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.

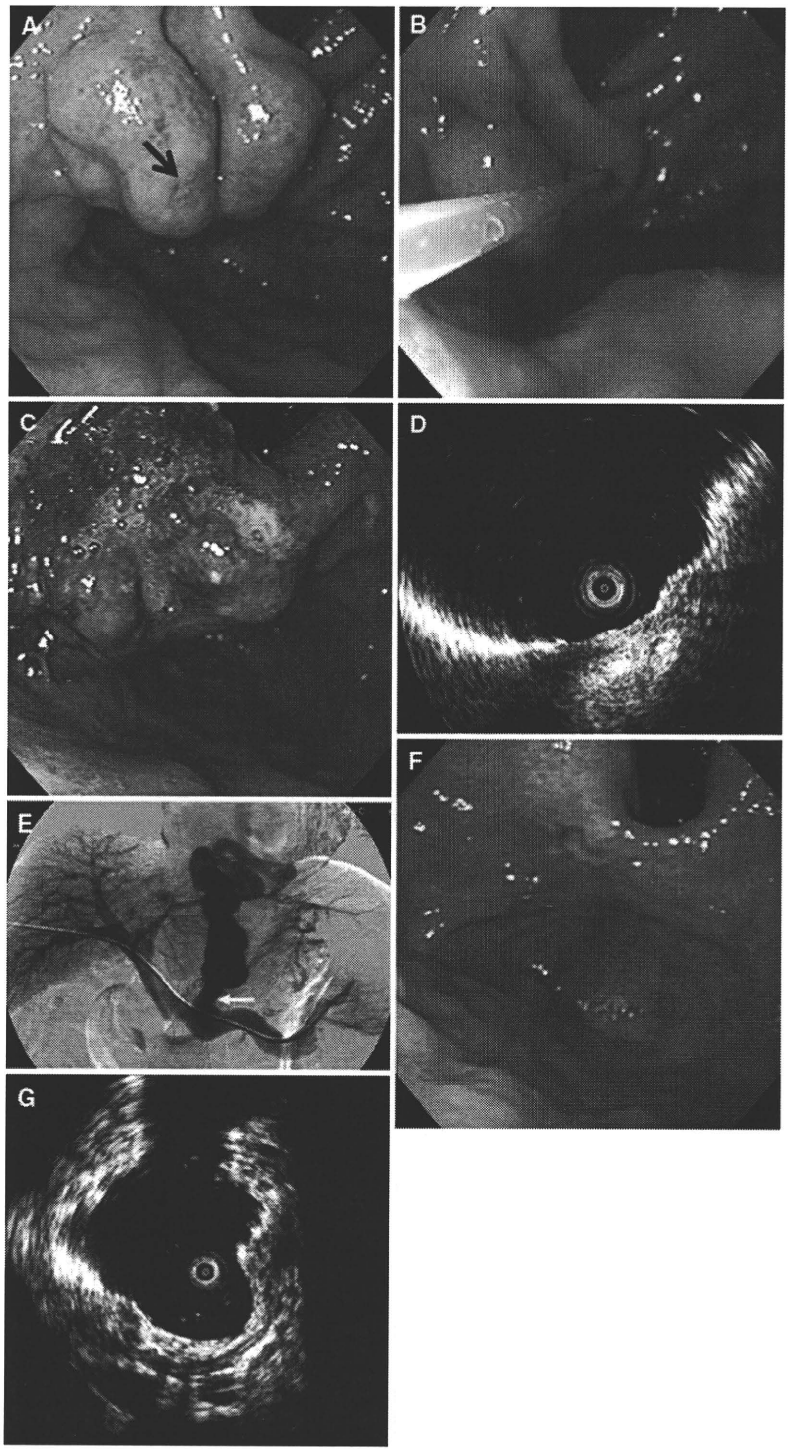

Fig. 1. 65-year-old female (case 10). **A** Before treatment. Endoscopy showed large-grade FV with bleeding point (*arrow*). **B** Puncture for FV. Withdrawn blood in the injection needle by suction indicated intravariceal puncture. **C** After seven endoscopic treatments. Protruding structure of FV 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

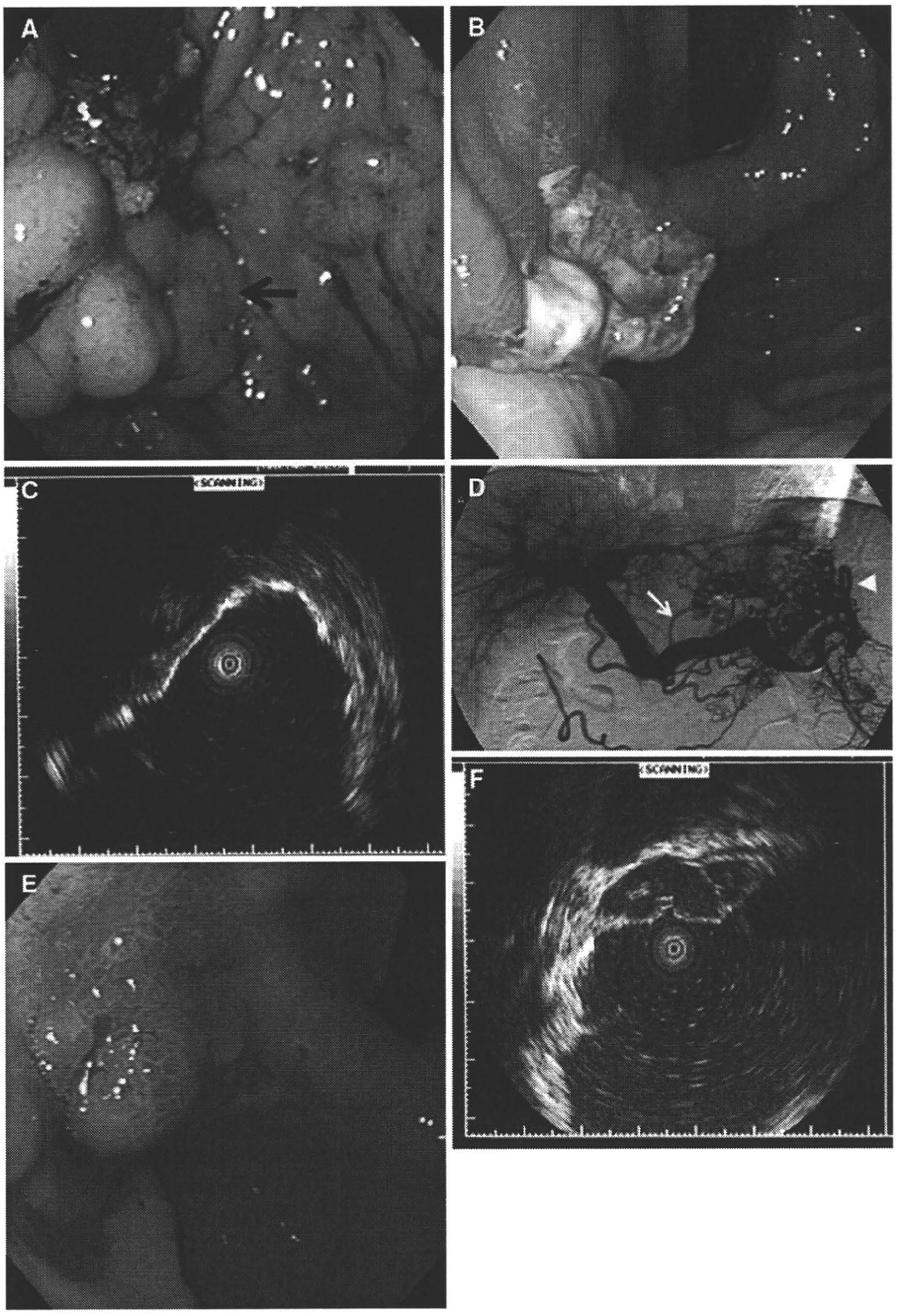

Fig. 2. 61-year-old male (case 7). **A** Before treatment. Endoscopy showed large-grade FV with bleeding point (*arrow*). **B** After 5 endoscopic treatments. Protruding structure of the varices disappeared. **C** EUS findings. EUS showed no vessel structure at the treated area. **D** Portogram. Portogram 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.

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

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ORIGINAL ARTICLE

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

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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.

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(Received 15 September 2009; accepted 27 October 2009)

ISSN 0036-5521 print/ISSN 1502-7708 online
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			*P*
	Total	HCC	Controls	
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.

Risk factor	All patients[a]		Patients aged > 40 years[b]		Patients with PLTs < 206,000 /μl[c]	
	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.

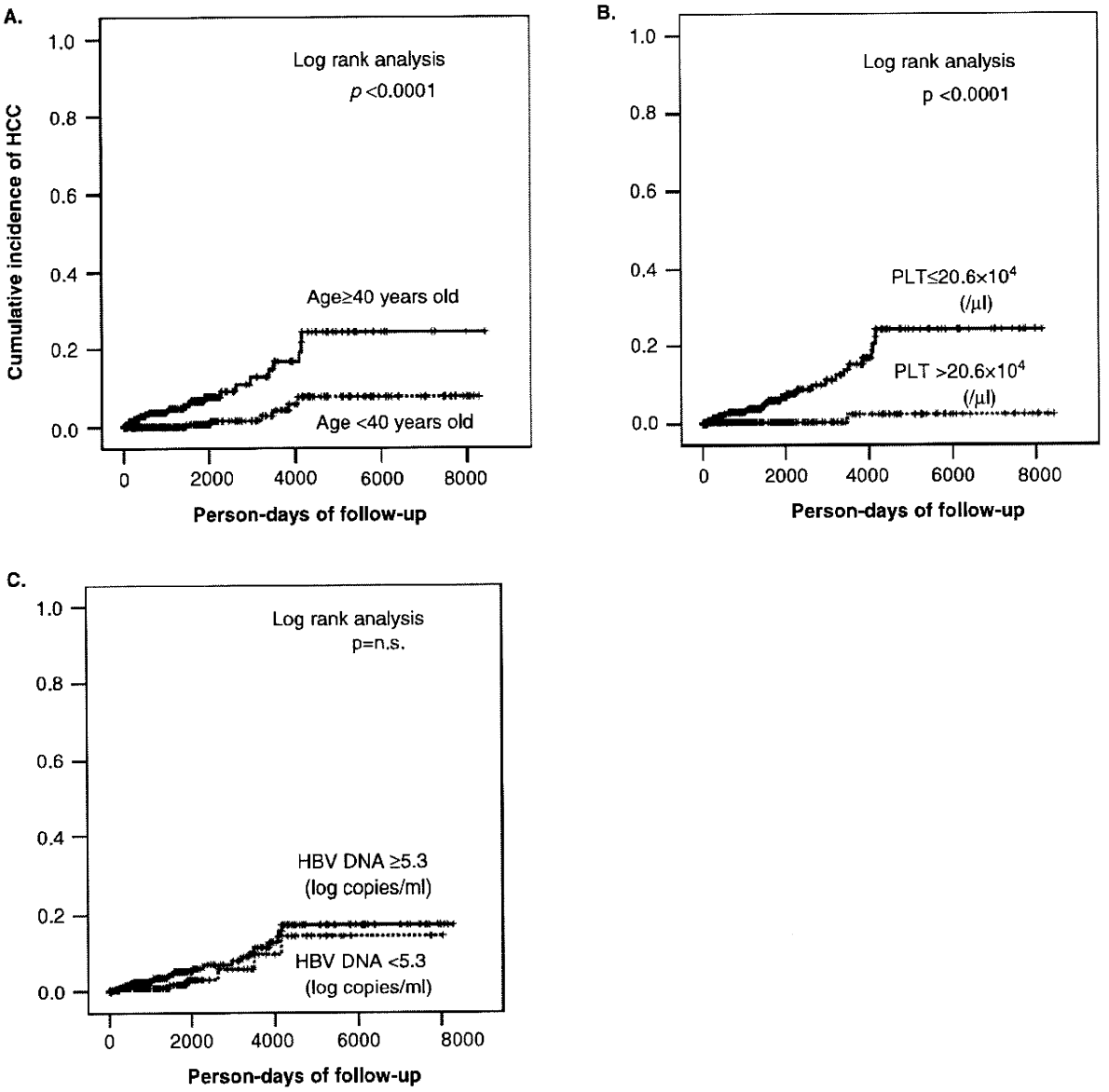

Figure 1. Cumulative occurrence of HCC based on (a) number of PLTs, (b) age, and (c) HBV DNA level. Thresholds for age, number of PLTs, and HBV DNA level were defined according to the average data for all patients. Dotted lines indicate the control group (high number of PLTs, younger age, and low HBV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

HCC was detected in 28 patients in the group with PLTs <206,000/μl ($n = 329$). The risk factors for HCC occurrence in the group with <206,000/μl 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/μl; 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

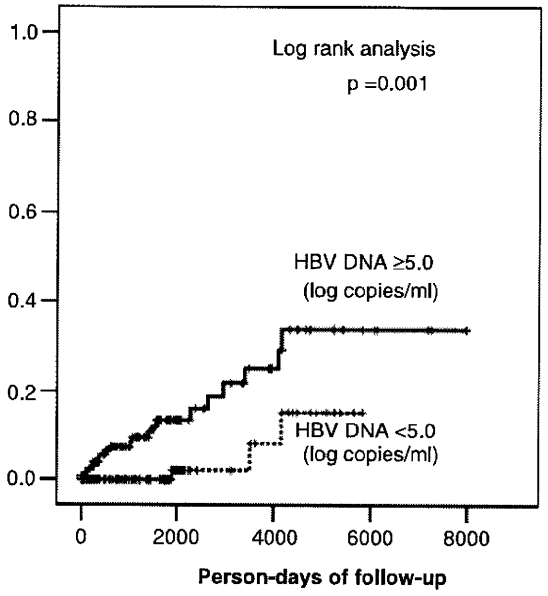

Figure 2. Cumulative occurrence of HCC based on the HBV DNA level in patients aged >40 years. The threshold for the HBV DNA level was defined according to the average data for the patients aged >40 years. A significant difference was observed by log-rank test. The dotted line indicates the control group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

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

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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,

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

-A11, -A24, -A26, -A31 or -A33; vi) an Eastern Cooperative Oncology Group performance status of 0-1, age between 20 and 75 years and adequate hematological function (white blood cell count ≥2,400/μl, hemoglobin level ≥8.0 g/dl and platelet counts ≥50,000/μl), renal function (serum creatinine ≤1.4 g/dl) and hepatic function (total bilirubin <2.5 mg/dl); and vii) negative status for hepatitis B antigens. The protocols for both series were approved by the Institutional Ethics Review Boards of Kurume University, and complete written informed consent was obtained from all patients at the time of enrollment. A total of 40 patients entered one of the two protocols at our institution between November 2003 and November 2008. In addition, the 39 patients who received more than six vaccinations were included in this follow-up study.

Peptides and vaccination. For the first protocol, four peptides capable of inducing both cytotoxic T lymphocyte (CTL) and humoral responses (IgG) in HLA-A24$^+$ patients were provided for vaccination as previously reported (11). These four peptides were derived from well-conserved regions of HCV1b as follows: protein E1-derived peptide from positions 213 to 221 (E1 213-221); protein E2-derived peptide from positions 488 to 496 (E2 488-496); non-structural region 3-derived peptide from positions 1081 to 1090 (NS3 1081-1090); and non-structural region 5A-derived peptide from positions 2132 to 2140 (NS5A 2132-2140). For the second protocol, we used a peptide for vaccination derived from HCV core protein; this peptide was capable of inducing both CTL and humoral responses (IgG) in nearly all HCV patients with different HLA-class IA alleles in Japan (12,14). This peptide originated from the HCV core protein at positions 35 to 44 (C 35-44), a well-known HLA-A2-restricted CTL epitope (15).

These five peptides were prepared under conditions of Good Manufacturing Practice by the American Peptide Company (San Diego, CA, USA). The peptide emulsion was injected into the subcutaneous region of the side of the abdomen or upper arm every 2 weeks from the 1st to the 24th vaccination, every 3 weeks from the 25th to the 48th vaccination and every 4 weeks thereafter. The first cycle consisted of six vaccinations; a second cycle of six vaccinations was conducted with patient consent in cases lacking any signs of severe toxicity. When patients wished to continue with the course of vaccinations, the series was extended, unless either disease progression or severe toxicity was observed.

Humoral responses to peptides. Peptide-specific IgG levels in the blood samples were measured using Luminex® systems as reported previously (11). Briefly, diluted plasma samples were incubated with peptide-coated microspheres. After the microspheres were washed, they were incubated with various antibodies (anti-human-IgG, -IgA, -IgM, -IgE, -IgG1, -IgG2, -IgG3 and -IgG4; purchased from Vector, Bethyl, Vector, Biosource, The Binding Site Ltd., Cappel, Cappel and The Binding Site Ltd., respectively). After being washed, the microspheres bound to each antibody were reacted with biotin-labeled detection antibody (Zymed or Cappel) and the corresponding R-phycoerythrin (Invitrogen) antibody, and the antibody levels were detected by a Luminex system as reported previously (11). All pre- and post-vaccination samples were measured simultaneously in order to avoid any possible biases associated with the *in vitro* assay. IgG against recombinant HCV core protein was also measured by means of a commercially available radioimmunoassay kit (SRL Laboratory, Tokyo, Japan).

Clinical laboratory data. Clinical laboratory values (e.g., serum ALT and AFP levels and blood platelet numbers) were measured by the Clinical Laboratory Division of the Kurume University Hospital. Quantitation of HCV-RNA, based on quantitative reverse transcription-polymerase chain reaction (qRT-PCR), was performed by a clinical lab company (SRL Laboratory).

Results

Patient characteristics and clinical responses. Thirty-nine HCV-positive patients (33 CH and 6 LC) who had received more than six rounds of HCV-derived peptide vaccinations were included in the analysis (Tables I-III). All patients, with the exception of 1 (pt. 16, HCV2a), were infected with HCV1b. At the time of entry into the study, 35 patients were non-responders to interferon-based therapy, and the remaining 4 patients refused treatment. All 6 LC patients had a history of HCC treatment, while 3 CH patients had space-occupying hepatic lesions (SOLs) suspected of being cancerous at the time they entered the study. The median frequency of vaccination was 26 rounds (range 6-89), and the median duration of the vaccination period was 16 months (range 2-69). No severe toxicity was observed throughout the vaccination period, but grade 1 or 2 local inflammation at the injection site was observed in most cases. Twelve patients were still receiving vaccinations at the time of this writing (September 2009), and the course of vaccinations had been terminated in the remaining 27 patients. Eleven patients received IFN-based therapy combined with vaccination followed by the vaccination alone, and 9 patients received IFN-based therapy after the end of vaccination (Tables I-III).

A significant decrease in ALT level (<70% ALT level at the end of vaccination or at the time of last vaccination vs. that before vaccination) was observed in 11 of 39 patients (7 of 28 patients treated with vaccination alone and 4 of 11 patients treated with vaccination alone followed by combined IFN-based therapy with vaccination). No significant decrease in platelet number (<70% at the end of vaccination or at the time of last vaccination vs. that before vaccination) was found in any of the 28 patients who received vaccination alone, but an increase (>130%) was noted in 1 patient. By contrast, a decrease in platelet number was observed in 4 of 11 patients with vaccination alone followed by combined IFN therapy. A significant decrease in the AFP level was found in 1 of 28 patients who received vaccination alone, whereas it was observed in 4 of 9 patients with vaccination alone followed by combined IFN therapy. HCV-RNA responders with >1 log decline were not found among the 28 patients who received the vaccination alone, while 4 of 11 patients who had received vaccination followed by combination therapy, including 3 sustained viral responders (SVR; pt. 1, 8 and 15), were HCV-RNA responders. As post-vaccination treatment, 9 patients received IFN-based therapy and only 1 (pt. 23) reached the status of SVR. The median observation period of the 39 patients was 47 months (range 10-69).

Table I. Patient characteristics before vaccination.

Patient	Age	Gender	Disease	Previous IFN	ALT	Plt	AFP	HCV-RNA
1	38	M	CH	-	139	11	8	465
2	43	M	CH (SOL)	IFN+RBV	139	17	9	3,820
3	52	F	LC (post HCC)	IFN+RBV	105	9	173	651
4	65	M	LC (post HCC)	IFN+RBV	292	5	7	4,030
5	55	M	CH	IFN+RBV	119	9	11	4,290
6	42	M	CH	IFN+RBV	73	12	4	621
7	49	M	CH	IFN+RBV	48	13	3	1,010
8	53	F	CH	IFN+RBV	27	21	4	2,090
9	70	F	CH	IFN+RBV	38	12	12	2,200
10	66	M	CH	IFN+RBV	154	15	11	139
11	58	F	CH	IFN+RBV	51	11	17	4,590
12	50	F	CH	IFN+RBV	62	14	10	1,730
13	61	F	CH	IFN+RBV	77	11	33	91
14	51	M	CH	IFN	41	22	3	3,420
15	50	M	CH	IFN+RBV	206	11	74	500
16	71	M	CH	IFN+RBV	114	9	4	114
17	51	M	CH	IFN	60	14	5	892
18	60	M	CH	IFN+RBV	108	8	9	3,100
19	49	F	CH	IFN+RBV	129	16	44	2,480
20	38	M	CH	IFN+RBV	358	6	29	2,870
21	61	F	LC (post HCC)	IFN+RBV	53	9	131	1,670
22	58	M	LC (post HCC)	RFA, IFN	45	8	7	2,130
23	71	M	CH	IFN	46	13	2	4,520
24	70	M	CH	IFN+RBV	47	25	5	2,470
25	68	M	CH	IFN+RBV	37	17	4	2,260
26	64	M	CH (SOL)	IFN+RBV	60	13	13	3,630
27	70	M	CH (SOL)	IFN+RBV	51	24	4	591
28	62	F	CH	-	24	19	5	583
29	53	F	CH	IFN	34	23	4	4,370
30	63	F	CH	-	73	12	3	59
31	58	F	CH	IFN+RBV	66	10	8	2,790
32	63	F	CH	IFN+RBV	52	12	3	2,230
33	57	F	CH	IFN+RBV	55	11	9	2,340
34	58	F	CH	IFN	80	12	7	2,150
35	58	F	CH	IFN+RBV	47	12	11	16,000
36	61	M	LC (post HCC)	IFN+RVB	104	10	27	32,000
37	58	M	CH	-	83	15	6	20
38	60	M	CH	IFN+RBV	144	16	5	25,000
39	73	M	LC (post HCC)	IFN+RBV	42	12	66	3,200

HCV, hepatitis C virus; CH, chronic hepatitis; SOL, space occupying lesions; LC, liver cirrhosis; HCC, hepatocellular carcinoma; IFN, interferon; RBV, ribavirin; ALT, alanine aminotransferase (IU/l); Plt, platelet numbers ($x10^4$ ml); AFP, α-fetoprotein (mg/ml); HCV-RNA, (kIU/ml).

Development of HCC. Under the circumstances described above, HCC became detectable during the vaccination period in 2 of 3 CH patients (pt. 26 and 27) with SOLs prior to vaccination. By contrast, HCC was undetectable throughout the vaccination period in the remaining 36 patients without a SOL prior to vaccination. However, HCC was diagnosed in 4 of these 36 patients after the course of vaccination, i.e., in 2 CH patients at 46 and 29 months after the end of vaccination (pt. 5 and 9), and in 2 LC patients at 49 and 18 months after the end of vaccination (pt. 4 and 21). Three of these 4 patients received IFN-based therapy combined with vaccination (pt. 5), or after the end of vaccination (pt. 9 and 21) and showed no viral response (Tables I-III).

Antibody responses. We measured the patient IgG responses to each of the peptide vaccines in plasma samples before,

Table II. Patient characteristics during vaccination.

Patient	Rounds	Duration (m)	Later addition of IFN	ALT	Plt	AFP	HCV-RNA	Onset of HCC (m)
1	39	18	+	54	10	4	-	
2	30	14	+	125	6	7	623	
3	25	11	+	110	5	300	1,120	
4	24	10	-	179	7	6	3,380	
5	28	13	+	125	8	5	5,000	
6	13	6	-	37	14	3	5,000	
7	44	22	+	67	9	2	1,360	
8	46	23	+	12	17	2	(-)	
9	18	8	-	29	15	10	4,430	
10	17	7	-	176	15	NA	301	
11	11	5	-	51	12	8	2,590	
12	15	7	-	83	12	12	2,120	
13	33	21	+	86	11	31	303	
14	19	8	-	68	23	4	4,470	
15	38	17	+	71	6	38	-	
16	64	30	+	93	10	6	(+)	
17	89+	61	-	39	14	6	1,600	
18	35	16	-	51	10	8	3,470	
19	26	15	+	193	10	28	2,890	
20	43	41	+	262	6	22	16,000	
21	22	12	-	63	9	134	3,870	
22	28	20	-	26	7	12	8,000	
23	6	2	-	51	13	2	2,310	
24	39+	35	-	108	22	2	32,000	
25	15	8	-	32	19	3	1,540	
26	19	10	-	68	13	39	3,650	+ (10)
27	33	24	-	71	23	6	200	+ (24)
28	33+	28	-	24	20	3	1,600	
29	29+	28	-	39	27	3	10,000	
30	33+	28	-	39	13	2	80	
31	32+	26	-	67	11	7	4,000	
32	32+	25	-	67	11	4	16,000	
33	17	9	-	44	12	11	5,000	
34	25+	21	-	55	12	7	2,000	
35	16+	13	-	67	9	19	8,000	
36	10	16	-	120	11	28	16,000	
37	23+	16	-	49	16	7	40	
38	14+	14	-	162	13	7	25,000	
39	16+	10	-	42	13	59	3,200	

HCV, hepatitis C virus; HCC, hepatocellular carcinoma; IFN, interferon; ALT, alanine aminotransferase (IU/l); Plt, platelet numbers ($x10^4$ ml); AFP, α-fetoprotein (mg/ml); HCV-RNA, (kIU/ml). m, months.

during and at the end of the vaccination period in all 39 patients. Moreover, when possible, we measured the IgG responses in patients after the end of the series of vaccinations (n=10). Representative results of all 39 cases are shown in Fig. 1A-C. In the first protocol for HLA-A24$^+$ patients (n=13), an increase in anti-peptide IgG to E1 213-221, E2 488-496, NS3 1081-1090 and NS5A 2132-2140 peptides at the end of the peptide vaccination was observed in 0 of 7, 12 of 13, 4 of 13 and 9 of 13 patients who received the corresponding peptides, respectively (Fig. 1A). An increase in IgG reactive to at least one of the vaccination peptides was observed in all 13 patients. The increased IgG reactive to the E2-488 or NS5A-2132, but not to the NS3-1081 peptide, was sustained for 6, 6, 49, 46, 17, 9, 6 and 6 months when the samples after the end of vaccination from pt. 1, 2, 4-6 and 9-11 were provided for measurement.

Table III. Patient characteristics post vaccination.

Patient	Post-vaccination IFN	Total OP (m)	Onset of HCC (m)
1			69
2			67
3			66
4		+ (49)	64
5		+ (46)	63
6	+		59
7	+		57
8			56
9	+	+ (29)	50
10	+		49
11	+		47
12			58
13			57
14	+		50
15			64
16			62
17			61
18	+		58
19			57
20			52
21	+	+ (18)	39
22			28
23	+		35
24			35
25			32
26			34
27			29
28			28
29			28
30			28
31			26
32			25
33			22
34			21
35			11
36			21
37			21
38			14
39			10

HCC, hepatocellular carcinoma; IFN, interferon; OP, observation periods. m, months.

In the second protocol for patients with different HLA-A alleles, the C 35-44 peptide was used for vaccination in all 26 patients, and an increase in anti-peptide IgG at the end of the peptide vaccination was observed in 22 of these patients (Fig. 1B and C). Ig isotypes and IgG subclasses of anti-C 35-44 peptide were also subjected to analysis in order to address whether Th1- or Th2-type immune responses were induced by peptide vaccination in the 26 patients who received C 35-44 peptide vaccinations. We found that all Ig isotypes (IgM, IgA, IgG and IgE), as well as all IgG subclasses (IgG1 to IgG4), were augmented by vaccination in all 22 patients exhibiting elevated IgG. Four representative cases (pt. 12, 19, 22 and 27) are shown in Fig. 2.

These results indicate that IgG responses to E2 488-496, NS5A 2132-2140 and C 35-44 peptides were boosted in the majority of vaccinated patients, i.e., IgG responses to NS3 1081-1090 were boosted in half of the patients, whereas the IgG response to the E1 213-221 peptide was not boosted in any of the patients. These results are consistent with those reported previously using samples from our Phase I study (11,12).

Measurement of cellular responses using post-vaccination samples was not carried out in this study, primarily due to the limited number of available peripheral blood mononuclear cells, although an increase in cellular responses to at least one of the vaccinated peptides during the Phase I studies was observed in the majority of patients, as reported previously (11,12).

Development of HCC and antibody responses. We then addressed the relationship between the development of HCC and patient immune responses.

HCC became detectable during the course of vaccination in 2 of 3 CH patients (pt. 26 and 27) with SOLs prior to vaccination. In these two patients, humoral responses to the vaccinated peptides were well augmented in the post-vaccination samples (Figs. 1 and 2). In the other patient (pt. 2) with a SOL but without HCC, the humoral responses to the vaccinated peptides were also augmented (Fig. 1).

HCC was undetectable in the remaining 36 patients throughout the vaccination period, but it became detectable in 4 patients post-vaccination, i.e., in 2 CH patients at 46 and 29 months after the end of the vaccination (pt. 5 and 9), and in 2 LC patients at 49 and 18 months after the end of the vaccination (pt. 4 and 21). The development of HCC in these four cases was associated with a disappearance of vaccination-induced humoral responses. Namely, the IgG boosting effect reactive to NS3-1081 and NS5A-2132, but not to E2-488, disappeared in pt. 4 and 5 when HCC became detectable 49 and 46 months after the end of vaccination, respectively (Fig. 1A). The IgG boosting effect reactive to NS5A-2132 and E2-488 also disappeared in pt. 9 when HCC became detectable 29 months after the end of vaccination. Similarly, the IgG boosting effect to the C-35 peptide in pt. 21 disappeared by the time HCC developed, i.e., 18 months after the end of the vaccination period (Fig. 1B).

The results presented above suggest an association between HCC development and the weakening of boosted IgG responses to the peptides used for vaccination. We then addressed whether IgG responses to the HCV core protein had any association with HCC development in pt. 21, who received core protein-derived peptide C-35 (Fig. 3). The increase in IgG levels in response to the HCV core protein was reduced to the pre-vaccination baseline level at the time of HCC development. The IgG responses in the remaining 3 patients who did not receive the C-35 peptide were also measured. As expected, there was no association in these 3 patients between IgG titers raised against the HCV core protein and HCC development (Fig. 3).

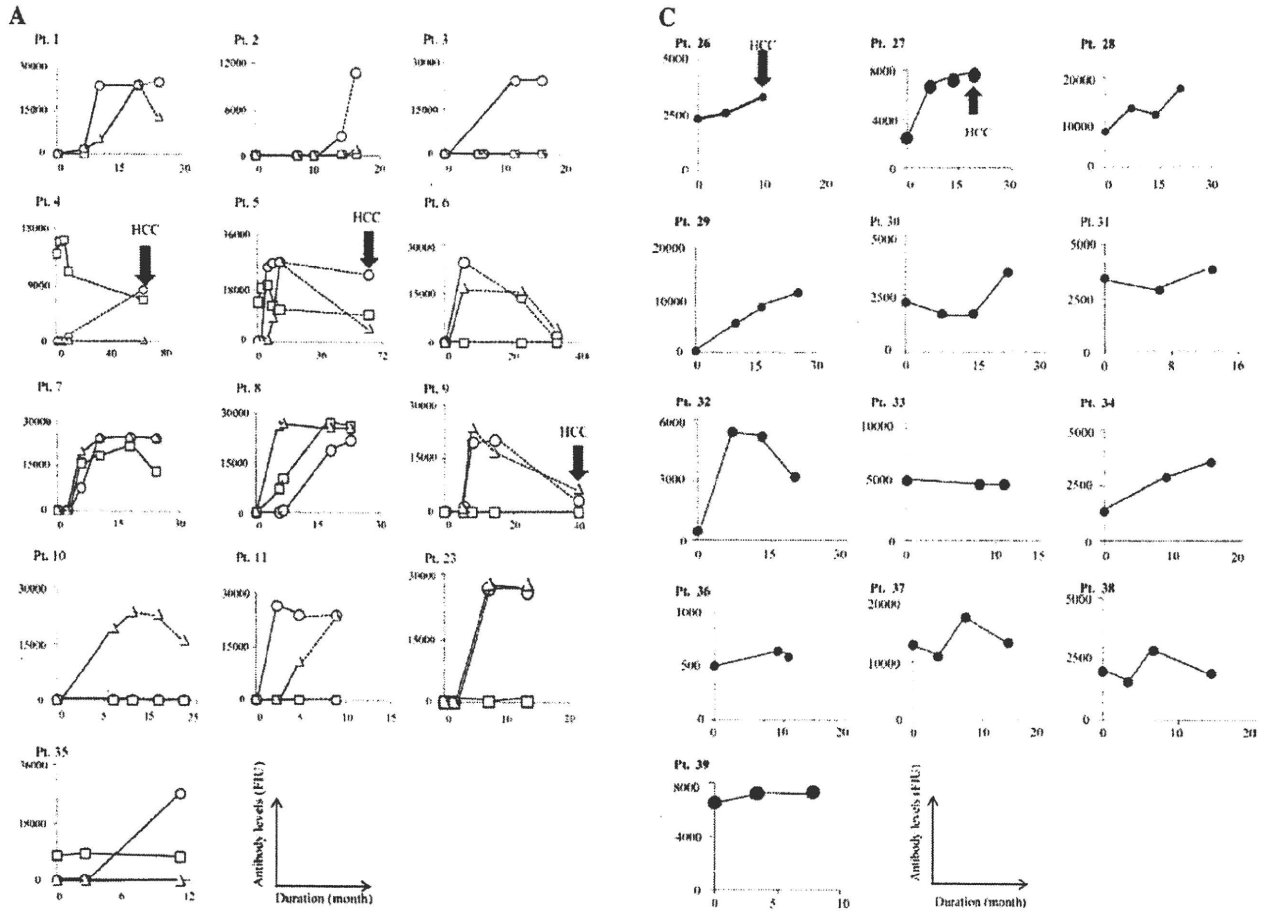

Figure 1. IgG responses to peptides. Kinetic measurement of IgG responses to each of the peptides used for vaccination in plasma samples before, during, and at the end of the vaccination period, as well as in samples obtained post-vaccination, when available. The vertical column indicates the levels of antibody expressed as fluorescence intensity units (FIU), as reported previously (11). The horizontal column indicates the duration of vaccination (——) and that of observation after the end of vaccination (-----). (A) o-o, IgG to E2 488-496; □-□, IgG to NS3 1081-1090; ▵-▵, IgG to NS5A 2132-2140 peptides. (B and C) ●-●, IgG to C 35-44 peptide.

Discussion

The reported annual occurrence rates of HCC in CH patients, LC patients, non-responders and patients with a history of HCC treatment vary largely from <1-5, 5-8, 1-7 and 10-20% per year, respectively, depending on the population studied (5,16-22). HCC has been shown to develop even in sustained viral responders at a rate of <1-2% per year, depending on the population studied (23,24). Only 39 vaccinated patients were included in the present follow-up study; thus, information regarding general HCC occurrence and recurrence rates at the Kurume University Hospital could be useful for gaining a better understanding of the results of this study. The 2-year occurrence rate of HCC in non-vaccinated HCV1b$^+$ patients with platelet numbers of <130,000 per mm^3 (stages F3 and F4) (5) who failed to respond to IFN-ribavirin therapy was 19%, while the 2-year recurrence rate in non-vaccinated HCV1b$^+$ patients after a history of HCC treatment was 47.5% at Kurume University Hospital (Sata *et al*, unpublished data). In this study, 16 and 6 patients were matched to the former and

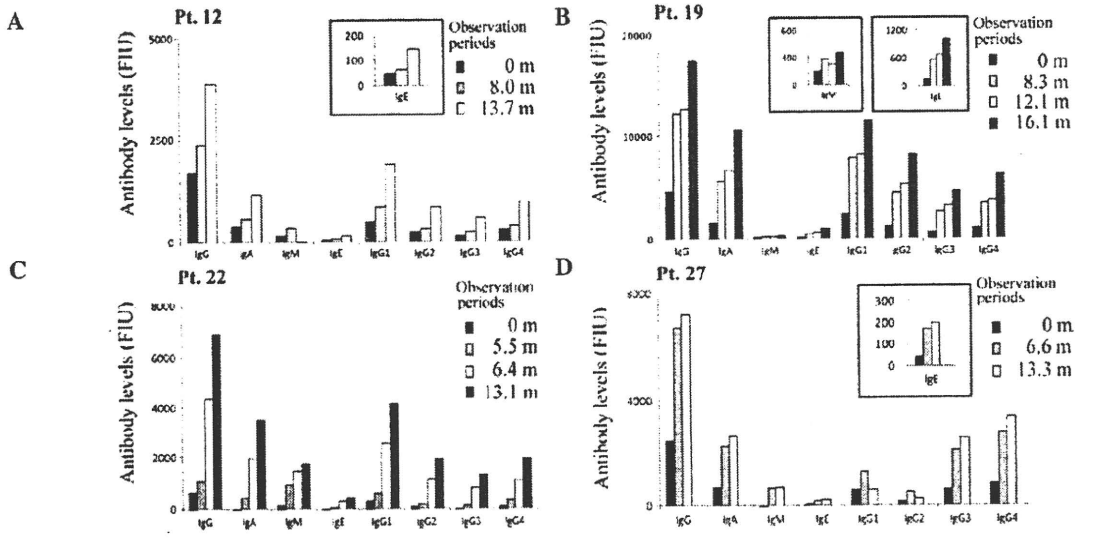

Figure 2. Isotypes and IgG subclasses. Kinetic measurement of Ig isotypes and IgG subclasses of anti-C 35-44 peptide was also studied in 22 patients. All isotypes (IgM, IgA, IgG and IgE), as well as all IgG subclasses (IgG1 to IgG4), were augmented by the C 35-44 vaccination in all 22 patients in whom IgG was elevated, as shown in Fig. 1. Four representative cases (pt. 12, 19, 22 and 27) are shown here.

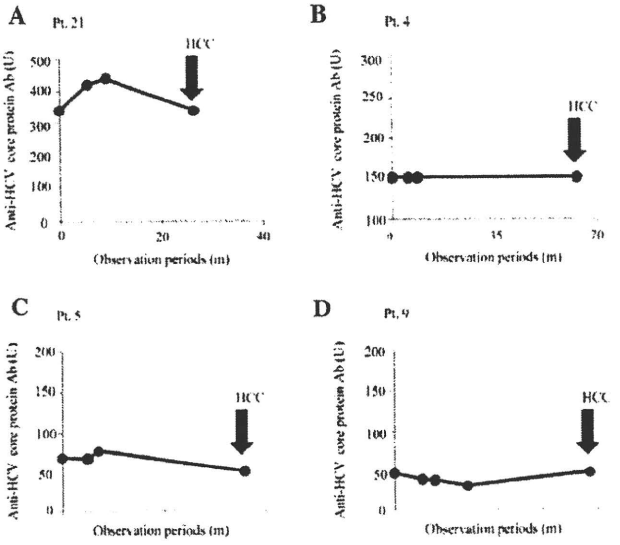

Figure 3. IgG responses to HCV core protein. Kinetic measurement of IgG responses against recombinant HCV core protein in plasma samples before, during and at the end of the vaccination, as well as after the end of vaccination in 4 patients who had developed HCC post-vaccination. The vertical column indicates the determined antibody level.

latter group of patients, respectively. A median observation period for these 22 patients after the initiation of vaccination was 55 months (range 10-69). HCC was undetectable in all of these 22 patients at least 30 months after the initiation of peptide vaccination. However, HCC became detectable 59, 58, 37 and 30 months after the initiation in pt. 4, 5, 9 and 21, respectively. With regard to post-vaccination, HCC became detectable at 49, 46, 29 and 18 months in pt. 4, 5, 9 and 21, respectively. These results suggest that peptide vaccination induced prophylaxis against HCC associated with HCV.

By contrast, HCC developed in 2 (pt. 26 and 27) of 3 CH patients with SOLs suspected of being cancerous at the start of the vaccination period (Tables I-III), suggesting that the peptide vaccine has no prophylactic effects in patients with pre-existing SOLs.

Our results suggest that the vaccination-induced increase in peptide-specific IgG lasts for 6 months after the end of a vaccination course. However, the duration of this response appears to depend on the specific peptides used for vaccination, as well as on the dose and frequency of vaccination. Thus, these issues need to be further investigated in future clinical trials with larger sample sizes. Moreover, HCC development was associated with a reduction in boosted immune responses showing reactivity to specific peptide vaccines, as well as to the corresponding core protein. Therefore, IgG reactivity to peptides used for vaccination may serve as a biomarker for predicting HCC development in HCV-positive patients who have received a peptide vaccine. It should be noted that only 4 patients were available for this aspect of the present study and therefore, further investigation is required in order to confirm our results. In addition, the biological roles of the peptide antibodies remain unclear at the present time and should be elucidated by future investigation.

Th1-type immune responses are thought to be involved in chronic-phase liver damage (25,26). Nelson *et al* found that interleukin-10 treatment resulted in the normalization of ALT levels in 19 of 22 CH patients who had been non-responders to INF-based treatment (27). Interleukin-10 promotes the production of IgA, IgG1 and IgG3 (28,29). We demonstrated an increase in all three of these Ig isotypes in post-vaccination samples, suggesting that the Th2-type immune response is indeed boosted by vaccination.

We also measured the CTL activity of PBMCs during the course of vaccination; our results were reported elsewhere (11,12). However, CTL activity was not measured in the post-vaccination follow-up study primarily due to the limited number of available samples.

In previously reported HCV vaccine trials (9-13), as well as in the present follow-up study, there has yet to be a sustained