

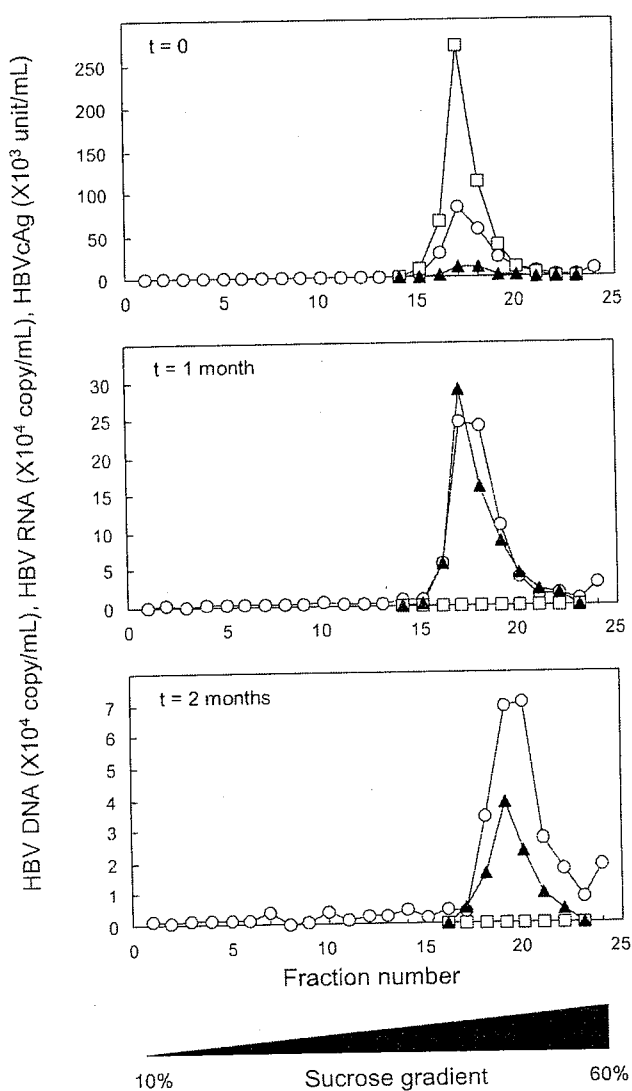
**Fig. 2.** Correlations between serum levels of HBV DNA and HBVcrAg with HBV RNA at the start of lamivudine therapy and 2 months afterward

RNA was also significantly correlated with that of HBVcrAg both at the start of treatment ( $r = 0.841$ ,  $P < 0.001$ ) and 2 months later ( $r = 0.777$ ,  $P < 0.001$ ). Ratios of HBV DNA and HBVcrAg levels to the HBV RNA level were calculated on log scale in patients who were positive for the above two parameters. The HBV DNA/HBV RNA ratio at the start of lamivudine therapy ( $n = 21$ ; median, 1.36; 25%–75% range, 1.20–1.71) decreased significantly ( $P < 0.001$ ) at 2 months after treatment commencement ( $n = 15$ ; median, 0.98; 25%–75% range, 0.86–1.11). On the other hand, the HBVcrAg per HBV RNA ratio at the start of treatment ( $n = 21$ ; median, 1.32; 25%–75% range, 1.21–1.52) did not change ( $P > 0.2$ ) after 2 months ( $n = 17$ ; median, 1.36; 25%–75% range, 1.15–1.54).

Serial serum samples obtained at the start of lamivudine therapy and at 1 and 2 months afterward were subjected to sucrose density gradient fractionation and tested for HBV DNA, HBV RNA, and HBVcrAg. Each of the three viral markers showed a single peak for the same fraction (Fig. 3), suggesting that HBV RNA was incorporated into the virus particles, similarly to HBV DNA. Viral particles containing HBV DNA were dominant at the start of treatment, while those containing HBV RNA became more prevalent 1 and 2 months afterward.

## Discussion

Replication of the HBV DNA genome proceeds via pregenomic RNA transcribed from the cccDNA present in the nuclei of infected hepatocytes.<sup>9,11,12</sup> The pregenomic RNA is then packaged into nucleocapsids and reverse transcribed to form minus-strand DNA. Plus-strand DNA synthesis is initiated following degradation of the pregenome. It has been reported that nucleocapsids containing only minus-strand DNA can be enveloped and then secreted from hepatocytes.<sup>19</sup> Thus, HBV virions in circulation have been considered to contain only mature viral genomes. In spite of this, HBV RNA could be detected in serum in the present study. HBV RNA was considered to be incorporated into virus particles because HBV RNA made a single peak for the same fraction where both HBV DNA and HBVcrAg made single peaks in sucrose gradient analyses conducted at three different time points during lamivudine therapy. Detection of HBV particles with RNA genome does not necessarily contradict the findings of the previous report,<sup>19</sup> since HBV RNA particles seemed to exist in only a small portion (0.1%–1%) of the HBV virions in patients without lamivudine administration. The possibility that our HBV RNA assay detected HBV DNA left undigested by the DNase was



**Fig. 3.** Distribution of HBV DNA, HBV RNA, and HBV core antigen (HBVcrAg) in sucrose density gradient fractions. Serum samples obtained at the start of lamivudine therapy and 1 and 2 months after were subjected to ultracentrifugation on a 10% to 60% (wt/wt) sucrose density gradient. *Open squares* indicate HBV DNA, *open circles* indicate HBVcrAg, and *closed triangles* indicate HBV RNA

considered to be negligible because HBV DNA was not detected by RTD-PCR in extracted HBV RNA samples, and the change of the HBV RNA level during lamivudine therapy differed significantly from that of HBV DNA.

Detection of HBV RNA in serum has rarely been reported to date. Su et al.<sup>20</sup> reported the presence of full-length and truncated RNAs in serum, though their results seem to be quite different in nature because the HBV RNA that they observed was not incorporated into core particles. Zhang et al.<sup>21</sup> reported the existence of an RNA genome with partially reverse-transcribed

minus-strand DNA in serum and speculated that the genome was in a virus particle. Such particles with HBV RNA accounted for about 1% of all HBV virions under untreated conditions, but became a major component under lamivudine administration. Although their analysis was done in a single patient, those observations are consistent with our observations that the rate of decrease of HBV DNA in serum was much faster than that of HBV RNA during lamivudine administration.

The HBVcrAg assay is a unique assay that measures the total amount of antigen coded by precore core genes such as HBV core and e antigens.<sup>14,15</sup> During the HBVcrAg assay, core antigen was released from the pretreated HBV virion and denatured, along with free e antigen and e antigen/antibody-complex. The HBVcrAg assay employs monoclonal antibodies that are reactive with the common epitopes of denatured core and e antigens. Under these conditions, the HBVcrAg assay simultaneously measures the core and e antigens within a given sample, irrespective of their source of origin. Serum HBVcrAg levels reflect the viral load in the untreated state because these levels correlate linearly with those of HBV DNA. On the other hand, it has been reported that HBVcrAg levels show different characteristics than HBV DNA levels under lamivudine administration.<sup>15,16</sup> It is noteworthy that HBVcrAg and HBV RNA levels both decreased significantly more slowly than HBV DNA after the beginning of lamivudine administration, but at a similar rate. This phenomenon is quite possible because synthesis of mRNA from cccDNA episomes and subsequent production of viral proteins is not inhibited by lamivudine like the synthesis of pregenomic RNA. Furthermore, it has been reported that the level of cccDNA decreases quite slowly during administration of nucleoside analogs,<sup>22</sup> indicating that serum levels of HBV RNA and HBVcrAg may be markers independent of the serum level of HBV DNA.

Measurement of serum HBV DNA is widely used for predicting and monitoring the effect of lamivudine therapy. However, a negative result of HBV DNA in serum does not necessarily indicate a good subsequent clinical course, because lamivudine-resistant strains often appear during drug administration and reactivation of HBV replication after discontinuation of treatment often occurs, even in patients who test negatively for serum HBV DNA during therapy.<sup>23-25</sup> Sung et al.<sup>13</sup> reported that the intrahepatic HBV cccDNA level at the end of lamivudine monotherapy or peginterferon and lamivudine combination therapy is a better predictor of a sustained virologic response than the serum HBV DNA level. Their conclusion seems quite reasonable because cccDNA in infected hepatocytes, not HBV in circulation, serves as a template for HBV pregenomic and messenger RNA in HBV replication. Although

cccDNA in the liver is a good marker for monitoring the effect of antiviral therapy, it is not easy to measure clinically because it requires a liver biopsy. Thus, serum markers that reflect the cccDNA level in hepatocytes are more suitable for clinical use. We previously reported that the serum HBVcrAg level is an independent marker, different from the serum HBV DNA level, for predicting the appearance of lamivudine resistance, and suggested that the HBVcrAg level reflects the level of cccDNA in hepatocytes.<sup>16</sup> In the present study, serum levels of HBVcrAg and HBV RNA correlated significantly, and the two levels decreased in a similar manner during lamivudine therapy (Figs. 1 and 2). These results further indicate that serum levels of both HBVcrAg and HBV RNA reflect the cccDNA level in hepatocytes since neither the synthesis of only messenger RNA, nor pregenomic RNA synthesized directly from cccDNA, which may be incorporated into viral particles, is inhibited by lamivudine.

In conclusion, HBV RNA that has been incorporated into viral particles is detectable in the serum of chronic HBV carriers. HBV RNA in serum is a new marker, which may reflect the cccDNA level in hepatocytes and may be useful for monitoring lamivudine therapy. We could not clarify the relationship between the HBV RNA level and clinical outcome in the present study. Thus, further studies are required to elucidate the clinical significance of HBV RNA in serum and its relationship to serum HBVcrAg.

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

## References

- Maddrey WC. Hepatitis B: an important public health issue. *J Med Virol* 2000;61:362–6.
- Beasley RP, Hwang LY, Lin CC, Chien CS. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22 707 men in Taiwan. *Lancet* 1981;2:1129–33.
- Liaw YF, Sung JJ, Chow WC, Farrell G, Lee CZ, Yuen H, et al. Lamivudine for patients with chronic hepatitis B and advanced liver disease. *N Engl J Med* 2004;351:1521–31.
- Matsumoto A, Tanaka E, Rokuhara A, Kiyosawa K, Kumada H, Omata M, et al. Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B: a multicenter retrospective study of 2795 patients. *Hepatol Res* 2005;32:173–84.
- Dienstag JL, Goldin RD, Heathcote EJ, Hann HW, Woessner M, Stephenson SL, et al. Histological outcome during long-term lamivudine therapy. *Gastroenterology* 2003;124:105–17.
- Dienstag JL, Perrillo RP, Schiff ER, Bartholomew M, Vicary C, Rubin M. A preliminary trial of lamivudine for chronic hepatitis B infection. *N Engl J Med* 1995;333:1657–61.
- Doong SL, Tsai CH, Schinazi RF, Liotta DC, Cheng YC. Inhibition of the replication of hepatitis B virus in vitro by 2',3'-dideoxy-3'-thiacytidine and related analogues. *Proc Natl Acad Sci U S A* 1991;88:8495–9.
- Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, 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.
- Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997;337:1733–45.
- Mason WS, Halpern MS, England JM, Seal G, Egan J, Coates L, et al. Experimental transmission of duck hepatitis B virus. *Virology* 1983;131:375–84.
- Summers J, Mason WS. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. *Cell* 1982;29:403–15.
- Tuttleman JS, Pourcel C, Summers J. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. *Cell* 1986;47:451–60.
- Sung JJ, Wong ML, Bowden S, Liew CT, Hui AY, Wong VW, et al. Intrahepatic hepatitis B virus covalently closed circular DNA can be a predictor of sustained response to therapy. *Gastroenterology* 2005;128:1890–7.
- Kimura T, Rokuhara A, Sakamoto Y, Yagi S, Tanaka E, Kiyosawa 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.
- 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 Hepat* 2003;10:324–30.
- Tanaka E, Matsumoto A, Suzuki F, Kobayashi M, Mizokami M, Tanaka Y, et al. Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance. *Liver Int* 2006;26:90–6.
- 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.
- Kimura T, Rokuhara A, Matsumoto A, Yagi S, Tanaka E, Kiyosawa K, et al. New enzyme immunoassay for detection of hepatitis B virus core antigen (HBcAg) and relation between levels of HBcAg and HBV DNA. *J Clin Microbiol* 2003;41:1901–6.
- Gerclsai Khan T, Tavis JE, Bruss V. Hepatitis B virus nucleocapsid envelopment does not occur without genomic DNA synthesis. *J Virol* 1996;70:4269–74.
- Su Q, Wang SF, Chang TE, Breitkreutz R, Hennig H, Takegoshi K, et al. Circulating hepatitis B virus nucleic acids in chronic infection: representation of differently polyadenylated viral transcripts during progression to nonreplicative stages. *Clin Cancer Res* 2001;7:2005–15.
- Zhang W, Hacker HJ, Tokus M, Bock T, Schroder CH. Patterns of circulating hepatitis B virus serum nucleic acids during lamivudine therapy. *J Med Virol* 2003;71:24–30.
- Werle-Lapostolle B, Bowden S, Locarnini S, Wurstthorn 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.
- Liaw YF, Chien RN, Yeh CT, Tsai SL, Chu CM. Acute exacerbation and hepatitis B virus clearance after emergence of YMDD motif mutation during lamivudine therapy. *Hepatology* 1999;30:567–72.
- Suzuki F, Tsubota A, Arase Y, Suzuki Y, 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.
- Zollner B, Schafer P, Feucht HH, 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.

## Fatal HBV Reactivation in a Subject with Anti-HBs and Anti-HBc

Takeji Umemura and Kendo Kiyosawa

**Key words:** hepatitis B virus, reactivation, fulminant hepatitis, rituximab

(DOI: 10.2169/internalmedicine.45.0158)

Approximately 3 billion people have been exposed to hepatitis B virus (HBV), and 350 million are chronic carriers worldwide. HBV infection is the primary cause of cirrhosis and hepatocellular carcinoma and one of the major causes of death globally. As medical services improve, many patients, including elderly, and those with cancer, autoimmune diseases or following transplantation for prevention of rejection undergo cytotoxic or immunosuppressive therapy. If these patients have chronic HBV infection, reactivation of HBV is a well-recognized complication (1, 2). HBV reactivation has a broad range of manifestations, ranging from slight, subclinical rises in transaminase activities to severe, potentially fatal fulminant hepatitis. This is a particularly critical issue in countries in the Far East and the tropics, where HBV is endemic.

The clearance of circulating hepatitis B surface antigen (HBsAg) and appearance of antibody to HBsAg (anti-HBs) with normalization of liver function have been generally accepted as evidence of clinical and serologic recovery from acute hepatitis B. However, even in HBsAg negative patients with resolved HBV infection (anti-HBs-positive and/or antibody to hepatitis B core antigen (anti-HBc)-positive), HBV replication has been shown to persist in the liver and in peripheral blood mononuclear cells for decades (3, 4). Hence, HBV reactivation has been reported after transplantation, immunosuppressive therapy, allogenic and autologous hematopoietic stem cell transplantation in this setting (1, 2, 5).

In the last issue of Internal Medicine, Sera et al (6) report a patient who developed hepatitis due to HBV reactivation and eventually died despite administration of lamivudine. This patient was resolved HBV infection such as, HBsAg-negative, and anti-HBs and anti-HBc-positive before immunosuppressive therapy. After chemotherapy including rituximab for malignant lymphoma, the patient developed severe hepatitis with HBsAg seroreversion and HBV DNA positivity in serum. Rituximab is a generally engineered chimeric murine/human monoclonal antibody against the CD20

antigen found on the surface of normal and malignant B lymphomas and is used alone or in combination with cytotoxic therapy. In 2001, Dervite et al (7) first reported a possible relationship between HBV reactivation and rituximab use in a patient with anti-HBs. After that report, some cases of reactivation of HBV in patients following treatment with rituximab that may prove fatal were reported (8-10). Hence, in October 2004, the U.S. Food and Drug Administration (FDA) reported a possible relationship between fulminant hepatitis and rituximab use. Furthermore, according to an abstract in the Proceeding of Shanghai Hong-Kong International Liver Congress, Dr. Lau's group evaluated the risk of developing HBV reactivation in HBsAg-negative patients after chemotherapy. They followed-up 244 HBsAg-negative patients with lymphoma treated with chemotherapy for a median 12 months. HBV reactivation developed in 8 (3%) of 244 patients and the risk was statistically higher in patients with rituximab-containing regimen. As rituximab has been found to induce profound and durable B cell depletion, CD20-positive cell was 0% in this case. B cells may act as antigen-presenting cells and prime cytotoxic T lymphocyte-specific responses in HBV infection (1). Thus, progressive B cell depletion may also account for the increasing incidence of HBV reactivation as well as cytomegalovirus (11) and parvovirus B19 (12). Hence careful attention must be employed when administering rituximab to patients with chronic hepatitis and resolved HBV infection. It is well known that anti-HBs is necessary to suppress HBV reactivation. However, the role of anti-HBs has not been demonstrated, especially in patients with occult HBV infection. Rituximab affects lymphoma B-cell as well as normal B-cell producing antibody.

The American Association of Study of Liver Diseases (13) has published the recommendations for antiviral prophylaxis of hepatitis B carriers who receive immunosuppressive or cytotoxic therapy as follows: "HBsAg testing should be performed in persons who have high risk of HBV infec-

Department of Internal Medicine, Division of Gastroenterology and Hepatology, Shinshu University School of Medicine, Matsumoto  
Correspondence to Kendo Kiyosawa, Department of Internal Medicine, Division of Gastroenterology and Hepatology, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621

tion, prior to initiation of chemotherapy or immunosuppressive therapy; Prophylactic antiviral therapy with lamivudine is recommended for HBV carriers at the onset of cancer chemotherapy or of a finite course of immunosuppressive therapy, and maintained for 6 months after completion of chemotherapy or immunosuppressive therapy." However, there is no available recommendation for individuals with resolved HBV infection who undergo cytotoxic or immunosuppressive therapy. Additionally, in subjects with past HBV infection the incidence and mortality of HBV reactivation have not been fully clarified in Japan. To clarify the mechanism of HBV reactivation receiving cytotoxic or immunosuppressive therapy, we have started a survey which was supported in part by a Grant-in-Aid from the Ministry of Health, Labour and Welfare of Japan. Here, we present the preliminary data from this retrospective study on the incidence and mortality of HBV reactivation in individuals with resolved HBV infection between January 2000 and December 2004. During 4 years, a total of 55 patients with HBV reactivation were seen at 90 hospitals certified by the Japan Society of Hepatology. During the same period, approximately 1,000 patients with acute hepatitis B were diagnosed in those hospitals. Among the patients with HBV reactivation, 27% developed fulminant hepatitis, compared with

only 7% in the acute hepatitis B group. Thus, the incidence of fulminant hepatitis in the HBV seroreversion group was significantly higher than in the acute hepatitis B group. Surprisingly, in patients with fulminant hepatitis, the HBV reactivation group had a significantly higher mortality than the acute hepatitis B group (100% vs. 44%). Taken together, these preliminary data of HBV reactivation in one-fourth of HBsAg-negative subjects causing fulminant hepatitis, and the high mortality of these patients, constitute important issues that need attention.

The patient reported in this issue (6) died due to fulminant hepatitis. Additionally, in our survey, many patients developed severe hepatitis and died. In contrast, some groups have reported that hepatic impairment was mild and no direct HBV-related mortality was observed in such cases (14, 15). Hence, we need to clarify this controversy. As Sera et al (6) found the mutations of core promoter and precore regions of HBV gene in their patient, viral characteristics including genotype and mutations should be investigated. We are planning further investigation of this group, including clinical backgrounds and mechanisms of high mortality. Furthermore, a guideline for reactivation prevention and treatment is necessary to manage this problem in patients undergoing or completing cancer therapy in Japan.

## References

1. Yeo W, Johnson PJ. Diagnosis, prevention and management of hepatitis B virus reactivation during anticancer therapy. *Hepatology* **43**: 209-220, 2006.
2. Xunrong L, Yan AW, Liang R, Lau GK. Hepatitis B virus (HBV) reactivation after cytotoxic or immunosuppressive therapy - pathogenesis and management. *Rev Med Virol* **11**: 287-299, 2001.
3. Rehmann B, Ferrari C, Pasquinelli C, Chisari FV. The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response. *Nat Med* **2**: 1104-1108, 1996.
4. Yuki N, Nagaoka T, Yamashiro M, et al. Long-term histologic and virologic outcomes of acute self-limited hepatitis B. *Hepatology* **37**: 1172-1179, 2003.
5. Kitano K, Kobayashi H, Hanamura M, et al. Fulminant hepatitis after allogeneic bone marrow transplantation caused by reactivation of hepatitis B virus with gene mutations in the core promoter region. *Eur J Haematol* 2006 (in press).
6. Sera T, Hiasa Y, Michitaka K, et al. Anti-HBs-positive liver failure due to hepatitis B virus reactivation induced by rituximab. *Intern Med* **45** (11): 721-724, 2006.
7. Dervite I, Hober D, Morel P. Acute hepatitis B in a patient with antibodies to hepatitis B surface antigen who was receiving rituximab. *N Engl J Med* **344**: 68-69, 2001.
8. Hamaki T, Kami M, Kusumi E, et al. Prophylaxis of hepatitis B reactivation using lamivudine in a patient receiving rituximab. *Am J Hematol* **68**: 292-294, 2001.
9. Westhoff TH, Jochimsen F, Schmittl A, et al. Fatal hepatitis B virus reactivation by an escape mutant following rituximab therapy. *Blood* **102**: 1930, 2003.
10. Tsutsumi Y, Tanaka J, Kawamura T, et al. Possible efficacy of lamivudine treatment to prevent hepatitis B virus reactivation due to rituximab therapy in a patient with non-Hodgkin's lymphoma. *Ann Hematol* **83**: 58-60, 2004.
11. Suzan F, Ammor M, Ribrag V, et al. Fatal reactivation of cytomegalovirus infection after use of rituximab for a post-transplantation lymphoproliferative disorder. *N Engl J Med* **345**: 1000, 2001.
12. Sharma VR, Fleming DR, Slone SP. Pure red cell aplasia due to parvovirus B19 in a patient treated with rituximab. *Blood* **96**: 1184-1186, 2000.
13. Lok AS, McMahon BJ. Chronic hepatitis B: update of recommendations. *Hepatology* **39**: 857-861, 2004.
14. Wands JR, Chura CM, Roll FJ, Maddrey WC. Serial studies of hepatitis-associated antigen and antibody in patients receiving antitumor chemotherapy for myeloproliferative and lymphoproliferative disorders. *Gastroenterology* **68**: 105-112, 1975.
15. Lok AS, Liang RH, Chiu EK, Wong KL, Chan TK, Todd D. Reactivation of hepatitis B virus replication in patients receiving cytotoxic therapy. Report of a prospective study. *Gastroenterology* **100**: 182-188, 1991.

# Patients With and Without Loss of Hepatitis B Virus DNA After Hepatitis B e Antigen Seroconversion Have Different Virological Characteristics

Noriko Misawa,<sup>1</sup> Akihiro Matsumoto,<sup>1</sup> Eiji Tanaka,<sup>1\*</sup> Akinori Rokuhara,<sup>1</sup> Kaname Yoshizawa,<sup>1</sup> Takeji Umemura,<sup>1</sup> Noboru Maki,<sup>2</sup> Tatsuji Kimura,<sup>2</sup> and Kendo Kiyosawa<sup>1,3</sup>

<sup>1</sup>Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan

<sup>2</sup>Advanced Life Science Institute, Inc., Wako, Japan

<sup>3</sup>Shinshu University Graduate School of Medicine, Institute of Organ Transplants, Reconstructive Medicine and Tissue Engineering, Matsumoto, Japan

The characteristic differences between patients with and without loss of hepatitis B virus (HBV) DNA after achieving hepatitis B e antigen seroconversion were analyzed by comparing changes in HBV DNA and HBV core-related antigen levels during a period from 3 years before to 3 years after the seroconversion. Of the 24 seroconverters, 6 (inactive replication group) showed continuous loss of HBV DNA in serum after the seroconversion and the remaining 18 did not lose HBV DNA (active replication group). The HBV DNA level was similar between the two groups, while the HBV core-related antigen level was significantly lower in the active replication group than in the inactive replication group before the seroconversion. The levels of both HBV DNA and HBV core-related antigen decreased remarkably around the time of seroconversion in the inactive replication group, while these levels did not change or decreased slightly in the active replication group. After the seroconversion, the HBV DNA level was significantly higher in the active replication group than in the inactive replication group, while the HBV core-related antigen level was similarly low between the two groups. Because the serum level of HBV core-related antigen mainly reflects that of HBe antigen, the low level of HBV core-related antigen seen after seroconversion in both groups might have contributed to the occurrence of seroconversion. The precore and core promoter mutations which cause diminished excretion of hepatitis B e antigen were significantly more frequent in the active replication group than in the inactive replication group. It was therefore considered that the seroconversion was caused mainly by a decrease in viral replication in the inactive replication group, and mainly by a decrease in HBe antigen production in the active replication group. **J. Med. Virol.** 78:68–73, 2006. © 2005 Wiley-Liss, Inc.

**KEY WORDS:** HBV DNA; seroconversion; HBV core-related antigen; precore mutation; core promoter mutation

## INTRODUCTION

A total of 350 million people worldwide are estimated to be carriers of hepatitis B virus (HBV) [Maynard, 1990; Maddrey, 2000]. HBV is important as a causative agent for liver diseases such as chronic hepatitis and hepatocellular carcinoma, especially in Asian countries [Lee, 1997]. In the natural history of chronic HBV infection, seroconversion from hepatitis B e (HBe) antigen to its antibody (anti-HBe) is usually accompanied by a decrease in HBV replication and remission of hepatitis [Realdi et al., 1980; Hoofnagle et al., 1981; Liaw et al., 1983]. Thus, HBe antigen seroconversion is a favorable sign for patients with chronic hepatitis B. However, there are some patients who continue to have elevated HBV DNA levels in the serum and active liver disease after the seroconversion [Bonino et al., 1986; Hsu et al., 2002].

Although the detailed mechanisms of HBe antigen seroconversion have not been fully clarified, several mutations in the HBV genome have been reported to be associated with the phenomenon. When the precore (pre-C) and core genes in the HBV genome are

Grant sponsor: Ministry of Health, Labour and Welfare of Japan; Grant number: 1640013-41.

\*Correspondence to: Eiji Tanaka, MD, Second Department of Internal Medicine, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan.

E-mail: etanaka@hsp.md.shinshu-u.ac.jp

Accepted 29 September 2005

DOI 10.1002/jmv.20505

Published online in Wiley InterScience  
(www.interscience.wiley.com)

transcribed and translated in tandem, HBe antigen is produced and secreted into circulation [Bruss and Gerlich, 1988; Garcia et al., 1988]. The G to A mutation at nucleotide (nt) 1896 in the pre-C region (G1896A), which converts codon 28 for tryptophan to a stop codon, is associated with the loss of HBe antigen [Carman et al., 1989; Okamoto et al., 1990]. The double mutation (A1762T and G1764A) in the core promoter (CP) has been shown to reduce the synthesis of HBe antigen by suppressing the transcription of precore mRNA [Okamoto et al., 1994; Takahashi et al., 1995; Buckword et al., 1996]. Convincing lines of evidence have indicated a close association of HBe antigen seroconversion with the appearance of precore and core promoter mutations [Okamoto et al., 1994; Takahashi et al., 1995; Buckword et al., 1996; Yamaura et al., 2003] as well as the severity of liver disease [Kosaka et al., 1991; Aritomi et al., 1998; Lindh et al., 1998].

A chemiluminescence enzyme immunoassay (CLEIA) was developed previously for the detection of HBV core-related antigen [Kimura et al., 2002; Rokuhara et al., 2003]. The HBV core-related antigen is expressed on HBe and core (HBc) antigens; both proteins are transcribed from the precore/core gene and their first 149 amino acids are identical. The HBVcrAg CLEIA measures the serum levels of HBe and HBc antigens simultaneously, using monoclonal antibodies, which recognize common epitopes of these two denatured antigens. However, the amount of HBV core-related antigen mainly reflects that of HBe antigen, because the concentration of HBe antigen in serum is much higher than that of HBc antigen [Kimura et al., 2002]. In the present study, the characteristic differences that may exist between patients with and without HBV DNA in serum after HBe antigen seroconversion were examined by comparing chronological changes of HBV DNA and HBV core-related antigen as well as by testing HBV genome mutations associated with the seroconversion.

## MATERIALS AND METHODS

### Patients

The present study is a retrospective one using stored sera from Japanese patients with chronic hepatitis B seen in Shinshu University Hospital. The clinical database was reviewed to identify all patients who had been followed from January 1985 to June 2001 and also showed seroconversion from HBe antigen to anti-HBe during the follow-up period. A total of 24 patients were recruited in the present study. The 24 patients consisted of 17 men and 7 women with a median age of 39 years. Seroconversion of HBe antigen was defined as disappearance of HBe antigen accompanied by the development of anti-HBe on at least two consecutive visits. All 24 patients met the following three criteria: (1) follow-up was performed for at least 3 years before and after the seroconversion; (2) chronic hepatitis without liver cirrhosis was confirmed by histological examination; and (3) serum samples were available for testing every 6 months during the follow-up period. Of the 24 patients,

12 patients received interferon administration of at most 4 weeks and none received nucleotide analogs such as lamivudine, adefovir, or entecavir during the follow-up period.

Serum concentrations of HBV DNA and HBV core-related antigen were determined every 6 months during the follow-up period, which ran from 3 years before to 3 years after the seroconversion. The presence or absence of the pre-C mutation of A1896 and the double mutation in the CP (T1762/A1764) was determined every year during the follow-up period. The serum samples had been stored at  $-20^{\circ}\text{C}$  or below until tested. Written informed consent was obtained from each patient.

### Serological Markers for HBV

Conventional HBV markers, including HBe antigen and anti-HBe, were tested using CLEIA kits (Fuji Rebio, Tokyo, Japan). Six major genotypes (A–F) of HBV were determined using the method reported by Mizokami et al. [1999], in which the surface gene sequence amplified by PCR was analyzed by restriction fragment length polymorphism.

The Pre-C and CP mutations were determined on nucleic acids extracted from 100  $\mu\text{l}$  of serum with a DNA/RNA extraction kit (Smitest EX-R and D; Genome Science Laboratories Co., Ltd., Tokyo, Japan). The stop codon mutation in the Pre-C region (A1896) was detected with an enzyme-linked mini-sequence assay kit (Smitest; Genome Science Laboratories). In principle, G1896 in the wild-type HBV and A1896 in the mutants were determined by mini-sequence reactions using labeled nucleotides that are complementary to either the wild-type or mutant. The results were expressed as a percent mutation rate according to the definition by Aritomi et al. [1998]. The sample was judged positive for the pre-C mutation when the mutation rate exceeded 50% in the present study, because the mutation rate steadily increase to 100% afterward once it exceed the rate of 50% [Yamaura et al., 2003]. The double mutation in the CP was detected using an HBV core promoter detection kit (Smitest; Genome Science Laboratories) [Aritomi et al., 1998]. This kit detects T1762/G1764 or A1762/T1764 by a polymerase chain reaction (PCR) with primers specific for either the wild-type or mutant. The results were recorded in three categories, that is, wild, mixed, and mutant types. In the present study, the sample was considered positive for the CP mutation when the results were in the mutant type category. The detection limits of the pre-C and the CP mutation kits are both 1,000 copies/ml according to the manufacturer. The pre-C mutation could be determined in 136 (99%) of 137 samples, which had HBV DNA levels higher than 1,000 copies/ml and in 30 (97%) of 31 samples which had levels lower than 1,000 copies/ml. Similarly, the CP mutation could be determined in 136 (99%) of 137 samples and in 28 (90%) of 31 samples.

The serum concentration of HBV DNA was determined using an Amplicor HBV monitor kit (Roche,

Tokyo, Japan) which had a quantitative range of 2.6–7.6 log copies/ml [Kessler et al., 1998]. Sera containing over 7.0 log copies/ml HBV DNA were diluted 10- or 100-fold in normal human serum and measured again to obtain the end titer.

The serum concentration of HBV core-related antigen was measured using the CLEIA reported previously [Kimura et al., 2002; Rokuhara et al., 2003]. In summary, 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 hr at room temperature and the wells were washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies against denatured HBc and HBe antigens (HB91 and HB110) were added to the well, and incubated for 1 hr 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 HBV core-related antigen concentration was read by comparison to a standard curve generated using recombinant pro-HBe antigen (amino acids, 10–183 of the precore/core gene product). The HBV core-related antigen 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 cut-off value was set tentatively at 3.0 log U/ml. Sera containing over 7.0 log U/ml HBV core-related antigen were diluted 10- or 100-fold in normal human serum and measured again to obtain the end titer.

### Statistical Analyses

The Mann–Whitney U test was used to analyze continuous variables. The Fisher's exact test was used in the analysis of categorical data. The Mantel-Haenszel chi-square test was used to evaluate positive rates for the pre-C and CP mutations. The Wilcoxon test was used to analyze the change in the level of HBV DNA and HBV core-related antigen. *P*-values less than 0.05 were considered significant. Statistical analyses were per-

formed using an SPSS 11.5 J statistical software package (SPSS, Inc., Chicago, IL).

## RESULTS

### Grouping of Seroconverters According to HBV DNA Outcome

The 24 seroconverters enrolled in the present study were classified into two groups according to changes in serum levels of HBV DNA. The HBV DNA level decreased substantially around the time of the seroconversion and then became continuously undetectable in one group (inactive replication group), and the level decreased slightly and did not become continuously undetectable even after the seroconversion in another group (active replication group). In the present study, the former group of patients were defined as those whose HBV DNA levels were lower than 2.6 log copies/ml at each of the time points of 1.5, 2, 2.5, and 3 years after the seroconversion, and the latter group of patients were defined as those whose HBV DNA levels were not. Of the 24 seroconverters, 6 belonged to the inactive replication group and the remaining 18 belonged to the active replication group.

The clinical backgrounds of the active and inactive replication groups are compared in Table I. The median age, gender ratio, and history of interferon therapy did not differ between the two groups. All patients were infected with genotype C HBV. Normalization of serum alanine aminotransferase (ALT) after seroconversion was considered to have occurred in cases in which ALT was normal at each of the time points of 2, 2.5, and 3 years after the seroconversion in the present study. The normalization of ALT was more frequent in the inactive replication group than in the active replication group, but the difference was not statistically significant.

### Changes in HBV DNA and HBV Core-Related Antigen Concentration

Changes in the serum level of HBV DNA are compared between the active and inactive replication groups in Figure 1A. At the start-point of the follow-up, the level was distributed within a similarly high range in both groups. In the inactive replication group, the median

TABLE I. Comparison of Clinical Backgrounds Between the Inactive and Active Replication Groups

Characteristics	Inactive replication group n = 6	Active replication group n = 18	<i>P</i>
Age at seroconversion (yr) <sup>a</sup>	37 (23–65)	39 (17–64)	>0.2*
Gender (M:F)	4:2	13:5	>0.2**
Genotype C <sup>b</sup>	6 (100%)	18 (100%)	>0.2**
History of interferon therapy <sup>b</sup>	3 (50%)	9 (50%)	>0.2**
ALT normalization <sup>c</sup>	4 (67%)	5 (28%)	0.150**

\*Mann–Whitney U test.

\*\*Fisher's exact test.

<sup>a</sup>Data are expressed as the median (range).

<sup>b</sup>Data are expressed as a positive number (percent).

<sup>c</sup>Normalization of serum ALT level after seroconversion (the ALT value was within the normal range at each of the time points of 2, 2.5, and 3 years after the seroconversion).



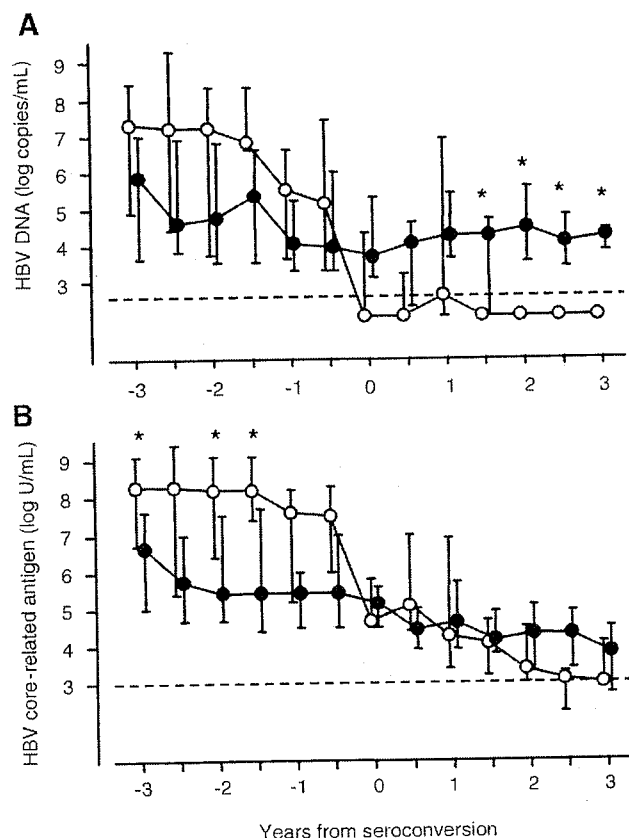


Fig. 1. Comparison of changes in HBV DNA (A) and HBV core-related antigen (B) levels between the inactive and active replication groups. Data are shown as the median  $\pm 25\%$  ranges. The broken lines indicate the detection limits of the HBV DNA and HBV core-related antigen assays, respectively. Open circles indicate inactive replication group and closed circles indicate active replication group. \* $P < 0.05$  between the inactive and active replication groups.

concentration decreased around the time of seroconversion and became continuously undetectable thereafter. In the active replication group, on the other hand, the median concentration tended to decrease around the time of seroconversion, but was not undetectable even at 3 years after seroconversion. The median HBV DNA level in the active replication group was significantly higher than that in the inactive replication group at 1.5 years after the seroconversion and each of the subsequent time points.

Changes in the serum concentration of HBV core-related antigen are compared between the active and inactive replication groups in Figure 1B. The concentration of HBV core-related antigen was significantly higher in the inactive replication group than in the active replication group at the start of the follow-up and at 1.5 and 2 years before the seroconversion point. The median concentration of HBV core-related antigen in the inactive replication group appeared to decrease around the time of seroconversion and reached a level comparable to that in the active replication group. The median HBV core-related antigen level was similar

between the inactive and active replication groups at all time points after the seroconversion, and it decreased slowly with time in both groups.

Changes in the log ratio of HBV core-related antigen/HBV DNA concentrations are compared between the inactive and active replication groups in Figure 2. The values of HBV core-related antigen and HBV DNA were substituted by their corresponding detection limit values when they were under the detection limit. The log ratio was similar between the two groups at points before the seroconversion. The log ratio decreased after the seroconversion in the active replication group, but did not change in the inactive replication group. The log ratio of HBV core-related antigen/HBV DNA was significantly lower in the active replication group than in the inactive replication group at all post-seroconversion time points except 1 year.

### Comparison of Pre-C and CP Mutations

The positive rates for the pre-C and CP mutations at the time points before and after the seroconversion are compared between the inactive and active replication groups in Figure 3. The pre-C mutation did not appear during the follow-up period in the inactive replication group. On the other hand, the positive rate for the pre-C mutation was around 30% before the seroconversion, and then increased to around 60% after the seroconversion in the active replication group. The difference in the positive rate was significant at the time points of 2 and 3 years after the seroconversion. The positive rate for the CP mutation was less than 40% in the inactive replication group during the follow-up period except at the last time point, while it was over 60% in the active replication group throughout the follow-up period. The difference in the positive rate was statistically significant at the time points of 2 and 3 years before the seroconversion and at 1 and 2 years after it.

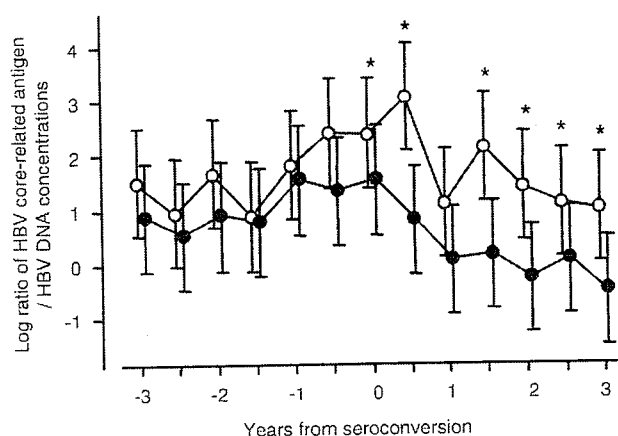


Fig. 2. Comparison of changes in the log ratio of HBV core-related antigen/HBV DNA levels between the inactive and active replication groups. Data are shown as the median  $\pm 25\%$  ranges. Open circles indicate inactive replication group and closed circles indicate active replication group. \* $P < 0.05$  between the inactive and active replication groups.

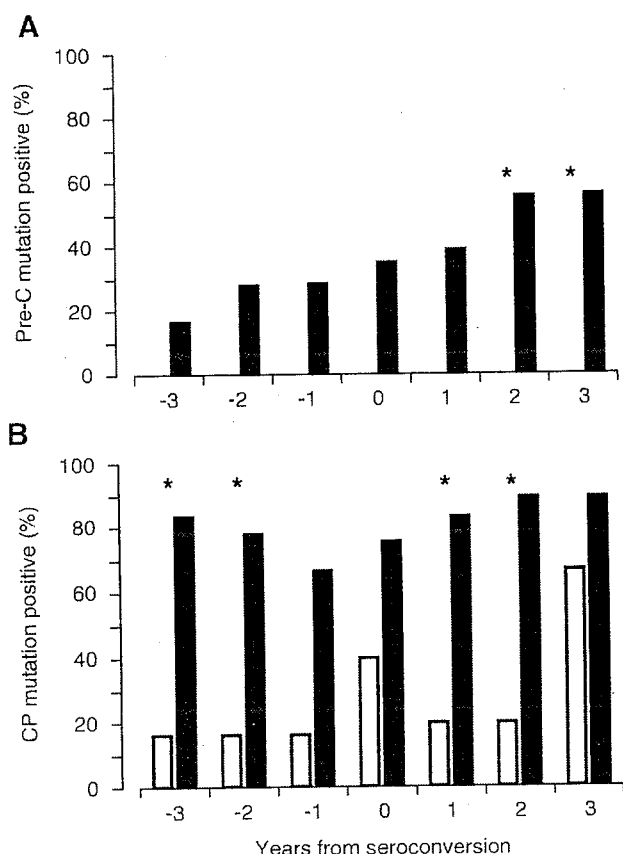


Fig. 3. Comparison of positive rates for the pre-C (A) and CP (B) mutations between the inactive and active replication groups. Open bars indicate inactive replication group and closed bars indicate active replication group. Number of patients in the inactive replication group is six at each time point except the followings: point 0 year (n = 5) in A, and points 0 year (n = 5), 1 year (n = 5), and 2 years (n = 5) in B. Number of patients in the active replication group is 18 at each time point except the followings: point 0 year (n = 17) in A and point 0 year (n = 17) in B. \* $P < 0.05$  between the inactive and active replication groups.

## DISCUSSION

Seroconverters were divided tentatively into two groups according to their levels of serum HBV DNA in the present study. It has been reported that older age and female gender are factors predicting occurrence of HBe antigen seroconversion in patients with chronic hepatitis B [Alward et al., 1985; Lok et al., 1987; McMahon et al., 2001]. On the other hand, in the present study, median age and gender distribution were similar between the inactive and active replication groups. A history of interferon treatment was recorded in half of the patients enrolled. The treatment history did not seem to be associated with the loss of HBV DNA after seroconversion, because the history was similarly distributed between the two groups and the duration of interferon therapy was as short as 4 weeks at most. Although the difference was not statistically significant, patients in the inactive replication group tended to show continuous normalization of ALT. Further, none of the

six patients in the inactive replication group developed end stage liver diseases such as cirrhosis and hepatocellular carcinoma after the follow-up period, while 4 of the 18 patients in the active replication group developed them (data not shown). High viral load, which is usually associated with active hepatitis, has been reported to be a risk factor for development of hepatocellular carcinoma even in patients with chronic hepatitis B who achieved HBe antigen seroconversion [Ikeda et al., 2003; Ohata et al., 2004]. We could not compare long-term prognosis between patients in the inactive and active replication groups in the present study. However, patients in the active replication group tended to show active hepatitis after the seroconversion and to develop end stage liver diseases. Thus, further analysis of patients whose active viral replication continues after the seroconversion would be of clinical significance.

Analysis of the changes in HBV DNA and HBV core-related antigen revealed a clear contrast between the two. Namely, the HBV DNA level was similar between the two groups, while HBV core-related antigen was significantly lower in the active replication group than in the inactive replication group before seroconversion. The levels of both HBV DNA and HBV core-related antigen decreased remarkably around the time of seroconversion in the inactive replication group, while these levels did not change or decreased slightly in the active replication group. After seroconversion, the HBV DNA level was significantly higher in the active replication group than in the inactive replication group, while the HBV core-related antigen level was similar between the two groups. Because the discrepancy in the log ratio of HBV core-related antigen/ HBV DNA between the two groups first appeared at the time of seroconversion and continued thereafter, the difference between the HBV DNA and HBV core-related antigen changes was suggested to be closely associated with the seroconversion. The results obtained in the present study indicate that the mechanism of seroconversion was different between the two groups.

Because the serum level of HBV core-related antigen mainly reflects that of HBe antigen [Kimura et al., 2002], the low level of HBV core-related antigen seen after seroconversion in both the inactive and active replication groups might have contributed to the occurrence of seroconversion. The pre-C and CP mutations, which were associated with the seroconversion, were frequent in the active replication group and rare in the inactive replication group, at least at around the time of seroconversion. The decrease of HBV core-related antigen excretion seen after seroconversion was thought to have been caused mainly by the decrease of viral replication in the inactive replication group, because viral replication did not resume in this group. On the other hand, the decrease of HBV core-related antigen was thought to have been caused mainly by the appearance of pre-C and/or CP mutations, because active viral replication continued in this group. These results suggested that the two groups had different mechanisms of seroconversion.

It has been reported that the frequency of the pre-C and the CP mutations differs among HBV genotypes. Orito et al. reported that the CP mutation was significantly associated with genotype C [Orito et al., 2001]. Yamaura et al. [2003] reported that the CP mutation was already commonly seen several years before the seroconversion in patients with genotype C. These results are consistent with the present finding that the majority of patients in the active replication group had the CP mutation from the start of follow-up. The fact that patients in the active replication group had a lower level of HBV core-related antigen before the seroconversion may be attributable to the frequent CP mutation seen in this group.

In conclusion, the present study showed that there were different mechanisms of HBe antigen seroconversion between patients in whom HBV viraemia continued after the seroconversion and those in whom it did not. Measurement of HBV core-related antigen in addition to HBV DNA was suggested to be useful in examining specific conditions of chronic hepatitis B.

#### ACKNOWLEDGMENTS

We thank Ms. Toyo Amaki and Ms. Asami Yamazaki for their technical assistance. This research was supported in part by a Grant-in Aid from the Ministry of Health, Labour and Welfare of Japan (no. 1640013-41).

#### REFERENCES

- Alward WL, McMahon BJ, Hall DB, Heyward WL, Francis DP, Bender TR. 1985. The long-term serological course of asymptomatic hepatitis B virus carriers and the development of primary hepatocellular carcinoma. *J Infect Dis* 151:604-609.
- Aritomi T, Yatsushashi H, Fujino T, Yamasaki K, Inoue O, Koga M, Kato Y, Yano M. 1998. Association of mutations in the core promoter and precore region of hepatitis virus with fulminant and severe acute hepatitis in Japan. *J Gastroenterol Hepatol* 13:1125-1132.
- Bonino F, Rosina F, Rizzetto M, Rizzi R, Chiaberge E, Tardanico R, Callea F, Verme G. 1986. Chronic hepatitis in HBsAg carriers with serum HBV-DNA and anti-HBe. *Gastroenterology* 90:1268-1273.
- Bruss V, Gerlich WH. 1988. Formation of transmembraneous hepatitis B e-antigen by cotranslational in vitro processing of the viral precore protein. *Virology* 163:268-275.
- Buckword VE, Xu Z, Chen M, Yen TS, Ou JH. 1996. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. *J Virol* 70:5845-5851.
- Cauman WF, Jacyna MR, Hadziyannis S, Karayiannis P, McGarvey MJ, Makris A, Thomas HC. 1989. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet* 2:588-591.
- Garcia PD, Ou JH, Rutter WJ, Walter P. 1988. 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* 106:1093-1104.
- Hoofnagle JH, Dusheiko GM, Seeff LB, Jones EA, Waggoner JG, Bales ZB. 1981. Seroconversion from hepatitis B e antigen to antibody in chronic type B hepatitis. *Ann Intern Med* 94:744-748.
- Hsu YS, Chien RN, Yeh CT, Sheen IS, Chiou HY, Chu CM, Liaw YF. 2002. Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. *Hepatology* 35:1522-1527.
- Ikeda K, Arase Y, Kobayashi M, Someya T, Saitoh S, Suzuki Y, Suzuki F, Tsubota A, Akuta N, Kumada H. 2003. Consistently low hepatitis B virus DNA saves patients from hepatocellular carcinogenesis in HBV-related cirrhosis. A nested case-control study using 96 untreated patients. *Intervirology* 46:96-104.
- Kessler HH, Pierer K, Dragon E, Lackner H, Santner B, Stunzner D, Stelzl E, Waitzl B, Marth E. 1998. Evaluation of a new assay for HBV DNA quantitation in patients with chronic hepatitis B. *Clin Diagn Virol* 9:37-43.
- Kimura T, Rokuhara A, Sakamoto Y, Yagi S, Tanaka E, Kiyosawa K, Maki N. 2002. Sensitive enzyme-immunoassay for hepatitis B virus core-related antigen and their correlation to virus load. *J Clin Microbiol* 40:439-445.
- Kosaka Y, Takase K, Kojima M, Shimizu M, Inoue K, Yoshida M, Tanaka S, Akahane Y, Okamoto H, Tsuda F, Miyakawa Y, Mayumi M. 1991. Fulminant HBV: Induction by hepatitis B virus mutants defective in the precore region and incapable of encoding e-antigen. *Gastroenterology* 100:1087-1093.
- Lee WM. 1997. Hepatitis B virus infection. *N Engl J Med* 337:1733-1745.
- Liaw YF, Chu CM, Su IJ, Huang MJ, Lin DY, Chang-Chien CS. 1983. Clinical and histological events preceding hepatitis B e antigen seroconversion to antibody in chronic type B hepatitis. *Gastroenterology* 84:216-219.
- Lindh M, Gustavson C, Mardberg K, Norkrans G, Dhillon AP, Horal P. 1998. Mutation of nucleotide 1762 in the core promoter region during hepatitis B e seroconversion and its relation to liver damage in hepatitis B e antigen carriers. *J Med Virol* 55:185-190.
- Lok ASF, Lai CL, Wu PC, Leung EKY, Lam TS. 1987. Spontaneous hepatitis B e antigen to antibody seroconversion and reversion in Chinese patients with chronic hepatitis B virus infection. *Gastroenterology* 92:1839-1843.
- Maddrey WC. 2000. Hepatitis B: An important public health issue. *J Med Virol* 61:362-366.
- Maynard JE. 1990. Hepatitis B: Global importance and need for control. *Vaccine* S18-S320.
- McMahon BJ, Holck P, Bulkow L, Snowball M. 2001. Serologic and clinical outcomes of 1536 Alaska natives chronically infected with hepatitis B virus. *Ann Intern Med* 135:759-768.
- Mizokami M, Nakano T, Orito E, Tanaka Y, Sakugawa H, Mukaide M, Robertson BH. 1999. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett* 450:66-71.
- Ohata K, Hamasaki K, Toriyama K, Ishikawa H, Nakao K, Eguchi K. 2004. High viral load is a risk factor for hepatocellular carcinoma in patients with chronic hepatitis B virus infection. *J Gastroenterol Hepatol* 19:670-675.
- Okamoto H, Yotsumoto S, Akahane Y, Yamanaka T, Miyazaki Y, Sugai Y, Tsuda F, Tanaka T, Miyakawa Y, Mayumi M. 1990. Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. *J Virol* 64:1298-1303.
- Okamoto H, Tsuda F, Akahane Y, Sugai Y, Yoshida M, Moriyama K, Tanaka T, Miyakawa Y, Mayumi M. 1994. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. *J Virol* 68:8102-8110.
- Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, Okanoue T, Yotsuyanagi H, Iino S. 2001. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. *Hepatology* 33:218-223.
- Realdi G, Alberti A, Rugge M, Bortolotti F, Rigoli AM, Tremolada F, Ruol A. 1980. Seroconversion from hepatitis B e antigen to anti-HBe in chronic hepatitis B infection. *Gastroenterology* 79:195-199.
- Rokuhara A, Tanaka E, Matsumoto A, Kimura T, Yamaura T, Orii K, Sun X, Yagi S, Maki N, Kiyosawa K. 2003. 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 Hepatitis* 10:1-7.
- Takahashi K, Aoyama K, Ohno N, et al. 1995. The precore/core promoter mutant (T1762A1764) of hepatitis B virus: Clinical significance and an easy method for detection. *J Gen Virol* 76:3159-3164.
- Yamaura T, Tanaka E, Matsumoto A, Rokuhara A, Orii K, Yoshizawa K, Miyakawa Y, Kiyosawa K. 2003. A case-control study for early prediction of hepatitis B e antigen seroconversion by hepatitis B virus DNA levels and mutations in the precore region and core promoter. *J Med Virol* 70:545-552.

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

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

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).

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

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 (HBe) 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 retro-

spectively 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 HBe 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 HBe 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

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		<i>P</i>
	Negative ( <i>n</i> = 54)	Positive ( <i>n</i> = 27)	
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. ‡ $\chi^2$ -test.

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).

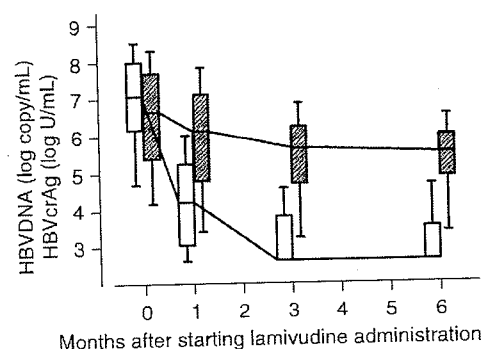


Fig. 1. Changes in the median levels of hepatitis B virus core-related antigen (HBVcrAg) and hepatitis B virus (HBV) DNA during lamivudine administration. The box plots show the 10th, 25th, 50th, 75th and 90th percentiles, with the open boxes indicating HBV DNA and shaded boxes indicating HBVcrAg. The 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.

## Prediction of lamivudine resistance

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 log copy/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

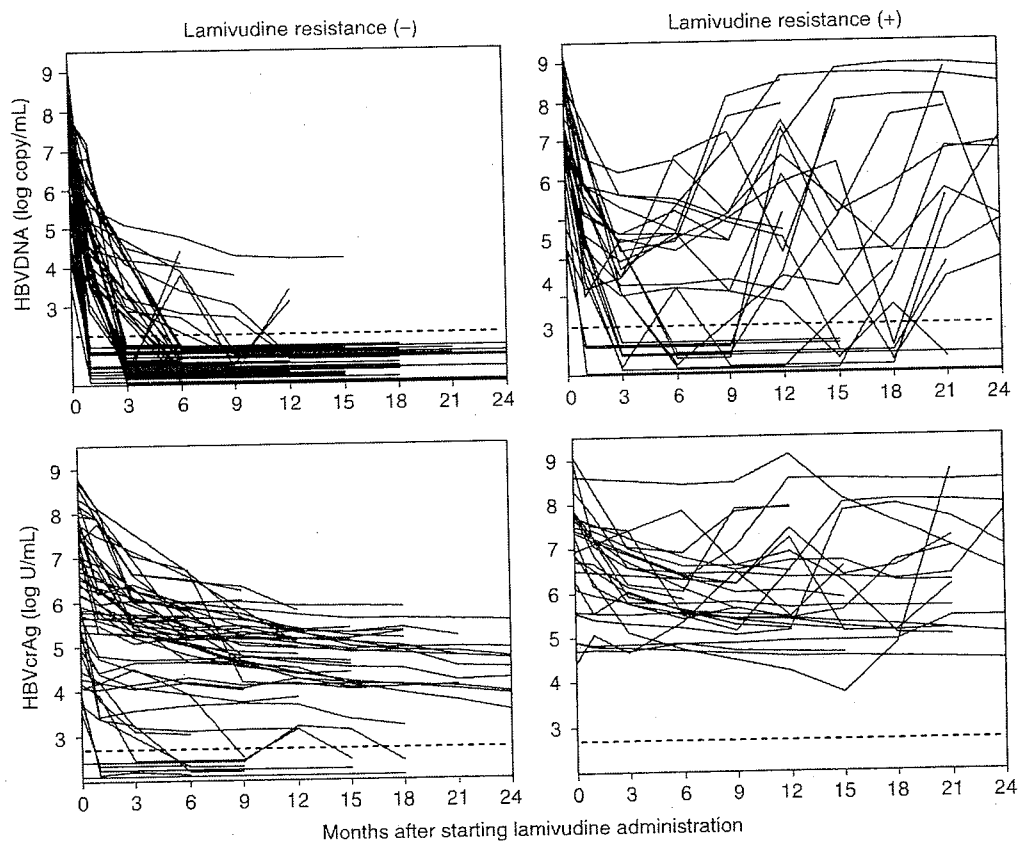


Fig. 2. Comparison of changes in serum hepatitis B virus (HBV) DNA and serum HBV core-related antigen (HBVcrAg) levels between patients who showed lamivudine resistance and those who did not. The broken lines indicate the detection limit of each assay.

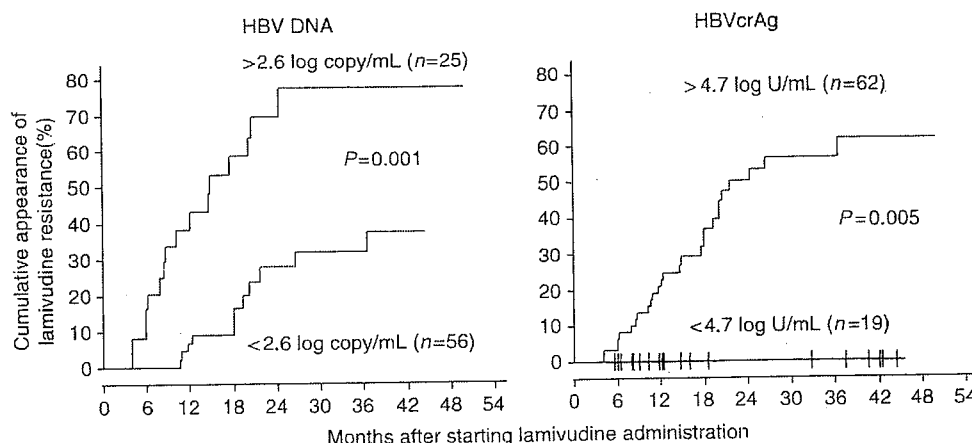


Fig. 3. Comparison of the cumulative occurrence of lamivudine resistance between patients who showed hepatitis B virus (HBV) DNA levels of less than the detection limit ( $2.6 \log \text{ copy/mL}$ ) at 6 months after starting lamivudine administration and those who did not (left figure), and similarly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

- 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.
- BENHAMOU Y, DOHIN E, LUNEL-FABIANI F, POYNARD T, HURAUX J M, KATLAMA C, et al. Efficacy of lamivudine on replication of hepatitis B virus in HIV-infected patients. *Lancet* 1995; 345: 396-7.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- TIPPES 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- LEE W M. Hepatitis B virus infection. *N Engl J Med* 1997; 337: 1733-45.
- 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.
- 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.
- 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.
- SUMMERS J, SMITH P M, HORWICH A L. Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification. *J Virol* 1990; 64: 2819-24.
- 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.

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

- 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.
- BENHAMOU Y, DOHIN E, LUNEL-FABIANI F, POYNARD T, HURAUX J M, KATLAMA C, et al. Efficacy of lamivudine on replication of hepatitis B virus in HIV-infected patients. *Lancet* 1995; 345: 396-7.
- 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.
- 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.
- 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.
- LIAN 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.
- 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.
- 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.
- 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.
- TIPPES 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.
- LIAN 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.
- 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.
- 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.
- 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.
- ROKUHARA A, TANAKA E, MATSUMOTO A, KIMURA T, YAMAMURA 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.
- 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.
- 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.
- LEE W M. Hepatitis B virus infection. *N Engl J Med* 1997; 337: 1733-45.
- 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.
- 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.
- 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.
- SUMMERS J, SMITH P M, HORWICH A L. Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification. *J Virol* 1990; 64: 2819-24.
- 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.

**Tanaka et al.**

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, WORS-THORN 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, DUO 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, DE-CRAEMER H, LAI C L. Factors associated with hepatitis B virus DNA breakthrough in patients receiving prolonged 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.

## Timing of interferon therapy and sources of infection in patients with acute hepatitis C

Kei Ogata<sup>a,\*</sup>, Tatsuya Ide<sup>a</sup>, Ryukichi Kumashiro<sup>a</sup>, Hiromitsu Kumada<sup>b</sup>,  
Hiroshi Yotsuyanagi<sup>c,1</sup>, Kiwamu Okita<sup>d</sup>, Yoshihiro Akahane<sup>e,2</sup>, Shuichi Kaneko<sup>f</sup>,  
Hirohito Tsubouchi<sup>g,3</sup>, Eiji Tanaka<sup>h</sup>, Hisataka Moriwaki<sup>i</sup>, Shuhei Nishiguchi<sup>j,4</sup>,  
Shinichi Kakumu<sup>k</sup>, Masashi Mizokami<sup>l</sup>, Shiro Iino<sup>m</sup>, Michio Sata<sup>a</sup>

<sup>a</sup> Second Department of Internal Medicine, Kurume University, Fukuoka, Japan

<sup>b</sup> Department of Gastroenterology, Toranomon Hospital, Tokyo, Japan

<sup>c</sup> Department of Internal Medicine, Division of Gastroenterology and Hepatology, St. Marianna University School of Medicine, Kawasaki, Japan

<sup>d</sup> Department of Gastroenterology and Hepatology, Yamaguchi University School of Medicine, Ube, Japan

<sup>e</sup> First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi, Yamanashi, Japan

<sup>f</sup> Department of Gastroenterology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan

<sup>g</sup> Second Department of Internal Medicine, Faculty of Medicine, The University of Miyazaki, Miyazaki, Japan

<sup>h</sup> Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan

<sup>i</sup> First Department of Internal Medicine, Gifu University School of Medicine, Gifu, Japan

<sup>j</sup> Department of Hepatology, Osaka City University, Graduate School of Medicine, Osaka, Japan

<sup>k</sup> Department of Internal Medicine, GI Division, Aichi Medical University School of Medicine, Aichi, Japan

<sup>l</sup> Department of Laboratory Medicine, Nagoya City University Medical School, Nagoya, Japan

<sup>m</sup> Seizankai Kiyokawa Hospital, Tokyo, Japan

Received 13 June 2005; received in revised form 22 August 2005; accepted 31 August 2005

Available online 15 December 2005

### Abstract

**Background/Aims:** Controversy over the selection of patients and optimum therapeutic method for acute hepatitis C has continued. The aims of this study were to investigate the source of infection, and to evaluate the timing of interferon (IFN) therapy in patients with acute hepatitis C in Japan.

**Methods:** The records of 102 patients from 12 facilities in Japan who developed acute hepatitis C after 1990 were investigated. In the patients treated with IFN, we performed multivariate analysis to investigate factors related to sustained virological response (SVR).

**Results:** Medical procedure was the most common source of infection, accounting for 32.4% in the 102 patients (33/102). Of 81 patients treated with IFN, 71 patients were followed after IFN therapy, and 57/71 (80.3%) had SVR. The SVR rate was significantly higher in patients treated with IFN within 24 weeks from onset of symptoms than the SVR rate in those treated after 25 weeks ( $P = 0.0016$ ). Multivariate analysis revealed that only the duration between onset of symptoms and initiation of IFN therapy (within 24 weeks) was related to SVR.

DOI of related article: 10.1016/j.hepres.2005.10.002.

**Abbreviations:** HCV, hepatitis C virus; IFN, interferon; ALT, alanine aminotransferase; SVR, sustained virological response; Peg-IFN, pegylated interferon

\* Corresponding author. Tel.: +81 942 31 7561; fax: +81 942 34 2623.

E-mail address: [keiogata@med.kurume-u.ac.jp](mailto:keiogata@med.kurume-u.ac.jp) (K. Ogata).

<sup>1</sup> Present address: Department of Infectious Diseases, Department of Infection Control and Prevention, Faculty of Medicine, University of Tokyo Hospital, Tokyo, Japan.

<sup>2</sup> Present address: Kofu Municipal Hospital, Yamanashi, Japan.

<sup>3</sup> Present address: Second Division of Internal Medicine, Medical Faculty, Kagoshima University, Kagoshima, Japan.

<sup>4</sup> Present address: Division of Hepatobiliary and Pancreatic Diseases, Department of Internal Medicine, Hyogo College of Medicine, Hyogo, Japan.

1386-6346/\$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.

doi:10.1016/j.hepres.2005.08.012