepatitis B virus and hepatitis C virus infections in the HIV-positive population sorted by the HIV risk group. (■), HBsAg, hepatitis B surface antigen; (▨), anti-HCV, antibody to hepatitis C virus. *Prevalence rates of anti-HCV are obtained from Koike K *et al.*[16]

## DISCUSSION

ALONG WITH THE increase in the number of HIV-infected patients in Japan, co-infection with HIV and hepatitis viruses has become a major medical issue. HBV infection of HIV-positive patients raises several difficult problems: HBV infection tends to develop into persistent infection, even in adults; some NRTI used in HAART also have inhibitory effects on the replication of HBV, the improper administration, or discontinuation of which may lead to drug resistance; and HIV–HBV co-infected patients on HAART have liver injuries more frequently than HIV-monoinfected patients. It is important to determine the status of HBV infection in HIV-positive patients.

According to the statistics of the Ministry of Health, Labor, and Welfare of Japan, the number of reported HIV-positive people was slightly over 14 000 in 2006.[14] In the present study, 6.4% of HIV-positive patients were positive for HBsAg, the most reliable marker for ongoing HBV infection. It might have been advantageous if

**Table 2** Number of hospitals categorized according to the number of patients infected with HIV and those co-infected with HIV and hepatitis B virus (HBV)

| No. HIV (+)/ HBV (+) | No. HIV(+) | | | | |
|---|---|---|---|---|---|
| | 0 | 1–19 | 20–49 | 50+ | Total |
| 0 | 53 | 76 | 13 | 1 | 143 |
| 1–9 | 0 | 38 | 11 | 6 | 55 |
| 10+ | 0 | 0 | 0 | 9 | 9 |
| Total | 53 | 114 | 24 | 16 | 207 |

serum HBV-DNA levels were determined, but unfortunately, HBV-DNA level determination was not a routine laboratory test in most hospitals. In addition, considering that the antibody to the hepatitis B core antigen might be the only marker of ongoing HBV infection in some immuno-compromised patients, it would also be advantageous if this viral marker were available. These issues should be investigated in future studies. Comments from hospitals to the questionnaire included one indicating that not all HIV-positive patients underwent a test for serum HBsAg, suggesting the actual prevalence of HBsAg in HIV-infected patients might be higher than 6.4%.

In a previous questionnaire study of HIV–HCV co-infection, the prevalence of HCV infection among HIV-infected patients was 19.2%;[16] the prevalence of HBV infection (6.4%), is one-third of it. The lower positivity for HBsAg than for the anti-HCV antibody among those who contracted HIV through blood products accounts for this difference: almost all (96.9%) of the patients who contracted HIV through blood products were also anti-HCV antibody positive.[16] It should be noted that among the homosexual male patients who were HIV positive, 8.3% were HBsAg positive, which is twice as high as that of the anti-HCV antibody in these populations. A higher prevalence of HBV infection as a sexually transmitted infection than that of HCV[17] may explain the high prevalence of HBV infection in HIV-positive homosexual men. Similarly, a HBV prevalence of 3.4% in heterosexually transmitted HIV-positive patients is higher than that of the general Japanese population of the same age.[15]

Of the 377 patients who were HBsAg positive, 122 (32.4%) had elevated serum ALT levels at least once in the 1-year observation period. In this type of study using a questionnaire, it is difficult to obtain the details of patients' data, including age, body weight, and the degrees of liver injuries and fibrosis. If detailed items were included in the questionnaire, then the collection rate would be low. This time, to obtain a high collection rate, we asked whether the patients with HBsAg showed an elevated ALT level higher than 100 IU/L at least once during the 1-year observation period. We thereby do not have details on liver disease in HIV–HBV co-infected patients in the current study. Nonetheless, one-third of HIV–HBV co-infected patients have moderate liver injuries, either chronic hepatitis B or adverse effects of drugs, and are waiting for an aid for the amelioration of liver disease. A detailed analysis of the progression and activity of liver disease in HIV–HBV co-infected patients is expected.

The collection rate of the present questionnaire from the hospitals belonging to the HIV/AIDS Network was 55.6% (207 of 372). This was higher than that (47.8%) in the HIV–HCV co-infection questionnaire study carried out in 2003. The reason for this increase is not clear, but presumably the questionnaire conducted in 2003 has raised awareness among hospital staff regarding the relevance of hepatitis virus and HIV co-infection in clinical practice.

In the current study, both Japanese patients and those of other nationalities/ethnicities were included in the study. Although the ratio of newly diagnosed HIV-positive foreign people has been declining to approximately 10% in 2006, the one in total HIV positive still accounts for approximately 25% in Japan. Because the rates of the HBV carrier are different among countries, it is ideal to analyze the HBV prevalence separately according to the nationalities/ethnicities. However, in the current survey to the hospitals in HIV/AIDS Network of Japan, nationality/ethnicity was not itemized in order to make the questionnaire simple. If we would attempt to obtain such data under the approval of the ethical committee in each hospital, the response rate to questionnaire would be extremely lowered.

To establish measures that decrease the morbidity and mortality of HIV–HBV co-infected patients, it is essential to determine the current status of co-infection. In the present study, the number and transmission routes of HIV–HBV co-infected patients in Japan were determined for the first time, although detailed information on the severity and progression of liver disease in HIV–HBV co-infected patients has not been obtained yet. Undoubtedly, this will be the first step towards improving the prognosis and quality of life of Japanese patients co-infected with HIV and HBV.

## ACKNOWLEDGMENTS

WE THANK MS. Ogawa for her assistance in the questionnaire inquiry. This work was supported in part by Health Sciences Research Grants from the Ministry of Health, Labor, and Welfare of Japan (Research on HIV/AIDS). We thank the hospitals in the HIV/AIDS Network of Japan for the responses to the questionnaire. The list of hospitals can be view at http://www.acc.go.jp/mLhw/mLhw_frame.htm.

## REFERENCES

1 Alter MJ. Epidemiology of viral hepatitis and HIV coinfection. *J Hepatol* 2006; 44: S6–9.

2 Sherman KE, Peters M, Koziel MJ. HIV and liver disease forum: conference proceedings. *Hepatology* 2007; 45: 1566–77.
3 Hoffmann CJ, Thio CL. Clinical implications of HIV and hepatitis B co-infection in Asia and Africa. *Lancet Infect Dis* 2007; 7: 402–9.
4 Maida I, Soriano V, Castellares C *et al.* Liver fibrosis in HIV-infected patients with chronic hepatitis B extensively exposed to antiretroviral therapy with anti-HBV activity. *HIV Clin Trials* 2006; 7: 246–50.
5 Bica I, McGovern B, Dhar R *et al.* Increasing mortality due to end-stage liver disease in patients with human immunodeficiency virus infection. *Clin Infect Dis* 2001; 32: 492–7.
6 Weinbaum CM, Sabin KM, Santibanez SS. Hepatitis B, hepatitis C, and HIV in correctional populations: a review of epidemiology and prevention. *AIDS* 2005; 19: S41–6.
7 Salmon-Ceron D, Lewden C, Morlat P *et al.* Liver disease as a major cause of death among HIV infected patients: role of hepatitis C and B viruses and alcohol. *J Hepatol* 2005; 42: 799–805.
8 Ozasa A, Tanaka Y, Orito E *et al.* Influence of genotypes and precore mutations on fulminant or chronic outcome of acute hepatitis B virus infection. *Hepatology* 2006; 44: 326–34.
9 Thio CL, Locarnini S. Treatment of HIV/HBV coinfection: clinical and virologic issues. *AIDS Rev* 2007; 9: 40–53.
10 Sulkowski MS. Hepatotoxicity associated with antiretroviral therapy containing HIV-1 protease inhibitors. *Semin Liver Dis* 2003; 23: 183–94.
11 Jain MK, Comanor L, White C *et al.* Treatment of hepatitis B with lamivudine and tenofovir in HIV/HBV-coinfected patients: factors associated with response. *J Viral Hepat* 2007; 14: 176–82.
12 Quarleri J, Moretti F, Bouzas MB *et al.* Hepatitis B virus genotype distribution and its lamivudine-resistant mutants in HIV-coinfected patients with chronic and occult hepatitis B. *AIDS Res Hum Retroviruses* 2007; 23: 525–31.
13 Tatsunami S, Taki M, Shirahata A, Mimaya J, Yamada K. Increasing incidence of critical liver disease among causes of death in Japanese hemophiliacs with HIV-1. *Acta Haematol* 2004; 111: 181–4.
14 The Ministry of Health, Labor and Welfare of Japan. *National AIDS Survey Report*, 2004. [Cited 2007.] Available from URL: http://www.wam.go.jp/wamappl/bb14GS50.nsf/vAdmPBigcategory40/727FDBF7F51718B5492572C800071D25?Open Document
15 Tanaka J, Kumagai J, Katayama K *et al.* Sex- and age-specific carriers of hepatitis B and C viruses in Japan estimated by the prevalence in the 3 485 648 first-time blood donors during 1995–2000. *Intervirology* 2004; 47: 32–40.
16 Koike K, Tsukada K, Yotsuyanagi H *et al.* Prevalence of coinfection with human immunodeficiency virus and hepatitis C virus in Japan. *Hepatol Res* 2007; 37: 2–5.
17 Denis F, Adjide CC, Rogez S, Delpeyroux C, Rogez JP, Weinbreck P. Seroprevalence of HBV, HCV and HDV hepatitis markers in 500 patients infected with the human immunodeficiency virus. *Pathol Biol (Paris)* 1997; 45: 701–8.

# B型慢性肝炎に対するインターフェロン治療
## ――現況と今後の展望
Interferon therapy for chronic hepatitis B

加藤道夫
Michio KATO
国立病院機構大阪医療センター消化器科

◎わが国でのB型慢性肝炎に対するIFN治療はHBe抗原陽性例のみが保険適用で，しかも投与期間が24週に限られている．その治療効果は若年，女性，ALT高値，HBV-DNA低値例などの好条件群では良好であるが，おしなべての成績は満足できるものではない．欧米からはPeg-IFN治療の良好な成績が多数報告されているが，わが国ではようやくPeg-IFN-α2aの治験が開始されたところである．若年例には核酸アナログ剤が使用困難で，Peg-IFN治療が大きな福音になると思われる．1日も早い保険適用が望まれる．

Key word：B型慢性肝炎，インターフェロン治療，HBステージ分類，ペグインターフェロン(Peg-IFN)

HBVキャリアは，その自然経過において80%以上がHBe抗原陰性，HBe抗体陽性，HBV-DNA低値の，いわゆる臨床的治癒の状態となる．しかし，少数ではあるが肝硬変に進展したり肝細胞癌を合併する例が存在し，B型肝細胞癌による死亡者数はこの20年間，約5,000名の状態が続いている．B型肝細胞癌発癌には，HBV増殖の多寡が密接に関係していることが明らかになり[1]，抗ウイルス薬によるHBV-DNA量の低下が発癌抑止にきわめて重要とされている．B型慢性肝炎に対する抗ウイルス薬としては，30～35歳以上の高年例には核酸アナログ剤が第一選択で，それより若年例ではインターフェロン(IFN)が用いられることが多い．

わが国でのB型慢性肝炎に対するIFN治療は1986年に1カ月投与が保険適用となり，2002年からは現行の6カ月投与が認可された．6カ月投与が可能となって，その有効性はある程度向上したが，HBe抗原陰性例は保険適用外である点など課題も多い．欧米で有用性が多く報告されているペグインターフェロン(Peg-IFN)は現在ようやく国内治験がはじまったばかりで市販までにはいまだ時間を要するが，近い将来，若年例に対する第一選択薬になると考えられる．

## B型慢性肝炎治療ガイドラインとステージ分類

表1は平成18年度厚生労働科学研究“B型およびC型肝炎ウイルスの感染者に対する治療の標準化に関する臨床的研究”によるB型慢性肝炎の治療ガイドラインである．35歳未満でHBe抗原陽性例にはIFN長期間欠投与が選択されている．また35歳以上でも，HBe抗原陽性で7 log copies/ml以上の高ウイルス例にはIFN長期間欠投与も考慮することが示されている．著者らが提唱したHBキャリアのステージ分類[2](表2)においても，HBステージⅠ(HBe抗原陽性：HBV-DNA 7.6 log copies/ml以上)の若年(Ⅰa)で肝線維化F2以上の例およびHBステージⅡ(HBe抗原陽性：HBV-DNA 7.6 log copies/ml未満)の若年(Ⅱa)例にはIFNを第一選択薬にあげている．また，HBステー