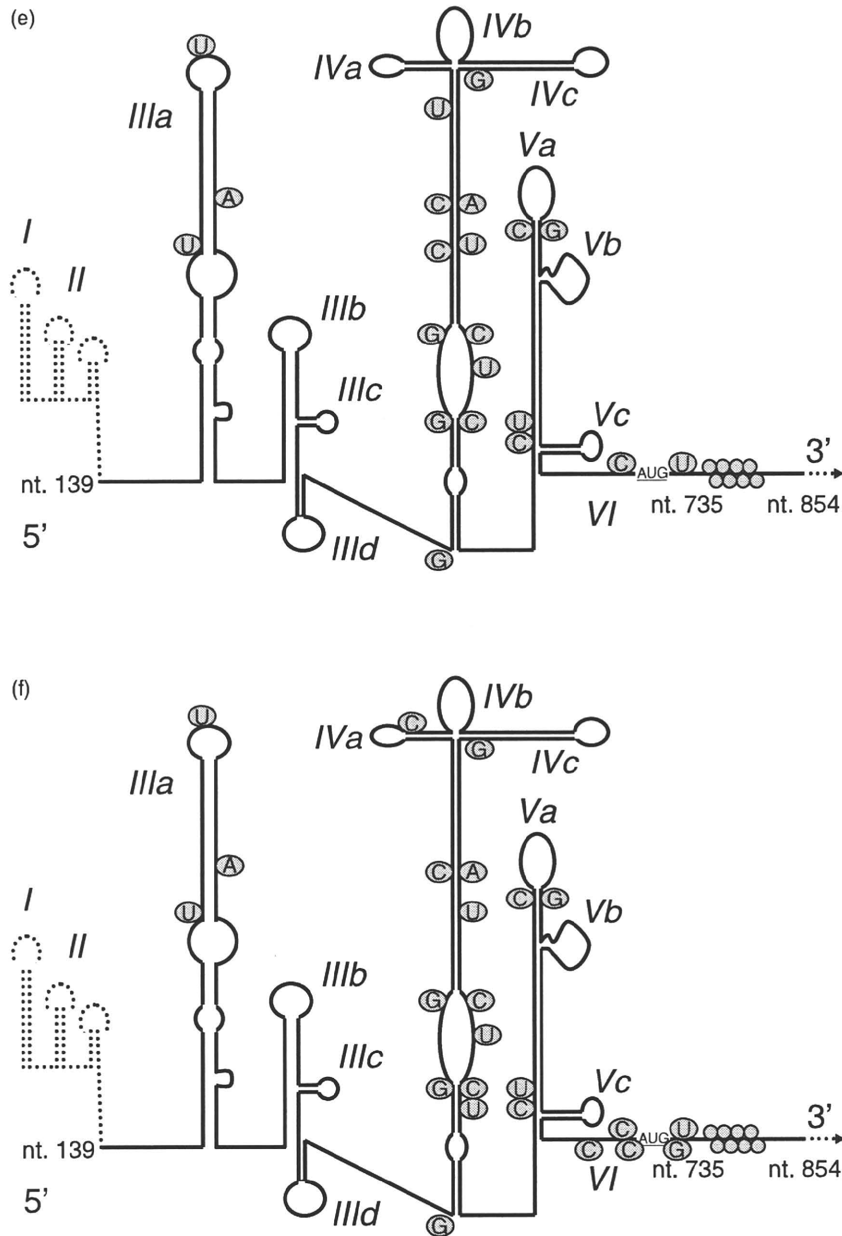

Figure 1 *Continued.*

dependent translation efficiencies) of HM175 were used as control. The HM175 strain of HAV was originally recovered from stool of a patient with hepatitis A in Melbourne, Australia. It is well-known that plasmid pHM175 was isolated directly from primary African green monkey kidney cells infected with this virus and *in vitro* transcribed RNA from this clone were cell-culture grown.[13,19] In Huh-7 cells, in which HAV can replicate and which are commonly used for HAV research,[27] the IRES activities of F1, F2, F3, A1, A2 and A3 were 1.05-, 0.39-, 3.76-, 0.048-, 2.19- and 0.6-fold, respectively, of that of HM175 (Fig. 2a). In HepG2, the IRES activities of F1, F2, F3, A1, A2 and A3 were 1.29-, 0.33-, 3.68-, 0.12-, 8.91- and 4.23-fold, respectively, of that of HM175 (Fig. 2b). In HLE, the IRES activities of F1, F2, F3, A1, A2 and A3 were 0.85-, 1.35-, 5.17-, 0.55-, 29.06- and 4.31-

© 2010 The Japan Society of Hepatology

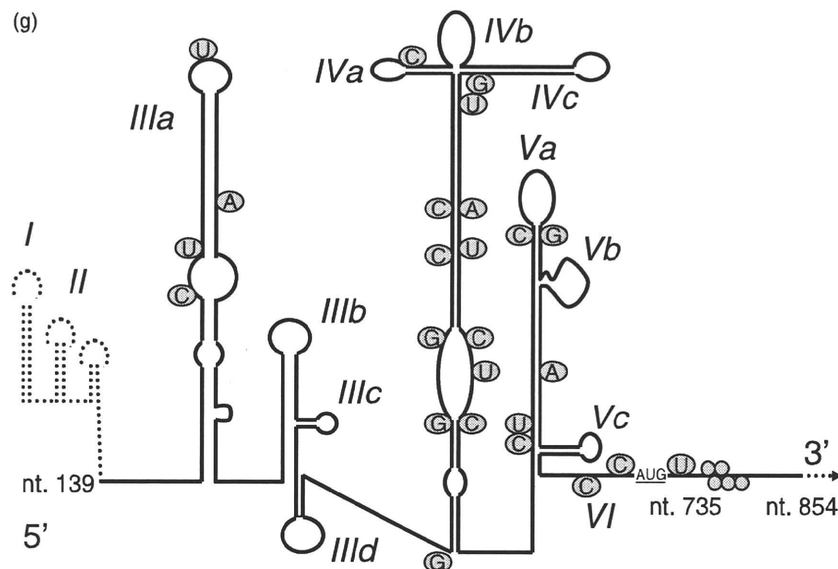

Figure 1 *Continued.*

fold, respectively, of that of HM175 (Fig. 2c). Compared with HM175, F3, A2 and A3 tended to have higher IRES activities in liver-derived cell lines, F1 had similar IRES activity to HM175, and F2 and A1 tended to have lower IRES activities than HM175.

F3 has a large deletion in domains III–IV (Fig. 1d). In A2, several nucleotide mutations around the AUG codon in domain VI of IRES were seen. These mutations possibly affected their IRES activities. A3 IRES activities in Huh-7, HepG2 and HLE were 0.6-, 4.23- and 4.31-fold, respectively. A2 showed very high activity in HLE compared to other IRES. These results may have been influenced by certain cellular factors.

HM175 HAV IRES activities not lower than those of clinical isolates in non-hepatocyte-derived cell lines

In HeLa, another permissive cell line[28] for HAV, the IRES activities of F1, F2, F3, A1, A2 and A3 were 0.33-, 0.31-, 1.0-, 0.11-, 0.87- and 0.32-fold, respectively, of that of HM175 (Fig. 2d).

Acute hepatitis A occasionally presents the manifestation of acute renal failure during the course of the disease.[10] We thus examined HAV IRES activities in African green monkey kidney cell lines BSC-1 and CV-1, as they were reported to be permissive for HAV replication,[29,30] and then we examined the HAV IRES activities in these two cell lines. In BSC-1, the IRES activities of F1, F2, F3, A1, A2 and A3 were 0.11-, 0.10-, 0.73-, 0.02-, 0.48- and 0.86-fold, respectively, of that of HM175 (Fig. 2e). In CV-1, the IRES activities of F1, F2, F3, A1, A2 and A3 were 0.04-, 0.20-, 0.46-, 0.05-, 0.76- and 0.62-fold, respectively, of that of HM175 (Fig. 2f). IRES activities of F3, A2 and A3, which tended to be higher in hepatocytes (Fig. 2a–c), did not differ much from that of HM175 in these non-hepatic cells (Fig. 2d–f). The IRES activities of HM175 in Huh-7, HepG2, HLE, HeLa, BSC-1 and CV-1 were 43.3, 469, 18.2, 618, 1814 and 1097, respectively.

Amantadine has inhibitory effect on clinical isolate-derived HAV-IRES-mediated translations

Amantadine has potential as an antiviral agent against HAV.[9,16–18] We previously reported that amantadine has an inhibitory effect on HAV HM175 IRES-mediated translation.[7] However, the effects of amantadine on clinical isolates from hepatitis A patients were still unknown. Concentrations of 1–100 μg/mL of amantadine were non-cytotoxic to hepatocytes.[7] Huh-7 cells were treated with 100 μg/mL of amantadine or PBS 24 h after transfection of reporter plasmids. Forty-eight hours after transfection, dual-reporter assay was performed for the evaluation of cap-dependent and cap-independent translation initiation (Fig. 3). In A2 isolates, IRES activity in the presence of amantadine was 0.93-fold that in its absence. However, amantadine at 100 μg/mL was effective against all fulminant hepatitis-derived IRES

© 2010 The Japan Society of Hepatology

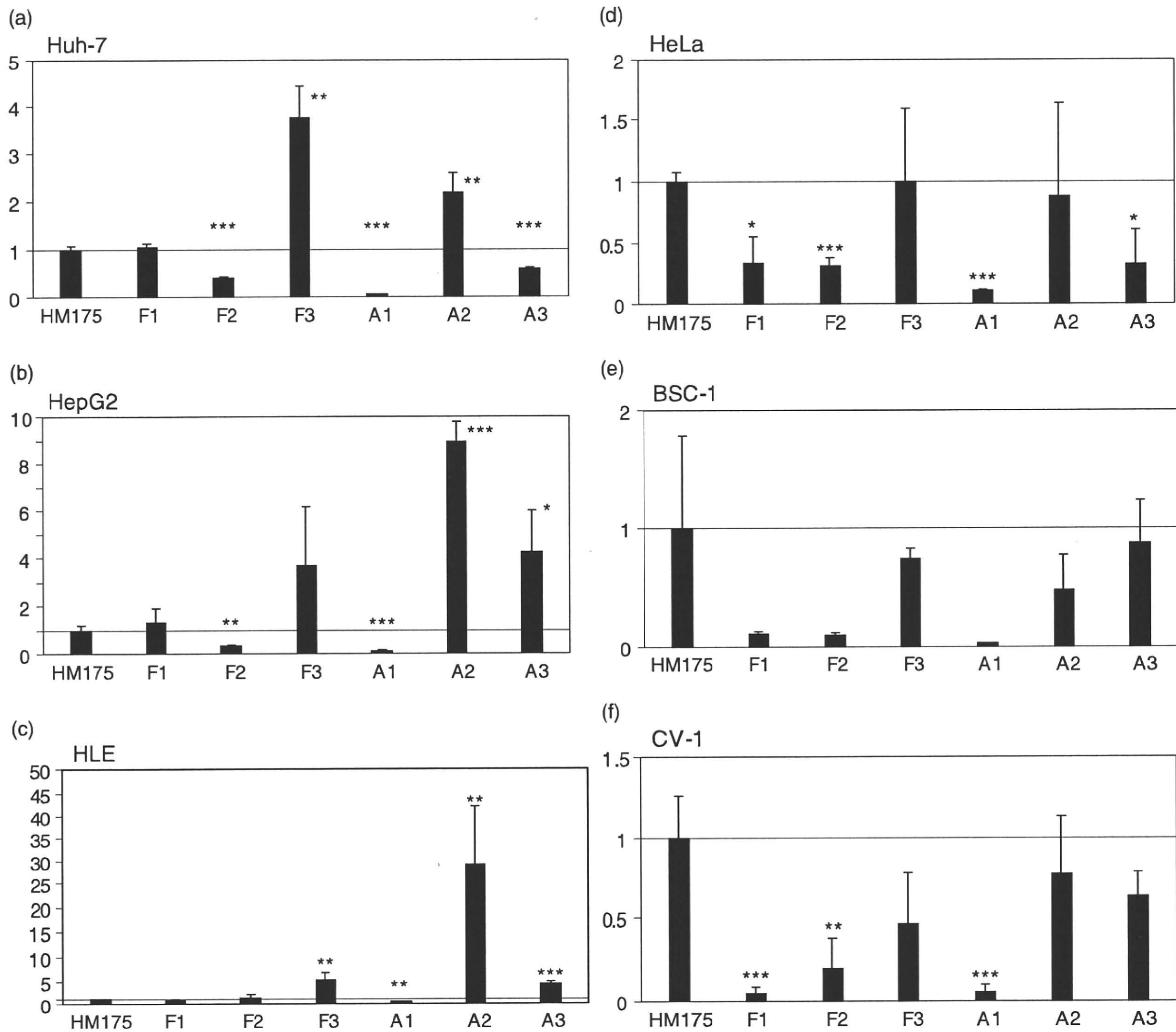

Figure 2 Clinical isolate-derived hepatitis A virus (HAV) internal ribosome entry site (IRES) activities in hepatocytes and non-hepatocytes. Plasmids of pSV40-HAV-IRES were transfected into hepatocytes: Huh-7 (a), HepG2 (b), HLE (c) and non-hepatocytes: HeLa (d), BSC-1 (e) and CV-1 (f). Forty-eight hours after transfection, dual-luciferase assays were performed. The IRES activities (firefly luciferase/*Renilla* luciferase) of pSV40-HAV-HM175-IRES were set at 1. F1, F2 and F3 and A1, A2 and A3 were derived from fulminant and acute self-limited hepatitis, respectively. $*P < 0.05$ vs HM-175; $**P < 0.01$ vs HM-175; $***P < 0.005$ vs HM-175.

activities (Fig. 3). Future studies will reveal whether fulminant hepatitis-derived HAV is more sensitive to amantadine than HAV from self-limited acute hepatitis.

Clinical manifestations among hepatitis A patients in the present study varied from self-limited acute hepatitis to severe fulminant hepatitis. It was reported that chronic liver diseases and older patients influenced the severity of hepatitis A, and the host immune response may vary at the cellular level in such patients. An absence of obvious correlation between genotypes and clinical status has been reported.[31] In this study, the fulminant hepatitis F3 clone had a relatively higher IRES activity in hepatocytes. We previously demonstrated that inhibition of IRES activities by small interfering RNA (siRNA) leads to the suppression of HAV viral replication in cell culture. Fujiwara *et al.*[32] reported that higher viral load in patients with fulminant and severe hepatitis A may be associated with the pathogenesis of disease severity. Further studies are needed.

© 2010 The Japan Society of Hepatology

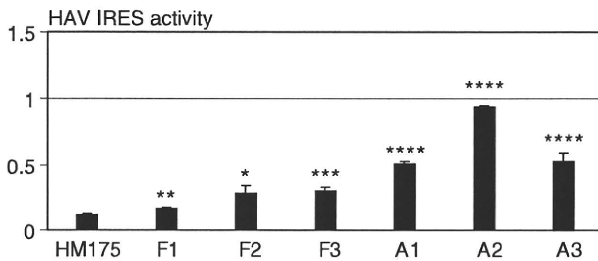

Figure 3 The effects of amantadine against clinical isolate-derived hepatitis A virus (HAV) internal ribosome entry site (IRES) activities. Twenty-four hours after transfection of reporter plasmids, Huh-7 cells were treated with 100 μg/mL of amantadine or phosphate buffered saline (PBS). The effects of amantadine against IRES activities (firefly luciferase/*Renilla* luciferase) are shown. Each value of IRES activity treated with PBS was set at 1. F1, F2 and F3 and A1, A2 and A3 were derived from fulminant and acute self-limited hepatitis, respectively. *$P < 0.05$ vs HM-175; **$P < 0.01$ vs HM-175; ***$P < 0.005$ vs HM-175; ****$P < 0.001$ vs HM-175.

It was reported that an antibiotic resistance titration assay (ARTA) is useful for HAV neutralization including virus-receptor interaction.[33,34] We also tested the effects of amantadine against HAV IRES of clinical isolates and confirmed the effects against fulminant hepatitis clones. Recently, it was reported that new acridines and hydrazones derived from cyclic β-diketone have stronger antiviral activities against HAV than amantadine.[9] Bicistronic reporter assay using clinical isolates may be another useful tool for testing antiviral activities like those of these drugs.

In conclusion, HAV IRES activities from clinical isolates vary in relation to different cell lines. For the development of antiviral agents against HAV, it seems important to investigate these effects on clinical isolates.

ACKNOWLEDGEMENTS

WE THANK DR S. U. Emerson for providing the plasmids, and Professor V. Gauss-Müller and Professor Omata for valuable discussion. This work was supported by grants for Scientific Research 15790338, 21590829, 21590828 and 21390225 from the Ministry of Education, Culture, Sports, Science and Technology, Japan (T. K., F. I. and O. Y.), a grant from the Ministry of Health, Labor and Welfare of Japan (O. Y.), and a grant from Chiba University Young Research-Oriented Faculty Member Development Program in Bioscience Areas (T. K.).

REFERENCES

1 Martin A, Lemon SM. Hepatitis A virus: from discovery to vaccines. *Hepatology* 2006; **43**: S164–S172.

2 Pinto RM, Aragones L, Costafreda MI, Ribes E, Bosch A. Codon usage and replicative strategies of hepatitis A virus. *Virus Res* 2007; **127**: 158–63.

3 Daniels D, Grytdal S, Wasley A. Centers for Disease Control and Prevention (CDC). Surveillance for acute viral hepatitis – United States, 2007. *MMWR Surveil Summ* 2009; **58**: 1–27.

4 Hernandez B, Hasson NK, Cheung R. Hepatitis C performance measure on hepatitis A and B vaccination: missed opportunities? *Am J Gastroenterol* 2009; **104**: 1961–7.

5 Kanda T, Kusov Y, Yokosuka O, Gauss-Müller V. Interference of hepatitis A virus replication by small interfering RNAs. *Biochem Biophys Res Commun* 2004; **318**: 341–5.

6 Kanda T, Zhang B, Kusov Y, Yokosuka O, Gauss-Müller V. Suppression of hepatitis A virus genome translation and replication by siRNAs targeting the internal ribosomal entry site. *Biochem Biophys Res Commun* 2005; **330**: 1217–23.

7 Kanda T, Yokosuka O, Imazeki F, Fujiwara K, Nagao K, Saisho H. Amantadine inhibits hepatitis A virus internal ribosomal entry site-mediated translation in human hepatoma cells. *Biochem Biophys Res Commun* 2005; **331**: 621–9.

8 Kusov Y, Kanda T, Palmenberg A, Sgro JY, Gauss-Müller V. Silencing of hepatitis A virus infection by small interfering RNAs. *J Virol* 2006; **80**: 5599–610.

9 El-Sabbagh OI, Rady HM. Synthesis of new acridines and hydrazones derived from cyclic b-diketone for cytotoxic and antiviral evaluation. *Eur J Med Chem* 2009; **44**: 3680–6.

10 Radha Krishna Y, Saraswat VA, Das K *et al.* Clinical features and predictors of outcome in acute hepatitis A and hepatitis E virus hepatitis on cirrhosis. *Liver Int* 2009; **29**: 392–8.

11 Schultz DE, Honda M, Whetter LE, Mcknight KL, Lemon SM. Mutations within the 5′ nontranslated RNA of cell culture-adapted hepatitis A virus which enhance cap-independent translation in cultured African green monkey kidney cells. *J Virol* 1996; **70**: 1041–9.

12 Brown EA, Zajac AJ, Lemon SM. *In vitro* characterization of an internal ribosomal entry site (IRES) present within the 5′ nontranslated region of hepatitis A virus RNA: comparison with the IRES of encephalomyocarditis virus. *J Virol* 1994; **68**: 1066–74.

13 Cohen JI, Ticehurst JR, Purcell RH, Buckler-White A, Baroudy BM. Complete nucleotide sequence of wild-type hepatitis A virus: comparison with different strains of hepatitis A virus and other picornaviruses. *J Virol* 1987; **61**: 50–9.

14 Lemon SM, Binn LN, Marchwicki R *et al. In vivo* replication and reversion to wild type of a neutralization-resistant antigenic variant of hepatitis A virus. *J Infect Dis* 1990; **161**: 7–13.

© 2010 The Japan Society of Hepatology

15 Totsuka A, Moritsugu Y. Hepatitis A virus protein. *Intervirology* 1999; **42**: 63–8.
16 Widell A, Hansson BG, Oberg B, Nordenfelt E. Influence of twenty potentially antiviral substances on *in vitro* multiplication of hepatitis A virus. *Antiviral Res* 1986; **6**: 103–12.
17 Crance JM, Biziagos E, Passagot J, van Cuyck-Gandre H, Deloince R. Inhibition of hepatitis A virus replication *in vitro* by antiviral compounds. *J Med Virol* 1990; **31**: 155–60.
18 Crance JM, Leveque F, Chousterman S, Jouan A, Trepo C, Deloince R. Antiviral activity of recombinant interferon-alpha on hepatitis A virus replication in human liver cells. *Antiviral Res* 1995; **28**: 69–80.
19 Emerson SU, Lewis M, Govindarajan S, Shapiro M, Moskal T, Purcell RH. cDNA clone of hepatitis A virus encoding a virulent virus: induction of viral hepatitis by direct nucleic acid transfection of Marmosets. *J Virol* 1992; **66**: 6649–54.
20 Fujiwara K, Yokosuka O, Ehata T *et al.* Frequent detection of hepatitis A viral RNA in serum during the early convalescent phase of acute hepatitis A. *Hepatology* 1997; **26**: 1634–9.
21 Fujiwara K, Yokosuka O, Ehata T *et al.* Association between severity of type A hepatitis and nucleotide variations in the 5′ non-translated region of hepatitis A virus RNA strains from fulminant hepatitis have fewer nucleotide substitutions. *Gut* 2002; **51**: 82–8.
22 Jansen RW, Siegl G, Lemon SM. Molecular epidemiology of human hepatitis A virus defined by an antigen-capture polymerase chain reaction method. *Proc Natl Acad Sci USA* 1990; **87**: 2867–71.
23 Robertson BH, Jansen RW, Khanna B *et al.* Genetic relatedness of hepatitis A virus strains recovered from different geographical regions. *J Gen Virol* 1992; **73**: 1365–77.
24 Kanda T, Yokosuka O, Ehata T *et al.* Detection of GBV-C RNA in patients with non-A-E fulminant hepatitis by reverse-transcription polymerase chain reaction. *Hepatology* 1997; **25**: 1261–5.
25 Brown EA, Day SP, Jansen RW, Lemon SM. The 5′ non-translated region of hepatitis virus RNA: Secondary structure and elements required for translation *in vitro*. *J Virol* 1991; **65**: 5828–38.
26 Glass MJ, Jia X-Y Summers DF. Identification of the hepatitis A virus internal ribosome entry site: *In vivo* and *in vitro* analysis of bicistronic RNAs containing the HAV 5′ non-coding region. *Virology* 1993; **193**: 842–52.
27 Gauss-Müller V, Kusov YY. Replication of hepatitis A virus replicon detected by genetic recombination in vivo. *J Gen Virol* 2002; **83**: 2183–92.
28 Ashida M, Hamada C. Molecular cloning of the hepatitis A virus receptor from a simian cell line. *J Gen Virol* 2002; **78**: 1565–9.
29 Kiernan RE, Marshall JA, Coulepis AG, Anderson DA, Gust ID. Cellular changes associated with persistent hepatitis A infection in vitro. *Arch Virol* 1987; **94**: 81–95.
30 Tsarev SA, Emerson SU, Balayan MS, Ticehurst J, Simian Purcell RH. Hepatitis A virus (HAV) strain AGM-27: comparison of genome structure and growth in cell culture with other HAV strains. *J Gen Virol* 1991; **72**: 1677–83.
31 Chitambar S, Joshi M, Lole K, Walimbe A, Vaidya S. Cocirculation of and coinfections with hepatitis A virus subgenotypes IIIA and IB in patients from Pune, western India. *Hepatol Res* 2007; **37**: 85–93.
32 Fujiwara K, Yokosuka O, Imazeki F, Saisho H, Miki M, Omata M. Do high levels of viral replication contribute to fulminant hepatitis A? *Liver Int* 2005; **25**: 194–5.
33 Tami C, Silberstein E, Manangeeswaran M *et al.* Immunoglobulin A (IgA) is a natural ligand of hepatitis A virus cellular receptor 1 (HAVCR1), and the association of IgA with HAVCR1 enhances virus-receptor interactions. *J Virol* 2007; **81**: 3437–46.
34 Konduru K, Virata-Theimer ML, Yu MY, Kaplan GG. A simple and rapid hepatitis A virus (HAV) titration assay based on antibiotic resistance of infected cells: evaluation of the HAV neutralization potency of human immune globulin preparations. *Virol J* 2008; **5**: 155.

© 2010 The Japan Society of Hepatology

Abdominal Imaging

© Springer Science+Business Media, LLC 2008
Published online: 9 January 2009

Abdom Imaging (2010) 35:8–14
DOI: 10.1007/s00261-008-9497-0

Long-term effect of endoscopic injection therapy with combined cyanoacrylate and ethanol for gastric fundal varices in relation to portal hemodynamics

Hitoshi Maruyama, Shinichiro Okabe, Takeshi Ishihara, Toshio Tsuyuguchi, Masaharu Yoshikawa, Shoichi Matsutani, Osamu Yokosuka

Department of Medicine and Clinical Oncology, Chiba University Graduate School of Medicine, 1-8-1, Inohana, Chuou-ku, Chiba 260-8670, Japan

Abstract

Background: The understanding on the long-term effect of endoscopic therapy for gastric fundal varices (FV) is still insufficient. The aim of this study was to evaluate the relationship between the long-term effect of the endoscopic injection therapy with combined cyanoacrylate (CA) and absolute ethanol (ET) for FV, and the portal hemodynamics.
Methods: The subjects of this retrospective study were ten consecutive cirrhotic patients with bleeding FV treated by endoscopic injection therapy with combined CA and ET. Percutaneous transhepatic portography was done after the completion of endoscopic treatment to assess portal hemodynamics.
Results: All the patients showed hemostasis by CA injection and complete obturation of FV by combined therapy of 5.6 ± 2.1 (3–9) times without any severe complications except for gastric ulcer in one case. Five patients had recurrence (50%), and three of them showed rebleeding (30%). The other five patients had no recurrence during a mean observation period of 5.58 years (1190–2735 days). Although recurrence did not correlate with portal venous pressure, it was significantly frequent in patients without advanced portosystemic collateral vessels (5/7, $P = 0.0384$) compared to patients with them (0/3).
Conclusions: Endoscopic injection therapy combining CA and ET may be effective for FV. Significant development of portosystemic collateral vessels would support long-term therapeutic effect after this treatment.

Correspondence to: Hitoshi Maruyama; *email:* maru-cib@umin.ac.jp

Key words: Endoscopic injection therapy—Gastric varices—Portal hypertension—Cyanoacrylate—Ethanol

Gastric fundal varices (FV) are a hemodynamic feature of major potential consequence in patients with portal hypertension [1]. The incidence and bleeding rate of FV are lower than those of esophageal varices (EV) [2, 3]. However, as bleeding from FV sometimes has a serious outcome, the control of FV bleeding is crucial in the management of patients with portal hypertension [4–6].

Endoscopy is an effective tool for attaining hemostasis in gastrointestinal bleeding cases, and intravariceal injection of cyanoacrylate (CA, Histoacryl; B. Braun, Melsungen AG, Germany) is a well-established technique for FV bleeding [7, 8]. However, CA injection alone does not always provide sufficient long-term protection against FV bleeding, as previous reports have shown a cumulative rebleeding rate from 18% to 28% per year [7–9]. Thus, the use of CA injection alone as a curative treatment for FV is controversial, and additional treatment may be required following CA injection.

Endoscopic injection therapy of CA combined with ethanolamine oleate (Grelan Pharmaceutical Co., Ltd., Tokyo, Japan) was reported by Akahoshi et al., but the outcome was less than satisfactory because of a cumulative nonbleeding rate of 52.7% at 5 years after the treatment, with a recurrent bleeding rate of 40% in a mean follow-up period of 28.1 months [10]. Meanwhile, absolute ethanol (ET) is also known as an effective sclerosant, and it was shown to be superior to ethanolamine oleate in a prospective randomized controlled trial in patients with EV [11]. On the basis of these backgrounds, we carried out

endoscopic injection therapy with combined CA and ET, a protocol that has not been performed elsewhere, in patients with bleeding FV. This study focused on the evaluation of the relationship between the long-term effect of the endoscopic injection therapy with combined CA and ET for bleeding FV and the portal hemodynamics as acquired by percutaneous transhepatic portography (PTP).

Materials and methods

Patients

Between 1994 and 1997, we had 22 consecutive patients with FV bleeding. Endoscopic CA injection was performed at the time of emergent endoscopic examination to achieve hemostasis. They received endoscopic ET injection as a consolidation therapy following the second CA injection given 5–7 days after the initial one. PTP was performed to evaluate the portal hemodynamics in 10 out of the 22 patients after completion of the endoscopic therapy, with the other 12 patients being ineligible for PTP, seven having moderate to severe ascites, and five severe impaired coagulation (prothorombin time <40%). Therefore, those 10 patients, six males and four females, aged 44–65 years (57.2 ± 6.7), were the subjects of this retrospective study (Table 1). All the patients were diagnosed as cirrhosis on the basis of imaging findings together with clinical symptoms and biochemistry findings. The cause of cirrhosis was viral in six patients (HCV 5, HBV 1), alcohol abuse in two, primary biliary cirrhosis in one and cryptogenic in one; the severity of liver damage as classified by the Child-Pugh scoring system was A in two, B in four, and C in four at the time of initial treatment [12]. Two patients had histologically proven hepatocellular carcinomas (HCC) that were controlled by non-surgical treatments, and none had thrombosis or tumor thrombosis in the portal vein according to both the ultrasound and contrast-enhanced computed tomography (CT). The end of the follow-up period was the date of final endoscopic observation without recurrence or the date of variceal bleeding or recurrence, with the mean period being 1547.2 ± 892.3 (210–2735) days. Informed written consent was obtained from all the patients, and the research was carried out in accordance with the Helsinki Declaration. This retrospective study was judged as having an appropriate design for publication by the ethics committee in our hospital.

Endoscopy

Endoscopic examination was performed using the Q200 or XQ200 system (Olympus Optical, Ltd., Tokyo, Japan). Endoscopic findings of FV and EV were classified according to the General Rules for Recording Endoscopic Findings set by the Japan Research Society for Portal Hypertension [13]: F1 (straight), F2 (winding), and F3 (nodule-beaded), corresponding to the grades of small, medium, and large, respectively. The grades of FV were F2 in six, and F3 in four, and six of the FV patients were accompanied by EV, F1 in four and F2 in two. According to the classification proposed by Sarin et al., there were gastroesophageal varices (GOV2) in six patients and isolated gastric varices (IGV1) in four patients [2].

All the patients were bleeders with symptoms of hematemesis or melena, and bleeding was confirmed by emergent endoscopic examination. Eight patients had primary FV, and two patients had secondary FV that developed after the endoscopic treatment for EV.

Endoscopic treatment

Puncture for FV was performed using a 23G injection needle (Sumitomo, Tokyo, Japan) via the biopsy channel. Before and after every injection, positive or negative appearance of withdrawn blood in the injection needle was checked by suction to ascertain whether the puncture was

Table 1. Clinical profile of the subjects

Case	Age/Sex[a]	PVP[b]	CV[c]	C/P[d]	Outcome[e]	Period[f]
1	63/M	288	PUV, LGV	<1	+/+	1460
2	64/M	270	LGV	<1	+/+	545
3	60/M	340	PUV	<1	+/−	2373
4	55/F	275	SGV	<1	+/−	700
5	50/F	310	LGV, PGV	<1	−/−	2735
6	55/M	226	IMV	<1	−/−	2606
7	61/M	300	LGV, SGV	<1	+/+	210
8	44/F	320	LGV	1≤	−/−	1190
9	55/M	300	LGV	1≤	−/−	1552
10	65/F	350	LGV	1≤	−/−	2101

[a]M, male; F, female
[b]Portal venous pressure (mmH_2O)
[c]Collateral vessel
[d]Ratio of diameter of main collateral vessel to portal trunk on the portogram
[e]Recurrence/rebleeding, +, positive; −, negative
[f]The period of observation from the end of treatment to the final endoscopic observation without recurrence, or the time of recurrence or rebleeding (days)

intravariceal or extravariceal. CA stock solution was injected at a dose of 0.5 mL per intravariceal puncture, and the injection was repeated around the bleeding point until hemostasis was obtained at the initial session. Five to seven days after the initial session, a second session was done for CA injection, which was repeated at every intravariceal puncture over the whole variceal lesion. Following these two sessions, ET injection was performed for both extravariceal and intravariceal punctures at a dose of 1 mL per puncture, with the total volume of ET being no more than 0.3 mL per body weight (kg) in one session. The sessions of ET injections were repeated once a week until obtaining the optimum therapeutic effect according to endoscopic ultrasonography (EUS). All endoscopic treatments were performed by H.M. without using fluoroscopy; blood pressure, heart rate and degree of oxygen saturation were continuously monitored during the procedure. Bilirubin, albumin, prothrombin time, AST, ALT, WBC, RBC, and hemoglobin were checked on the following day of each treatment.

EUS

EUS was performed using the Sonoprobe System SP701 (Fujinon, Tokyo, Japan) with a 15 MHz radial scanning device. EUS was done before every session of endoscopic injection therapy except for the initial emergency examination to evaluate the therapeutic effect of the previous session. The operators of EUS were H.M. for all the patients, and the recorded images on VHS videotapes were reviewed by T.I. and T.T. The absence of intramural vessels and/or presence of intramural vessels with blood clot formation were considered to be evidence of a favorable effect.

PTP

All the patients underwent portal vein catheterization by US-guided procedure after completion of the endoscopic therapy [14], and portal venous pressure (PVP) was measured at the middle part of the portal trunk. Then, a portogram was taken during the injection of contrast medium (35 mL, 7 mL/s, Omnipaque300, Daiichi, Tokyo, Japan) into the splenic hilum, and portal trunk and extrahepatic collateral vessels were evaluated. The diameter of the most significant collateral vessel was compared with that of the portal trunk on the image, and the ratio of the collateral vessel diameter (C) to the portal trunk diameter (P) was defined as C/P in this study. PTP was performed by H.M. and M.Y., and the X-ray images were reviewed by S.M.

Contrast-enhanced CT

Contrast-enhanced CT was performed after the completion of all endoscopic treatment sessions evaluated by EUS, and before PTP. The images were taken using Vertex 3000 Formula (GE Yokokawa Medical Systems, Hino, Japan) after the injection of 100 mL of iodinated contrast material (Omnipaque300, Daiichi, Tokyo, Japan) at 3.0 mL/s by a mechanical power injector. Scanning was done with a 30-s delay between contrast material administration and the start of scanning for the hepatic artery-dominant phase, an 80-s delay for the portal vein-dominant phase, and a 180-s delay for the equilibrium phase. The images were read by O.S. to evaluate the therapeutic effect on FV.

Statistical analysis

All data were expressed as mean ± SD or percentage. Statistical significance was determined using the chi-square test and significance was taken at $P < 0.05$. The statistical analysis was performed using the SPSS package (version 13.0 J; SPSS Inc., Chicago, Illinois, USA).

Results

Effect of endoscopic injection therapy for FV

CA injection achieved hemostasis in all the patients (10/10). The number of sessions of endoscopic injection therapy with combined CA and ET was 5.6 ± 2.1 (3–9), resulting in a complete obturation effect which was confirmed by both EUS and contrast-enhanced CT findings in all the patients. As for short-term complications, six patients had abdominal pain after the ET injection, three had mild fever for 2 days following the treatment, and one patient had a severe gastric ulcer requiring three months to heal. None had remarkable post-treatment changes in blood tests, or in vital signs during and after the treatment. As a long-term complication, three patients had aggravation of EV, found 6, 12, and 12 months, respectively, after the treatment.

PTP findings at the end of endoscopic injection therapy

PVP ranged from 226 to 350 (297.9 ± 36.1) mmH_2O. Collateral vessels demonstrated on the portograms were left gastric vein (LGV) in four patients, paraumbilical vein (PUV) in one patient, short gastric vein (SGV) in one patient, inferior mesenteric vein (IMV) in one patient, both PUV and LGV in one patient, both LGV and posterior gastric vein (PGV) in one patient, and both LGV and SGV in one patient. The portograms did not demonstrate FV because they were completely embolized, and none of the collateral vessels were associated with FV. Seven patients had C/P less than 1.0 and three patients had C/P equal to or more than 1.0. No complications were noted during and after the PTP procedures.

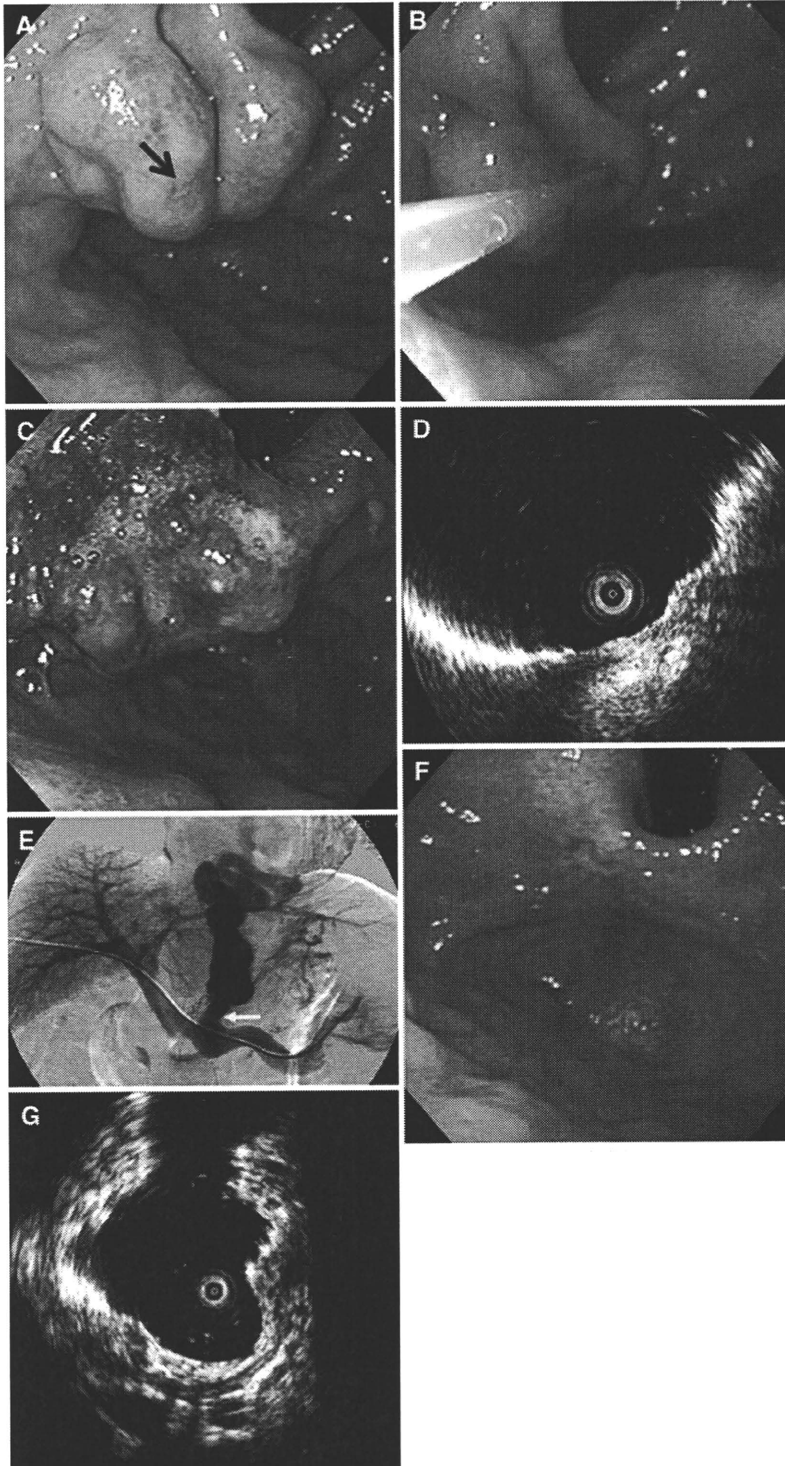

Fig. 1. 65-year-old female (case 10). **A** Before treatment. Endoscopy showed large-grade FV with bleeding point (*arrow*). **B** Puncture for FV. Withdrawn blood in the injection needle by suction indicated intravariceal puncture. **C** After seven endoscopic treatments. Protruding structure of FV disappeared. **D** EUS findings after seven endoscopic treatments. EUS showed no vessel structure at the treated area. **E** Portogram. Portogram at the end of endoscopic treatment showed marked development of collateral vessel (*arrow*), the diameter of which was not less than that of the portal trunk (C/P ≥ 1.0). PVP was 350 mmH_2O. **F** Endoscopy 5 years after treatment. Endoscopy showed no recurrence in the stomach. **G** EUS 5 years after treatment. EUS showed no vessel structure at the treated area.

Relationship between portal hemodynamics and recurrence of FV

Five patients (5/10, 50%) did not have any recurrent findings during the mean course of 5.58 years (1190–2735 days, Fig. 1), while the other five (5/10, 50%) had recurrence, and three of them (3/10, 30%) showed rebleeding (Fig. 2). The recurrence of FV was found in five out of the seven patients with C/P less than 1.0, while in none of the three with C/P equal to or more than 1.0 ($P = 0.0384$) in the clinical course of 1190, 1552, and

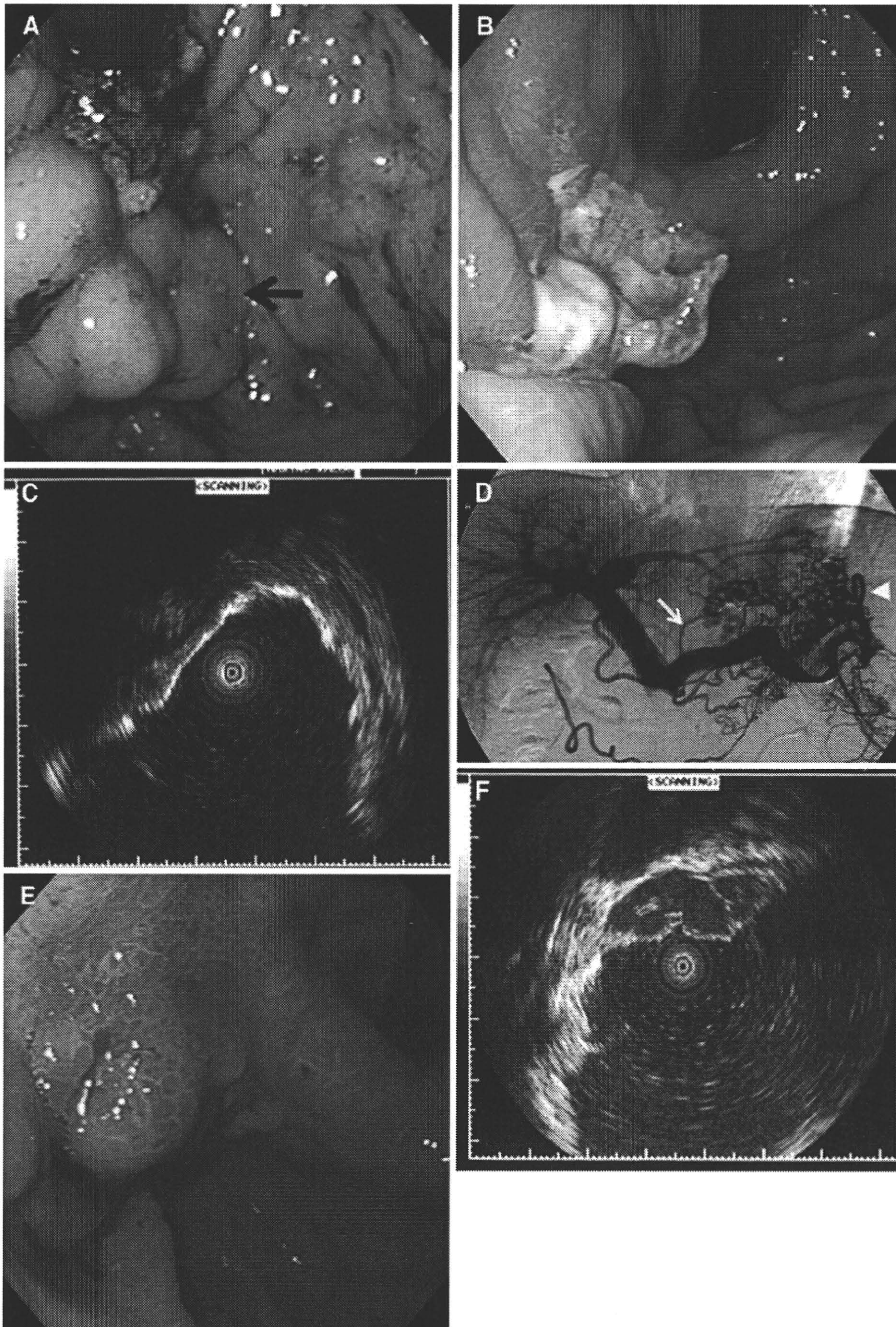

Fig. 2. 61-year-old male (case 7). **A** Before treatment. Endoscopy showed large-grade FV with bleeding point (*arrow*). **B** After 5 endoscopic treatments. Protruding structure of the varices disappeared. **C** EUS findings. EUS showed no vessel structure at the treated area. **D** Portogram. Portogram at the end of endoscopic treatment showed collateral vessels, LGV (arrow) and SGV (*arrow head*), the diameters of which were apparently less than that of the portal trunk (C/P < 1.0). PVP was 300 mmH_2O. **E** Endoscopy 210 days after treatment. This case had melena, and emergent endoscopy showed a protruding appearance that was recurrent finding of FV. **F** EUS 210 days after treatment. EUS showed apparent vessel structure at the protruding lesion.

2101 days, respectively. There was no relationship between recurrence of FV and PVP, Child-Pugh score and presence of esophageal varices.

Discussion

This study may be the first to report on the clinical evaluation of the therapeutic effect on bleedings FV by endoscopic injection therapy with combined CA and ET, a simple and safe procedure. The initial step of our technique was the intravariceal injection of CA stock solution without using fluoroscopy, and bleeding from FV were completely controlled in all the cases. Some previous studies, however, reportedly used CA together with lipiodol as a mixture [8, 15]. In fact, fluoroscopy may be useful for checking the appearance of the injected agent, which has a potential risk of migration into systemic circulation through the inferior vena cava via the outflow route [16]. However, this monitoring might not be necessarily required because the trajectory of the in-

jected agent is uncontrollable. Furthermore, the diluted mixture of CA and lipiodol might have an easy-flowing property compared with the CA stock solution. In any event, a minimal-dose injection of CA may be essential for attaining hemostasis in spite of the different ways of the clinical usage of CA.

The next particular feature of our technique was the second step, that is, ET injection at both the intra- and extra-variceal punctures as a consolidation therapy. According to the report by Sarin et al., ET is easy to use for rapid injection and has an economically favorable aspect [11]. As the EUS findings of FV after the two sessions of CA injection showed incomplete therapeutic effect in all the cases, additional consolidation therapy was considered appropriate. However, the mean number of treatment sessions was 5.6 times, not an inconsiderable number, and the rebleeding rate was 30%, fairly similar to the results in the previous reports [7–10]. Therefore, there may be still room for improvement in the technical aspects of ET injection. Meanwhile, five patients had a clinical course of more than 5 years without any recurrence, and three of them had advanced collateral vessels. The most suitable selection may provide quite sufficient long-term efficacy by the endoscopic treatment in patients with bleeding FV.

The relationship between the pathophysiology of FV and PVP is still unclear. Watanabe et al. found that PVP was significantly lower in patients with FV (240 ± 37 mmH_2O) than in patients with EV (326 ± 66 mmH_2O), and PVP in patients with FV decreased according to the development of gastrorenal shunt [17]. Another study reported that PVP in patients with large FV was lower than that in patients with EV, probably because of the development of gastrorenal shunts [18]. According to the report by Tripathi et al., FV bleeding accounts for many cases in bleeding patients with a portal pressure gradient of ≤ 12 mmHg pre-transjugular intrahepatic portosystemic stent shunt [19]. They also added that, though it is not clear why patients bleed at a portal pressure of < 12 mmHg, other factors such as the presence of red spots, gastritis, and variceal size may be important. These results suggest that high PVP is not always a cause for FV bleeding, and might support our finding that PVP at the end of FV treatments was not a significant factor for FV recurrence. Meanwhile, as the grade of collateral vessels on portograms was closely related to FV recurrence, development of collateral vessels may play a role as a suppression factor for FV recurrence.

Balloon-occluded retrograde transvenous obliteration (B-RTO) is a quite effective method for the embolization of FV [20]. However, this technique requires the presence of gastrorenal shunt or gastrocaval shunt as drainage route from FV and radiation exposure [21, 22]. Furthermore, as the aggravation of EV is a frequent occurrence after B-RTO [23], its application may not always be the best choice for the treatment of FV.

There were some limitations to our study. The first is that all the patients underwent PTP only once after completion of the endoscopic treatment. As their initial clinical presentation was FV bleeding and a series of endoscopic treatments was required from the beginning, application of PTP before treatment was considered to be inappropriate for them. Therefore, PVP might not represent the pathophysiology of the pre-treatment condition in each patient, and the influence of a series of endoscopic treatments on the development of these collateral vessels is uncertain. Although PTP has the advantage of a reliable assessment method due to direct opacification, the repeated evaluation of portal hemodynamics by the other non-invasive method might be preferable during the treatment course. Another limitation is that our study was done with a small number of patients in a retrospective manner. Subsequent clinical examinations with large numbers of patients may be necessary to confirm our results.

In conclusion, endoscopic injection therapy with combined CA and ET may be an effective treatment method for patients with bleeding FV. Development of portosystemic collateral vessels would support long-term therapeutic effect after this treatment.

References

1. Bosch J, Abraldes JG, Groszmann R (2003) Current management of portal hypertension. J Hepatol 38:S54–S68
2. Sarin SK, Lahoti D, Saxena SP, et al. (1992) Prevalence, classification and natural history of gastric varices: a long-term follow-up study in 568 portal hypertension patients. Hepatology 16:1343–1349
3. Kim T, Shijo H, Kokawa H, et al. (1997) Risk factors for hemorrhage from gastric fundal varices. Hepatology 25:307–312
4. Ryan BM, Stockbrugger RW, Ryan JM (2004) A pathophysiologic, gastroenterologic, and radiologic approach to the management of gastric varices. Gastroenterology 126:1175–1189
5. Lubel JS, Angus PW (2005) Modern management of portal hypertension. Intern Med J 35:45–49
6. Williams SG, Westaby D (1994) Management of variceal haemorrhage. BMJ 308:1213–1217
7. Greenwald BD, Caldwell SH, Hespenheide EE, et al. (2003) N-2-butyl-cyanoacrylate for bleeding gastric varices: a United States pilot study and cost analysis. Am J Gastroenterol 98:1982–1988
8. Oho K, Iwao T, Sumino M, Toyonaga A, Tanikawa K (1995) Ethanolamine oleate versus butyl cyanoacrylate for bleeding gastric varices: a nonrandomized study. Endoscopy 27:349–354
9. Sarin SK, Jain AK, Jain M, Gupta R (2002) A randomized controlled trial of cyanoacrylate versus alcohol injection in patients with isolated fundic varices. Am J Gastroenterol 97:1010–1015
10. Akahoshi T, Hashizume M, Shimabukuro R, et al. (2002) Long-term results of endoscopic histoacryl injection sclerotherapy for gastric variceal bleeding. A 10-year experience. Surgery 131:S176–S181
11. Sarin SK, Mishra SP, Sachdev GK, et al. (1988) Ethanolamine oleate versus absolute alcohol as a variceal sclerosant: a prospective randomized controlled trial. Am J Gastroenterol 83:526–530
12. Pugh RN, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R (1973) Transection of the esophagus for bleeding esophageal varices. Br J Surg 60:646–649
13. The general rules for study of portal hypertension (2004) The Japan society for portal hypertension, 2nd edn. pp. 37–50
14. Kimura K, Tsuchiya Y, Ohto M, et al. (1981) Single-puncture method for percutaneous transhepatic portography using a thin needle. Radiology 139:748–749

15. Ramond MJ, Valla D, Mosnier JF, et al. (1989) Successful endoscopic obliteration of gastric varices with butyl cyanoacrylate. Hepatology 10:488–493
16. Irisawa A, Obara K, Sato Y, et al. (2000) Adherence of cyanoacrylate which leaked from gastric varices to the left renal vein during endoscopic injection sclerotherapy: a histopathologic study. Endoscopy 32:804–806
17. Watanabe K, Kimura K, Matsutani S, Ohto M, Okuda K (1988) Portal hemodynamics in patients with gastric varices: a study in 230 patients with esophageal and/or gastric varices using portal vein catheterization. Gastroenterology 95:434–440
18. Chao Y, Lin HC, Lee FY, et al. (1993) Hepatic hemodynamic features in patients with esophageal or gastric varices. J Hepatol 19:85–89
19. Tripathi D, Therapondos G, Jackson E, Redhead DN, Hayes PC (2002) The role of the transjugular intrahepatic portosystemic stent shunt (TIPSS) in the management of bleeding gastric varices: clinical and haemodynamic correlations. Gut 51:270–274
20. Kanagawa H, Mima S, Kouyama H, et al. (1996) Treatment of gastric fundal varices by balloon-occluded retrograde transvenous obliteration. J Gastroenterol Hepatol 11:51–58
21. Koito K, Namieno T, Nagakawa T, Morita K (1996) Balloon-occluded retrograde transvenous obliteration for gastric varices with gastrorenal or gastrocaval collaterals. AJR Am J Roentgenol 167:1317–1320
22. Hirota S, Matsumoto S, Tomita M, Sakom M, Kono M (1999) Retrograde transvenous obliteration of gastric varices. Radiology 211:349–356
23. Ninoi T, Nishida N, Kaminou T, et al. (2005) Balloon-occluded retrograde transvenous obliteration of gastric varices with gastrorenal shunt: long-term follow-up in 78 patients. AJR Am J Roentgenol 184:1340–1346

Scandinavian Journal of Gastroenterology, 2010; 45: 243–249

informa
healthcare

ORIGINAL ARTICLE

Risk of Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Virus Infection

KENICHI ITO[1], MAKOTO ARAI[1], FUMIO IMAZEKI[1], YUTAKA YONEMITSU[1], DAN BEKKU[1], TATSUO KANDA[1], KEIICHI FUJIWARA[1], KENICHI FUKAI[1], KENICHI SATO[2], SAKAE ITOGA[2], FUMIO NOMURA[2] & OSAMU YOKOSUKA[1]

[1]*Departments of Medicine and Clinical Oncology, and* [2]*Departments of Molecular Diagnosis, Graduate School of Medicine, Chiba University, Chiba, Japan*

Abstract

Objective. To determine the risk factors for the occurrence of hepatocellular carcinoma (HCC) in patients with hepatitis B virus (HBV) infection. ***Material and methods.*** A total of 620 patients who tested positive for hepatitis B surface antigen and were referred to Chiba University Hospital between February 1985 and March 2008 were included in the study and the following characteristics were analyzed: age, gender, status of hepatitis B e antigen, alanine aminotransferase level, HBV DNA level, and number of platelets (PLTs). ***Results.*** HCC was detected in 30 cases during the follow-up period (5.4 ± 5.1 years). Multivariate analysis revealed that age >40 years [compared with patients aged <40 years; odds ratio (OR) = 4.28; 95% confidence interval (CI) = 1.68–10.9] and PLT level <206,000/μl (compared with patients with a higher PLT level; OR = 8.50; 95% CI = 1.98–36.2) were predictive factors for HCC occurrence. In patients aged >40 years, the HBV DNA level (compared with <5.0 log copies/ml; OR = 4.22, 95% CI = 1.13–15.8) and PLT level (compared with patients with >196,000/μl PLTs; OR = 15.6, 95% CI = 2.06–118.3) were predictive factors for HCC occurrence. ***Conclusions.*** Advanced age and low PLT level were risk factors for HCC occurrence in patients with HBV infection. In patients aged >40 years, viral load was also a risk factor for HCC.

Key Words: *Hepatitis B virus, hepatocellular carcinoma*

Introduction

The clinical course of patients with hepatitis B virus (HBV) infection varies considerably [1]. Therefore, long-term follow-up studies of patients with HBV infection are quite complex and difficult. In most of the patients, the disease is either non-progressive or shows a slow progression and is usually accompanied by the loss of serum HBV DNA after seroconversion of hepatitis B e antigen (HBeAg) [2]. Some patients show continuous elevation of the alanine aminotransferase (ALT) level, which leads to cirrhosis [3]. HBV infection is also associated with an increased risk of developing hepatocellular carcinoma (HCC), which is one of the most common human cancers and causes of death. Although previous studies have attempted to determine factors influencing the prognosis of patients with HBV infection, the key factors remain to be identified. Recent studies have indicated that the serum level of HBV DNA correlates with the progression of liver diseases [1,4–6]. However, viral load alone cannot predict the occurrence of HCC in the future [7]. In this study, multivariate analyses of the risk factors for HCC occurrence were performed for data obtained from 620 patients with HBV infection who were referred to a single institute in Japan.

Correspondence: Makoto Arai, MD, Department of Medicine and Clinical Oncology (K1), Graduate School of Medicine, Chiba University, Inohana 1-8-1, Chiba-City, 260-8670, Japan. Tel: +81-43-226-2083. Fax: +81-43-226-2088. E-mail: araim-cib@umin.ac.jp

(Received 15 September 2009; accepted 27 October 2009)

ISSN 0036-5521 print/ISSN 1502-7708 online © 2010 Informa UK Ltd.
DOI: 10.3109/00365520903450113

Material and methods

Patients

This was a retrospective analysis. The study was approved by the ethical committee of Chiba University and written informed consent was obtained from all the patients. Of the hepatitis B surface antigen (HBsAg)-positive carriers ($n = 676$) who were referred to Chiba University Hospital between February 1985 and March 2008, those who tested positive for hepatitis C virus (HCV) antibody (anti-HCV) or had autoimmune liver disease and those who had another potential cause of chronic liver disease were excluded. The characteristics of the excluded HBsAg-positive carriers were as follows: anti-HCV positivity in 12, autoimmune liver disease in four and primary biliary cirrhosis in one. Five patients who had previously received lamivudine treatment were also excluded. Thirty-nine patients consulted a physician only once and were excluded from further analysis. Thus, a total of 620 patients were further analyzed. Serum samples were collected during diagnosis and stored at −20°C until analysis.

Serologic markers, HBV DNA quantitative assay, and genotyping

HBsAg, HBeAg, and anti-HBe levels were determined by enzyme-linked immunosorbent assay (ELISA; Abbott Laboratories, Chicago, IL) and anti-HCV was also measured by ELISA (Ortho Diagnostics, Tokyo, Japan). Serum HBV DNA levels were quantified by polymerase chain reaction (PCR) assay (Amplicor HBV Monitor; Roche Diagnostics, Basle, Switzerland); the linear range of this assay was 2.6–7.6 log copies (LC)/ml. The six major genotypes of HBV (A–F) were determined by EIA (HBV Genotype EIA; Institute of Immunology Co., Ltd., Tokyo, Japan). Aspartate aminotransferase (AST), ALT, and the number of platelets were determined and the aminotransferase to platelet ratio index (APRI) was calculated [8].

Statistical analysis

The baseline data are presented as mean ± SD. The difference in the values of clinical parameters between the two groups was analyzed by unpaired t-test, Welch's t-test, and chi-square test. The Cox proportional hazards model was used to identify factors predictive of HCC occurrence using the SPSS version 16.1 software package (SPSS Inc., Chicago, IL).

Results

Demographic characteristics of HCC and control patients

None of the study participants had HCC at entry. In total, 30 incident HCC cases (HCC group) occurred during the follow-up period. During the follow-up period, most of the patients were re-evaluated at least once a year for liver function and detection of HCC. Screening for detection of HCC was performed on the basis of typical findings of abdominal ultrasonography, dynamic CT, angiography, and/or MRI. For all patients suspected of having HCC by imaging analysis, the diagnosis of HCC was confirmed by pathological analysis. If the patient had HCC or was being treated with an antiviral drug (lamivudine or entecavir), we terminated the follow-up. At baseline, significant differences were observed in age, gender, status of HBeAg, ALT and HBV DNA levels, number of platelets (PLTs), and APRI between the HCC (n = 30) and control (n = 590) groups (Table I). The 590 patients in whom HCC was not detected during the follow-up period constituted the control group. The average follow-up period was 5.1 ± 4.1 and 5.4 ± 5.2 years in the HCC and control groups, respectively, and this difference was not significant.

Patients with HBV

The differences in age, sex, PLT and ALT levels, status of HBeAg, and HBV DNA level between the HCC and control groups were investigated. We defined threshold levels as age 40 years, HBV DNA 5.3 LC/ml, ALT 72.9 IU/l, and PLTs 206,000/μl according to the average data of all patients. Univariate analysis revealed that age, number of PLTs, and HBV DNA level at baseline were predictive factors for HCC occurrence. Multivariate analysis revealed that age >40 years [compared with patients aged <40 years; odds ratio (OR) = 4.28; 95% confidence interval (CI) = 1.68–10.9] and PLT level <206,000/μl (compared with patients with a higher PLT level; OR = 8.50, 95% CI = 1.98–36.2) were predictive factors for HCC occurrence (Table II). Thus, these analyses revealed that age and PLT level were the most important factors influencing future occurrence of HCC. Kaplan–Meier curves were constructed for age ($P < 0.0001$; log-rank test; Figure 1a), PLT level ($P < 0.0001$; log-rank test; Figure 1b), and HBV DNA (P = NS; log-rank test; Figure 1c). Next, we categorized the HBV patients into two subgroups according to the thresholds of age and PLT level based on the average data, and performed further analysis. Because there was only one HCC patient aged <40 years and

Table I. Characteristics of study subjects and their association with HCC.

Parameter	Group: Total	HCC	Controls	P
No. of patients	620	30	590	
Gender; *n* (%)				<0.001[a]
Male	364 (59)	20 (67)	344 (58)	
Female	256 (41)	10 (33)	246 (42)	
Age (years); mean ± SD	40.0 ± 14.2	50.0 ± 11.6	40.0 ± 14.2	<0.001[b]
HBeAg status; *n* (%)				<0.001[a]
Positive	269 (43)	17 (57)	252 (43)	
Negative	351 (57)	13 (43)	338 (57)	
HBV DNA (LC/mL); mean ± SD	5.3 ± 2.0	6.4 ± 1.3	5.3 ± 2.0	0.002[b]
ALT (IU/l); mean ± SD	72.9 ± 89.3	105.0 ± 129.3	71.0 ± 86.6	0.041[c]
PLTs (/μl); mean ± SD	206,000 ± 66,000	130,000 ± 51,160	210,000 ± 64,410	<0.001[c]
APRI >0.5; *n* (%)	294 (47.4)	27 (90)	267 (45.3)	<0.001[a]
Interval between two consecutive visits (years); mean ± SD	5.4 ± 5.1	5.1 ± 4.1	5.4 ± 5.2	NS[c]
Genotype A/B/C/D/not determined; *n*	7/38/333/0/242	1/0/24/0/5	6/38/309/0/237	NS[a]

[a]Chi-square test.
[b]Welch's *t*-test.
[c]Unpaired *t*-test.

only two cases had a PLT level >206,000/μl, we did not analyze these groups.

Analysis of the subgroup of HBV patients aged >40 years

HCC was detected in 29 patients in the group aged >40 years (*n* = 372). Significant differences were observed in the status of HBeAg, HBV DNA, and PLT levels at baseline between the HCC (*n* = 29) and control groups (*n* = 343). The average follow-up period was 5.1 ± 4.1 and 5.0 ± 4.7 years in the HCC and control groups, respectively, and this difference was not significant. We defined thresholds as age 49 years, HBV DNA 5.0 LC/ml, ALT 66.0 IU/l, and PLTs 196,000/μl, according to the average data for the patients aged >40 years. The risk factors for HCC occurrence in patients aged >40 years were analyzed by Cox regression analysis. Univariate analysis revealed that ALT, PLT, and HBV DNA levels at baseline were predictive factors for HCC occurrence. Multivariate analysis revealed that the HBV DNA

Table II. Multivariate analysis of risk factors associated with HCC in patients with HBV infection.

	All patients[a]		Patients aged >40 years[b]		Patients with PLTs <206,000 /μl[c]	
Risk factor	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Age	4.28 (1.68–10.9)	0.002	2.16 (0.88–5.29)	NS	1.75 (0.71–4.34)	NS
Male gender	1.48 (0.67–3.26)	NS	2.25 (0.86–5.90)	NS	1.43 (0.61–3.35)	NS
HBeAg-positive	1.34 (0.59–3.06)	NS	0.98 (0.41–2.33)	NS	1.06 (0.45–2.51)	NS
HBV-DNA	1.59 (0.62–4.13)	NS	4.22 (1.13–15.8)	0.032	1.20 (0.49–2.94)	NS
ALT	0.86 (0.40–1.87)	NS	1.44 (0.61–3.44)	NS	0.923 (0.40–2.11)	NS
PLTs	8.50 (1.98–36.2)	0.004	15.6 (2.06–118.3)	0.008	4.49 (1.62–12.5)	0.004

[a]The thresholds of age, HBV-DNA, ALT, and PLTs were defined as 40 years, 5.3 LC/ml, 72.9 IU/l, and 206,000 /μl, respectively.
[b]The thresholds of age, HBV-DNA, ALT, and PLTs were defined as 49 years, 5.0 LC /ml, 66.0 IU/l, and 196,000 /μl, respectively.
[c]The thresholds of age, HBV-DNA, ALT, and PLTs were defined as 42 years, 5.8 LC /ml, 84 IU/l, and 159,000 /μl, respectively.
HR = hazard ratio.

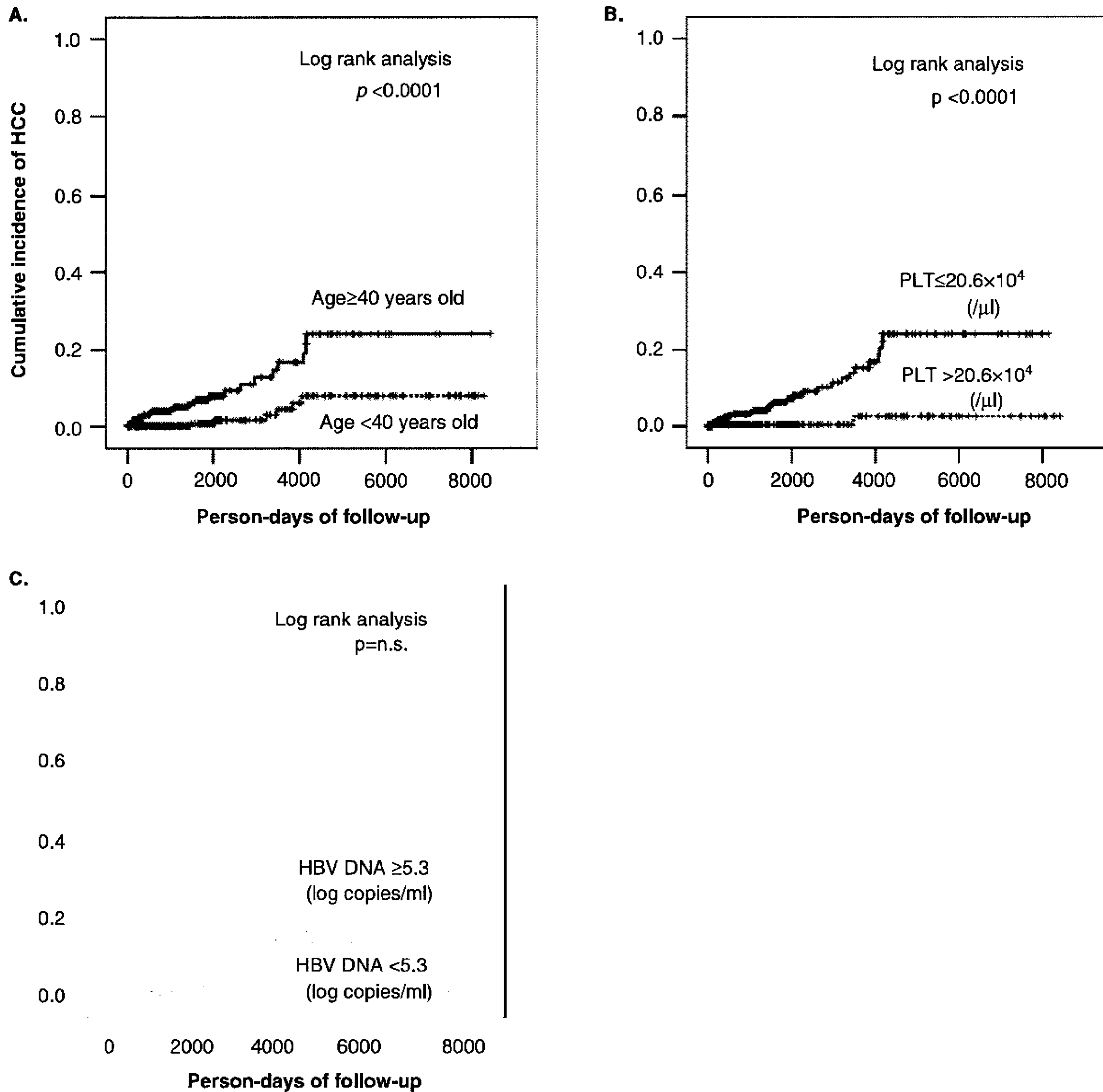

Figure 1. Cumulative occurrence of HCC based on (a) number of PLTs, (b) age, and (c) HBV DNA level. Thresholds for age, number of PLTs, and HBV DNA level were defined according to the average data for all patients. Dotted lines indicate the control group (high number of PLTs, younger age, and low HBV DNA level).

level (compared with < 5.0 LC/ml; OR = 4.22; 95% CI = 1.13–15.8) and PLT level (compared with $> 196{,}000/\mu l$; OR = 15.6; 95% CI = 2.06–118.3) were predictive factors for HCC occurrence (Table II). Kaplan–Meier curves were constructed for HBV DNA ($P = 0.001$; log-rank test; Figure 2).

Analysis of the subgroup of HBV patients with PLTs $< 206{,}000/\mu l$

HCC was detected in 28 patients in the group with PLTs $< 206{,}000/\mu l$ ($n = 329$). The risk factors for HCC occurrence in the group with $< 206{,}000/\mu l$ PLTs were analyzed by Cox regression analysis. Univariate analysis revealed that age and PLT level at baseline were predictive factors for HCC occurrence. Multivariate analysis revealed that PLT level (compared with patients with $> 159{,}000/\mu l$; OR = 4.49; 95% CI = 1.62–12.5) was the only predictive factor for HCC occurrence (Table II).

Discussion

In Japan, HBV infection is one of the most important factors determining HCC occurrence [9]. Moreover, HCC is one of the most important determinants for

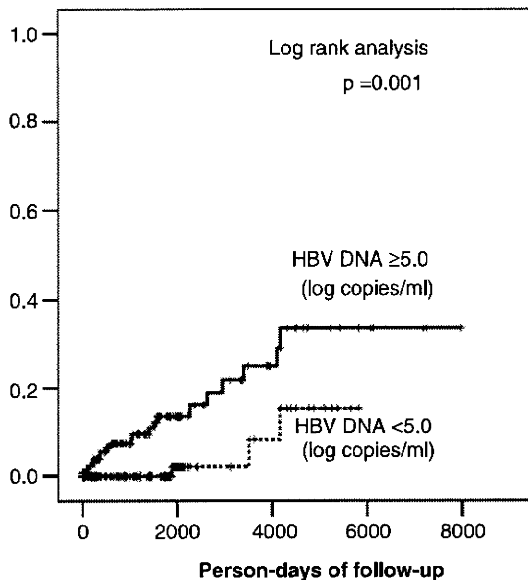

Figure 2. Cumulative occurrence of HCC based on the HBV DNA level in patients aged > 40 years. The threshold for the HBV DNA level was defined according to the average data for the patients aged > 40 years. A significant difference was observed by log-rank test. The dotted line indicates the control group (low HBV DNA level).

the prognosis of patients with HBV infection. In previous studies, factors associated with an increased risk of HCC among people with chronic HBV infection included demographic characteristics, lifestyle, and environmental, viral and clinical factors. Among these, male gender, older age, HBV genotype, cirrhosis, elevated ALT, and high viral load were found to be factors associated with HCC [6,10–19]. We focused on clinical factors which may be tested easily and for which tests are available all over the world. This report clarifies the relative risk for HCC in all patients with HBV who were referred to a single institute in Japan and provides important information for physicians.

In this study, the relative risk of HCC was found to be increased to 4.28 (95% CI 1.68–10.9) times higher for patients aged > 40 years compared with those aged < 40 years. In addition, a low PLT level, which indicates advanced fibrosis in the liver, including cirrhosis, was a risk factor for HCC: the relative risk was found to be increased to 8.50 (95% CI 1.98–36.2) times higher for patients with a PLT level < 206,000/μl compared with higher levels. The HBV DNA level was not selected as a risk factor for HCC occurrence in all patients with HBV infection by multivariate analysis. Previous follow-up studies have shown that viral load is an important and independent factor for HCC occurrence [4,5,20]. However, in the present study, although various thresholds of HBV DNA level were used for analysis, none of the thresholds showed statistical significance in multivariate analysis (data not shown). In contrast, the analysis intended for patients aged > 40 years revealed that high HBV viral load was added as a risk factor for HCC. By changing the threshold of HBV DNA from 4.5 to 5.3 LC/ml in 0.1-log increments, 5.0 or 5.1 LC/ml were found to be the best (data not shown); therefore we designated the threshold of HBV DNA level as > 5.0 LC/ml. In our study, HBV carriers aged > 40 years with HBV DNA levels > 5.0 LC/ml had a 4.22-times higher risk of HCC compared to HBV carriers with lower viral loads. In previous studies in Japan regarding predictive factors for HCC, Ohata et al. [5] reported that age, HBV DNA, and staging of fibrosis were the important factors, while Murata et al. [21] reported that the number of PLTs was the only factor after HBeAg seroconversion. On the other hand, in an analysis of patients with liver cirrhosis in Japan, levels of HBV DNA and/or ALT were the predictive factors for HCC [12,19]. Taken together with the present study, these reports suggest that the HBV DNA level may not be an absolute factor for predicting HCC in the analysis, irrespective of the age of the patients and the number of PLTs, but that in patients with advanced age or low numbers of PLTs, indicating advanced fibrosis of the liver, HBV DNA could be a predictive factor for the occurrence of HCC. The PLT level negatively reflects the extent of liver fibrosis [22], therefore it is very difficult to achieve an improvement in liver fibrosis and to recover the PLT level concomitantly, but a high viral load can be lowered by antiviral drug treatment. Therefore, in patients aged > 40 years, lowering the viral load using an antiviral drug might be an important way to avoid the occurrence of HCC but, in younger patients, lowering the HBV DNA level may not result in direct inhibition of HCC occurrence, although the activity of hepatitis could be suppressed.

The decrease in the number of PLTs in patients with liver disease reflects advanced fibrosis of the liver, which is strongly related to HCC occurrence. In fact, the patients in the HCC group of our study were suggested to show advanced fibrosis because they had higher values of APRI than the controls. In addition to being a marker of liver fibrosis, the influence of PLTs on cytotoxic T lymphocytes (CTLs) has been studied with keen interest. Chronic HBV infection is characterized by an inefficient CTL response, which often results in continuous destruction of hepatocytes. A recent study indicated that PLTs are required for virus-specific CTLs to accumulate within the liver and perform pathogenetic and/or antiviral roles [23]. In our study, low PLT number was a strong risk factor for HCC in all the HBV carriers, irrespective of age or PLT number at baseline. Especially in the HBV

carriers aged >40 years, low PLT number has the strongest association with HCC occurrence. Therefore, older HBV carriers with low PLT levels should be followed closely because of a high possibility of HCC occurrence, as for HCV carriers with low PLT levels [24].

The presence of HBeAg is often associated with active liver disease, whereas HBeAg seroconversion often coincides with loss of HBV DNA in serum, normalization of the ALT level, and clinical remission [25]. Spontaneous HBeAg seroconversion confers a good long-term outcome on most patients. In this study, the status of HBeAg at baseline differed significantly between the HCC and control groups; however, the status of HBeAg was not identified by univariate analysis as a predictive factor for HCC occurrence. From these results, we speculated that the HBe protein was not the direct precursor of HCC, although the HBe antigen status often reflects the replication of HBV DNA.

In this study, we evaluated parameters for predicting HCC only at first admission. A previous study reported that changes in ALT or HBV DNA levels during the follow-up period were important for predicting advanced liver disease and HCC [26]. We need to evaluate the importance of following changes in these parameters.

There was only one HCC patient aged <40 years. This patient was male and was followed up from the age of 27 years; his ALT, HBV DNA, and PLT levels and the status of HBeAg at baseline were 34 IU/l, 7.7 LC/ml, 203,000/μl, and positive, respectively. It was difficult to predict the occurrence of HCC in this case only on the basis of the risk factors for HCC indicated in this study. Hence, we need to find an adequate risk factor to predict HCC in such a case.

In conclusion, advanced age and low PLT level were the risk factors for HCC in patients with HBV infection, irrespective of the PLT level at baseline. In patients aged >40 years, viral load was added as a risk factor for HCC.

Declaration of interests: The authors indicated no potential conflict of interest.

References

[1] Yang HI, Yeh SH, Chen PJ, Iloeje UH, Jen CL, Su J, et al. Associations between hepatitis B virus genotype and mutants and the risk of hepatocellular carcinoma. J Natl Cancer Inst 2008;100:1134–43.

[2] Chu CJ, Hussain M, Lok AS. Quantitative serum HBV DNA levels during different stages of chronic hepatitis B infection. Hepatology 2002;36:1408–15.

[3] Fujiwara A, Sakaguchi K, Fujioka S, Iwasaki Y, Senoh T, Nishimura M, et al. Fibrosis progression rates between chronic hepatitis B and C patients with elevated alanine aminotransferase levels. J Gastroenterol 2008;43:484–91.

[4] Chen CJ, Yang HI, Su J, Jen CL, You SL, Lu SN, et al. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. JAMA 2006;295:65–73.

[5] Ohata K, Hamasaki K, Toriyama K, Ishikawa H, Nakao K, Eguchi K. High viral load is a risk factor for hepatocellular carcinoma in patients with chronic hepatitis B virus infection. J Gastroenterol Hepatol 2004;19:670–5.

[6] Wu CF, Yu MW, Lin CL, Liu CJ, Shih WL, Tsai KS, et al. Long-term tracking of hepatitis B viral load and the relationship with risk for hepatocellular carcinoma in men. Carcinogenesis 2008;29:106–12.

[7] Yuen MF, Tanaka Y, Fong DY, Fung J, Wong DK, Yuen JC, et al. Independent risk factors and predictive score for the development of hepatocellular carcinoma in chronic hepatitis B. J Hepatol 2009;50:80–8.

[8] Shaheen AA, Myers RP. Diagnostic accuracy of the aspartate aminotransferase-to-platelet ratio index for the prediction of hepatitis C-related fibrosis: a systematic review. Hepatology 2007;46:912–21.

[9] Befeler AS, Di Bisceglie AM. Hepatocellular carcinoma: diagnosis and treatment. Gastroenterology 2002;122: 1609–19.

[10] Liu CJ, Chen BF, Chen PJ, Lai MY, Huang WL, Kao JH, et al. Role of hepatitis B viral load and basal core promoter mutation in hepatocellular carcinoma in hepatitis B carriers. J Infect Dis 2006;193:1258–65.

[11] Yu MW, Hsu FC, Sheen IS, Chu CM, Lin DY, Chen CJ, et al. Prospective study of hepatocellular carcinoma and liver cirrhosis in asymptomatic chronic hepatitis B virus carriers. Am J Epidemiol 1997;145:1039–47.

[12] Mahmood S, Niiyama G, Kamei A, Izumi A, Nakata K, Ikeda H, et al. Influence of viral load and genotype in the progression of Hepatitis B-associated liver cirrhosis to hepatocellular carcinoma. Liver Int 2005;25:220–5.

[13] Sumi H, Yokosuka O, Seki N, Arai M, Imazeki F, Kurihara T, et al. Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. Hepatology 2003;37:19–26.

[14] Fattovich G, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. Gastroenterology 2004;127:S35–50.

[15] Park BK, Park YN, Ahn SH, Lee KS, Chon CY, Moon YM, et al. Long-term outcome of chronic hepatitis B based on histological grade and stage. J Gastroenterol Hepatol 2007;22:383–8.

[16] Chen CJ, Liang KY, Chang AS, Chang YC, Lu SN, Liaw YF, et al. Effects of hepatitis B virus, alcohol drinking, cigarette smoking and familial tendency on hepatocellular carcinoma. Hepatology 1991;13:398–406.

[17] McMahon BJ, Holck P, Bulkow L, Snowball M. Serologic and clinical outcomes of 1536 Alaska Natives chronically infected with hepatitis B virus. Ann Intern Med 2001;135:759–68.

[18] Tang B, Kruger WD, Chen G, Shen F, Lin WY, Mboup S, et al. Hepatitis B viremia is associated with increased risk of hepatocellular carcinoma in chronic carriers. J Med Virol 2004;72:35–40.

[19] Ishikawa T, Ichida T, Yamagiwa S, Sugahara S, Uehara K, Okoshi S, et al. High viral loads, serum alanine aminotransferase and gender are predictive factors for the development

of hepatocellular carcinoma from viral compensated liver cirrhosis. J Gastroenterol Hepatol 2001;16:1274–81.
[20] Kumar M, Kumar R, Hissar SS, Saraswat MK, Sharma BC, Sakhuja P, et al. Risk factors analysis for hepatocellular carcinoma in patients with and without cirrhosis: a case–control study of 213 hepatocellular carcinoma patients from India. J Gastroenterol Hepatol 2007;22:1104–11.
[21] Murata K, Sugimoto K, Shiraki K, Nakano T. Relative predictive factors for hepatocellular carcinoma after HBeAg seroconversion in HBV infection. World J Gastroenterol 2005;11:6848–52.
[22] Karasu Z, Tekin F, Ersoz G, Gunsar F, Batur Y, Ilter T, et al. Liver fibrosis is associated with decreased peripheral platelet count in patients with chronic hepatitis B and C. Dig Dis Sci 2007;52:1535–9.
[23] Iannacone M, Sitia G, Isogawa M, Marchese P, Castro MG, Lowenstein PR, et al. Platelets mediate cytotoxic T lymphocyte-induced liver damage. Nat Med 2005;11:1167–9.
[24] Lok AS, Seeff LB, Morgan TR, di Bisceglie AM, Sterling RK, Curto TM, et al. Incidence of hepatocellular carcinoma and associated risk factors in hepatitis C-related advanced liver disease. Gastroenterology 2009;136:138–48.
[25] Ma H, Wei L, Guo F, Zhu S, Sun Y, Wang H. Clinical features and survival in Chinese patients with hepatitis B e antigen-negative hepatitis B virus-related cirrhosis. J Gastroenterol Hepatol 2008;23:1250–8.
[26] Ikeda K, Arase Y, Kobayashi M, Someya T, Hosaka T, Saitoh S, et al. Hepatitis B virus-related hepatocellular carcinogenesis and its prevention. Intervirology 2005;48: 29–38.

Prophylactic effect of peptide vaccination against hepatocellular carcinoma associated with hepatitis C virus

NOBUKAZU KOMATSU[1,3], SHIGERU YUTANI[1,2], AKIRA YAMADA[1,3], SHIGEKI SHICHIJO[1], KAZUMI YOSHIDA[1], MINORU ITOU[2], RYOKO KUROMATSU[2], TATSUYA IDE[2], MASATOSHI TANAKA[2], MICHIO SATA[2] and KYOGO ITOH[1,3]

Departments of [1]Immunology and Immunotherapy, and [2]Internal Medicine, Kurume University School of Medicine, [3]Research Center for Innovative Cancer Therapy, Kurume University, Kurume, Fukuoka 830-0011, Japan

Received March 9, 2010; Accepted April 30, 2010

DOI: 10.3892/etm_00000097

Abstract. The purpose of the present study was to investigate the prophylactic effects of peptide vaccination against hepatocellular carcinoma (HCC) associated with hepatitis C virus (HCV). Two different Phase I clinical trials of HCV-derived peptides for 40 HCV-positive patients with chronic hepatitis (CH) and liver cirrhosis (LC) were conducted from November 2003 to November 2008. Among the patients, 39 (33 CH and 6 LC) received prolonged peptide vaccination with a median vaccination of 26 rounds (range 6-89). Median vaccination and observation periods were 16 months (range 2-61) and 47 months (range 10-69), respectively. Three CH and all 6 LC patients had space-occupying lesions (SOLs) or a history of HCC, respectively. HCC became detectable during the vaccination period in 2 of the 3 CH patients with SOLs prior to vaccination. By contrast, HCC was undetectable throughout the vaccination period in the remaining 36 patients without SOLs. However, HCC became detectable in 4 of these 36 patients, i.e., 2 CH patients at 46 and 29 months after the end of the vaccination period, and 2 LC patients at 49 and 18 months after the end of vaccination. The development of HCC was associated with a reduction in boosted IgG responses to the vaccinated peptides. These results may provide new information on peptide vaccination for HCV-positive CH or LC patients lacking SOLs. Further studies are recommended to confirm the prophylactic effects of peptide vaccination against HCC associated with HCV.

Introduction

Hepatitis C virus (HCV) is prevalent worldwide, with nearly 180 million infected individuals all carrying a risk of hepatocellular carcinoma (HCC) at later stages of the disease (1,2). Interferon (IFN)-based therapies are effective in 80% of patients infected with the HCV2 and 3 genotypes and also in 50% of patients with the HCV1b genotype. However, IFN therapy has several limitations, including medical and physical contra-indications, adverse events and high cost (1-4). HCV1b, the most frequently observed strain in Japan, is also a common strain in the US (3,4).

Certain HCV patients show a spontaneous clearance of the virus along with acquisition of specific immunity, which encourages hopes of developing a clinically effective vaccine (5-7). However, the development of either prophylactic or therapeutic HCV vaccines is expected to be very difficult, since HCVs are very heterogeneous and their antigens are highly mutable (6-8). Indeed, in regards to a sustained viral response (SVR), no clinical benefit has yet been reported from HCV vaccines for either IFN-naive or IFN-resistant patients in recent clinical trials, including our own, in spite of successful immunological responses in a substantial number of patients (9-13). However, we recently identified a decrease in α-fetoprotein (AFP), a biomarker for HCC, in a percentage of vaccinated patients who showed elevated AFP levels prior to vaccination (12). These results suggest that the HCV vaccine is effective as a cancer prophylaxis in chronic hepatitis (CH) and liver cirrhosis (LC) patients. Subsequently, we report the results of a follow-up study of cancer prophylaxis in patients who had received a prolonged course of peptide vaccinations at our university.

Materials and methods

Patients. Patients received the HCV-derived peptides under one of two recent Phase I clinical studies held at the Kurume University Hospital; one study was conducted with HLA-A24+ patients (11) and the other with patients bearing multiple HLA-class I alleles (HLA-A2, -A3, -A11, -A24 -A26, -A31 or -A33) (12). The inclusion criteria were as follows: i) persistent HCV infection confirmed by serological HCV-RNA tests; ii) diagnosis of CH or LC; iii) non-response to previous IFN-based treatment or refusal to receive such treatment; iv) no detectable HCC at the time of entry into the study; v) positive status for one of the following alleles: HLA-A2, -A3,

Correspondence to: Dr Nobukazu Komatsu, Department of Immunology and Immunotherapy, Kurume University School of Medicine, Kurume University, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan
E-mail: kom@med.kurume-u.ac.jp

Key words: hepatitis C virus, peptide vaccine, clinical study, cancer