demonstrate hypervascular hepatic lesions seen on contrast-enhanced CT but not by non-contrast US in our department. The following criteria were used for study enrolment: (i) cirrhotic patients with solitary or multiple focal hypervascular lesions found by contrast-enhanced CT taken for the surveillance of HCC, (ii) radiological diagnosis of hepatic lesions was HCC on CT images, (iii) non-contrast US could not recognize the hepatic lesions and (iv) patients without egg allergy, a contraindication of Sonazoid™. In this study period, there were 1286 patients who received a contrast-enhanced CT examination for the surveillance of HCC in our department. Among them, the subjects of this study were 44 cirrhotic patients with 55 hypervascular lesions, and they consisted of 28 males and 16 females, aged 68.2 ± 9.2 years (range 33–78). The diagnosis of liver cirrhosis was based on imaging findings with clinical symptoms and biochemistry findings in all patients, with six patients positive for hepatitis B virus surface antigen, 33 positive for hepatitis C virus antibody, two with alcohol abuse and three patients cryptogenic. The total number of hypervascular lesions in all patients was 55 (one in 37 patients, two in four patients, three in two patients and four in one patients) with a size ranging from 5 to 24 mm (12.7 ± 4.5 mm) on CT images. The serum α-fetoprotein level ranged from 1.9 to 991.1 ng/ml, being normal in 20 patients and abnormal in 24 patients (109.6 ± 209.8). Nine patients had no previous HCC diagnosis or treatment, and the other 35 patients had treatment histories for HCC: percutaneous ethanol injection (PEI) in five, radiofrequency ablation (RFA) in 17, transarterial chemoembolization (TACE) in five and TACE followed by PEI in eight. Hypervascular lesions were located at treated sites in 43 lesions in 34 patients and at untreated sites in 12 lesions in 10 patients. As non-contrast-enhanced greyscale US examination had failed to detect any of the lesions, a percutaneous needle biopsy was not performed at that time. This study was approved by the ethics committee of our institute, and informed written consent was obtained from all patients.

Ultrasound examination

US examination was performed using SSA-770A and 790A (APLIO; Toshiba, Tokyo, Japan) with a 3.75 MHz convex or microconvex probe. After non-contrast greyscale US (tissue harmonic imaging, 2.5/5.0, 14–27 Hz) and colour Doppler imaging, contrast-enhanced US was carried out in the pulse subtraction harmonic imaging mode with an MI level from 0.24 to 0.3 to observe the suspected tumour location area as estimated from the contrast-enhanced CT images. Gain was adjusted at an optimal level, and the dynamic range was set at 65 dB for non-contrast US and 45-55 dB for contrast-enhanced US. Observation of non-contrast images and the late-phase (5–10 min after the injection of Sonazoid™) images was performed by both an intercostal scan and a subcostal scan for the right lobe, and both a sagittal scan and a transverse scan for the left lobe, under possible breath holding. Each scan was completed with gentle and reciprocatory movement of the probe, right side to left side and left side to right side, or caudal side to cranial side and cranial side to caudal side. As for the early phase (from onset of contrast enhancement to 1 min), a scan plane that allowed the most stable demonstration was carefully selected and contrast-enhanced findings were observed by tilting the probe under possible breath holding.

The contrast agent Sonazoid™ (perflubutane microbubbles with a median diameter of 2–3 μm) was used at a dose of 0.0075 ml/kg by a manual bolus injection following a flush with 3.0 ml of normal saline solution, once for the observation of each lesion (once in 37 patients, twice in four patients, three times in two patients and four times in one patient). The subsequent injection was given after the disappearance of the previous enhancement. The operators for US examinations were H. M. (18-year experience) in 20 patients, M. T. (6-year experience) in 14 patients, H. I. (6-year experience) in six patients and H. O. (8-year experience) in four patients. All US images recorded digitally were reviewed at a later date by H. O., and contrast-enhanced findings in the hepatic lesions were noted in comparison with those in surrounding liver parenchyma as positive, equal or negative enhancement. Hepatic lesions with either positive enhancement in the early-phase image or negative enhancement in the late-phase image were considered to be localized target lesions. Furthermore, the presence or absence of contrast enhancement in the intrahepatic portal vein was also noted at the late phase, based on the microbubble-disappearance time (20).

Contrast-enhanced computed tomography and computed tomography angiography

Contrast-enhanced CT with a dynamic study was performed in all patients using Lightspeed ultra16 (GE Yokogawa Medical Systems, Hino, Japan) with an injection of 100 ml of a contrast medium (Iopamiron 350; Nihon Schering, Osaka, Japan) at 3 ml/s from the antecubital vein by a mechanical power injector. Imaging was performed with a 30-s delay between contrast medium administration and start of imaging 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. Contrast-enhanced CT was performed again in patients who received treatment for hepatic lesions to evaluate the therapeutic response. Findings of contrast-enhanced CT both before and after the treatment were evaluated blindly by S. O. to confirm the therapeutic effect and whether the lesion contrast-enhanced US demonstrated was the lesion detected on contrast-enhanced CT.

CT angiography was performed using Infinix Active Aquilion Type (Toshiba) with an injection of 15 ml of contrast medium (Iopamiron 300; Nihon Schering) at 3 ml/s via a catheter placed at the common hepatic artery by a mechanical power injector. Imaging was performed with a 2-s and an 8-s delay between contrast medium administration and start of imaging for the artery-dominant phase. This imaging was planned after a contrast-enhanced US examination and applied in 27 patients according to the standard protocol in our department: in patients with the initial treatment for HCC, patients with solitary or multiple lesions requiring TACE because of the lesions considered to be technically difficult for percutaneous needle advancement and patients with hepatic lesions not demonstrated by contrast-enhanced US. Findings of CT angiography were evaluated blindly by M. Y.

Ultrasound-guided puncture for hepatic lesions

Biopsy for hepatic lesions was performed by a 21-gauge needle (Sonopsy; Hakko, Tokyo, Japan), PEI was performed with a 22-gauge Chiba needle (Top, Tokyo, Japan) and RFA was performed with a 17-gauge cool-tip radio frequency electrode (Radionics, Burlington, MA, USA). All US-guided procedures were performed using a convex or microconvex probe with a specially designed attachment. Percutaneous needle biopsy was applied in patients with the initial diagnosis or treatment for

HCC, according to the standard protocol in our department. When hepatic lesions became recognized on the non-contrast sonogram in reference to the contrast-enhanced US findings, US-guided punctures were conducted under non-contrast US. However, when hepatic lesions were not clearly recognized on the non-contrast sonogram regardless of the reference of contrast-enhanced US findings, these US-guided procedures were conducted under contrast-enhanced US with an additional injection of Sonazoid™.

Statistical analysis

All data were expressed as mean ± standard deviation or percentage. Statistical significance was determined using Fisher's exact test, and significance was considered at $P < 0.05$. Statistical analysis was performed using the SPSS package (version 13.0J; SPSS Inc., Chicago, IL, USA).

Results

Detection of focal hepatic lesions by contrast-enhanced ultrasound

Fifty-two lesions showed a positive enhancement in the early phase and 44 lesions showed a negative enhancement in the late phase (Table 1), with phasic detectability being significantly higher in the early phase (52/55) than that in the late phase (44/55, $P = 0.0221$). Consequently, 53 lesions were demonstrated by contrast-enhanced US with Sonazoid™, yielding a detection rate of 96.4% (Figs 1 and 2). Twenty-one of the 55 lesions (38.2%) were < 10 mm, with the detection rate between lesions < 10 mm and ≥ 10 mm not being significantly different (Fig. 3). In addition, 13 of the 55 lesions (23.6%) lesions were located deeper than 8 cm from the skin surface (Fig. 3). Contrast enhancement in the intrahepatic portal vein at the late phase was still visible in 33 (60%) patients with 37 lesions and in 11 (29.7%) of the 37 lesions showing an equal enhancement to the surrounding liver parenchyma in this phase. Percutaneous needle biopsy was performed for 10 of the successfully demonstrated 53 lesions under the non-contrast sonogram. Their diagnosis was seven moderately differentiated HCC (7, 10, 13, 15, 18, 20 and 24 mm), two well-differentiated HCC (10, 12 mm) and one benign nodule (13 mm) found in alcoholic liver disease.

Clinical course after contrast-enhanced ultrasound

Percutaneous US-guided treatments were successfully performed in 42 lesions (PEI in 20 and RFA in 22) in 32 patients, and all hypervascular hepatic lesions changed to hypovascular appearances on contrast-enhanced CT images after the treatment. Thus, treatment effectiveness was confirmed on contrast-enhanced CT images and this was the evidence that the lesion contrast-enhanced US demonstrated was the lesion detected on contrast-enhanced CT. Six patients, each patient having one hypervascular lesion on contrast-enhanced CT, were treated by TACE alone because CT angiography showed more than three lesions in the liver. In the remaining seven lesions in six patients, six were diagnosed as non-HCC lesions. Two lesions were diagnosed as arterioportal communication by both CT angiography and contrast-enhanced US. Two lesions undetected by contrast-enhanced US were diagnosed as a vascular abnormality such as arterioportal communication because HCC was ruled out by CT angiography and the findings on subsequent contrast-enhanced CT showed no change in their clinical course, one case after 11 months and the other after 1 year (Fig. 2). One lesion was diagnosed as arteriovenous communication by contrast-enhanced US, and the other case with a benign lesion in alcoholic liver disease by a percutaneous needle biopsy. These six lesions and one lesion with severe liver damage were followed up without any treatment, and the findings of the former six lesions on contrast-enhanced CT did not change during the clinical course of 8.4 ± 1.9 (5–14) months.

Table 1. Contrast-enhanced ultrasound findings of hepatic lesions in each phase

Enhanced findings	Early phase	Late phase
Positive	52	0
Equal	3	11
Negative	0	44

Fifty-two lesions showed positive enhancement in the early phase and 44 lesions showed negative enhancement in the late phase, with phasic detectability being significantly higher in the early phase (52/55) than in the late phase (44/55, $P = 0.0221$). Consequently, 53 lesions were demonstrated by contrast-enhanced ultrasound with Sonazoid™, a detection rate of 96.4%.

Discussion

Percutaneous US-guided treatments that require obvious demonstration of focal hepatic lesions on sonograms are minimally invasive and effective for HCC. However, a potential pitfall sometimes hinders the wider application of the technique, because visualization of focal hepatic lesions is not easy in cirrhotic patients (10, 11). To overcome this problem for the application of percutaneous US-guided treatments, some ingenious development is awaited.

As shown in the present study, the majority of ultrasonically unrecognized small hepatic lesions with a hypervascular appearance on contrast-enhanced CT were successfully demonstrated by contrast-enhanced US with Sonazoid™. Furthermore, the results suggested that our technique might be less dependent on the size or the location of the hepatic lesions.

Although all the hepatic lesions had a hypervascular appearance on contrast-enhanced CT, three of the 55 lesions showed equal enhancement on the early-phase sonograms. This might be explained by the saturation by contrast enhancement of the surrounding parenchyma owing to the inappropriate scan timing for the hepatic lesion, because the onset of contrast enhancement after the injection of the microbubble agent varies case by case and breath holding is sometimes inadequate. Furthermore, we had to observe early-phase images while searching for the lesions with positive enhancement by tilting the probe. Therefore, an inevitable delayed observation might account for the equal enhancement on the early-phase sonograms in these three hepatic lesions.

Meanwhile, the late-phase observation was relatively easy to perform, as with repeated observation it was possible to depict the negatively enhanced lesions in the liver parenchyma with homogeneous enhancement. However, detectability of hepatic lesions in the late phase was significantly lower than that in the early phase, and 11 of the 55 lesions had equal enhancement in the late phase. It is reported that Sonazoid™ microbubbles are captured by Kupffer cells in the liver (18), while retained intrahepatic microbubble circulation was found in approximately half of the subjects in the late phase in the present study.

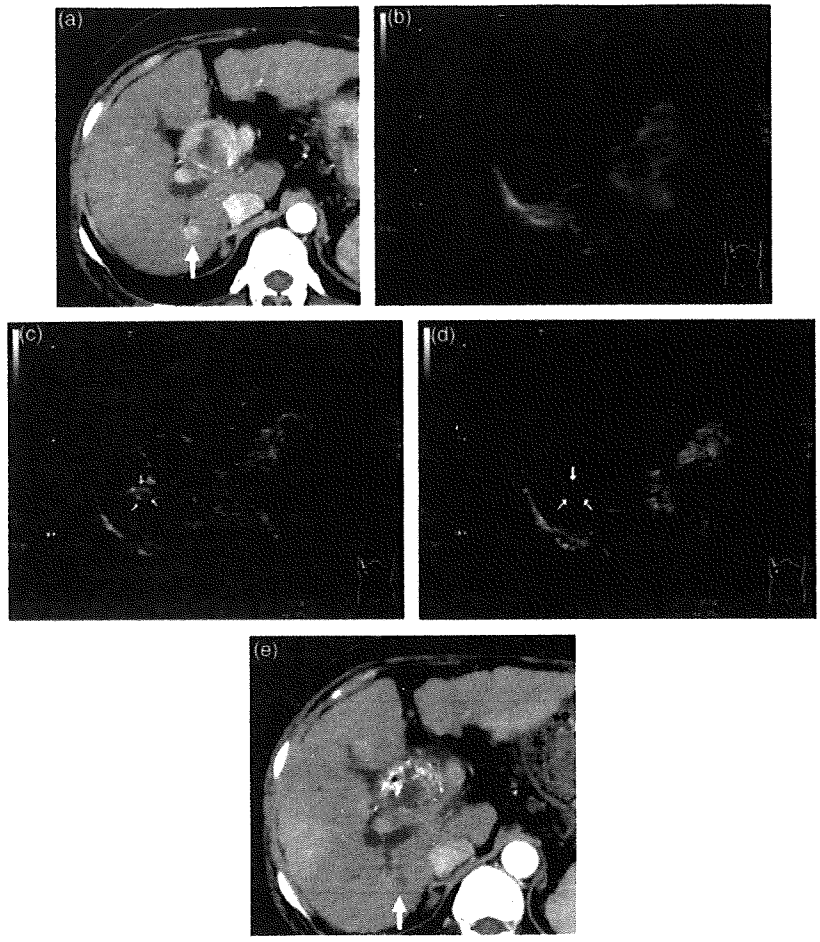

Fig. 1. Fifty-seven-year-old female, hepatitis B virus-related liver cirrhosis. (a) Contrast-enhanced computed tomography (CT) image. A hypervascular lesion (arrow) considered to be hepatocellular carcinoma was demonstrated on CT image. (b) Non-contrast sonogram. Hepatic lesion corresponding to CT findings was not recognized on the sonogram before enhancement. (c) Contrast-enhanced sonogram in the early phase. A hypervascular lesion was demonstrated on the sonogram after enhancement (arrows). (d) Contrast-enhanced sonogram in the late phase. The lesion showed negative enhancement in this phase (arrows). (e) Contrast-enhanced CT image after a percutaneous ethanol injection. Hepatic lesion showed a hypovascular appearance after the treatment (arrow).

Therefore, the phase from 5 to 10 min after the injection of Sonazoid™ might not be produced by accumulated microbubbles alone, and the equal enhancement findings in some hepatic lesions in the late phase might be explained by the residual intravascular enhancement. In any event, observation both in the early phase and in the late phase would be necessary for detection of these hepatic lesions.

The previous study showed that Levovist (Schering AG, Berlin, Germany), a first-generation US contrast agent, improved the localization of ultrasonically unrecognized hypervascular lesions in the liver (12). However, the detection rate of 75% was lower than that with Sonazoid™. An appropriate imaging mode for Levovist was based on Doppler mode, which suffers from artefacts, and it requires the setting of regions of interest whose size vary inversely to the frame rate. In addition, contrast-enhanced US images were observed by a low frame rate of 4–8 Hz in the early phase and intermittent scanning with 1 frame/s in the late phase, imaging sequences suitable for Levovist. Thus, improved signal-to-noise ratio and real-time performance may be the advantages of our technique with Sonazoid™, which resulted in increased detectability of hepatic lesions in comparison with the results using Levovist. Another study showed that RFA was sufficiently achieved using Sonazoid™ for undetected lesions by conventional US (19). However, that study had some differences from ours, because it included large-sized tumours and the use of a combined method with low acoustic power and high acoustic power according to the phase. Although an optimal acoustical condition for Sonazoid™ has still not been established, our technique under a low-MI condition throughout the examination may be more simple and convenient. Obviously, the establishment of appropriate imaging sequences would be helpful for the standardization and popularization of contrast-enhanced US with Sonazoid™.

We failed to demonstrate two focal hepatic lesions in our study, which were fortunately considered to be arterioportal communications. In fact, there were five patients with vascular abnormalities, so-called 'pseudo-lesions', that sometimes confuse the differentiation from hypervascular HCC on contrast-enhanced CT (21–23). The reasons for detection failure of these

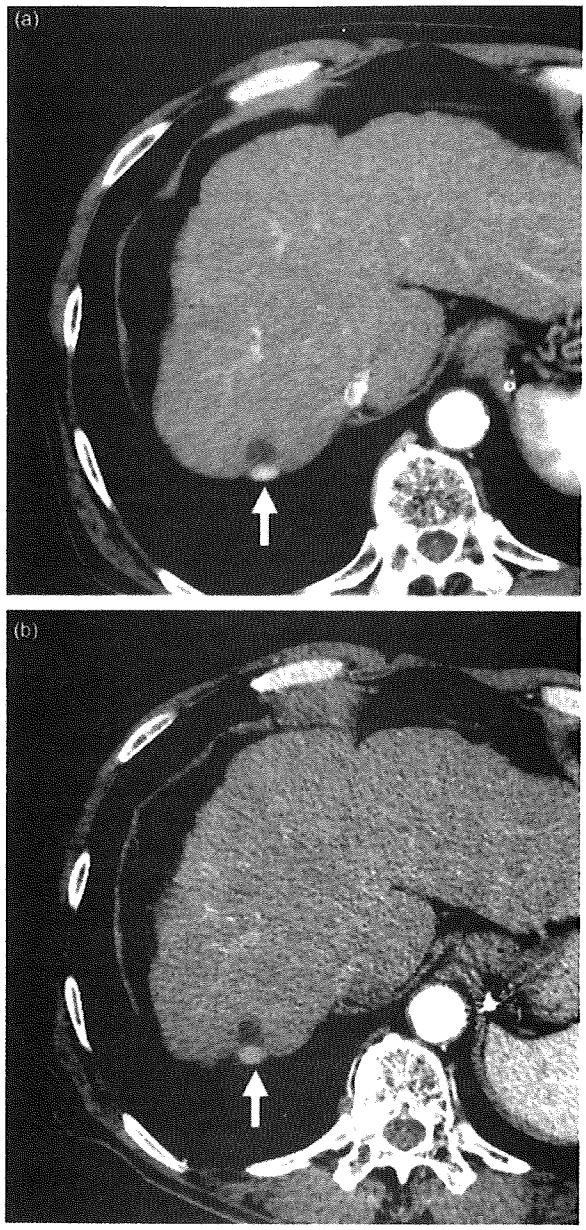

Fig. 2. Sixty-eight-year-old male, liver cirrhosis caused by alcohol abuse. (a) Contrast-enhanced computed tomography (CT) image on 10 March 2007. Contrast-enhanced CT image showed a hypervascular lesion (arrow) considered to be recurrence of hepatocellular carcinoma around the treated area. This lesion was not demonstrated by either non-contrast ultrasound (US) or contrast-enhanced US. (b) Contrast-enhanced CT image on 22 February 2008. Hypervascular lesion (arrow) did not show remarkable changes in form and size in comparison with that in the previous CT image. This hypervascular lesion was considered to be a vascular abnormality such as arterioportal communication.

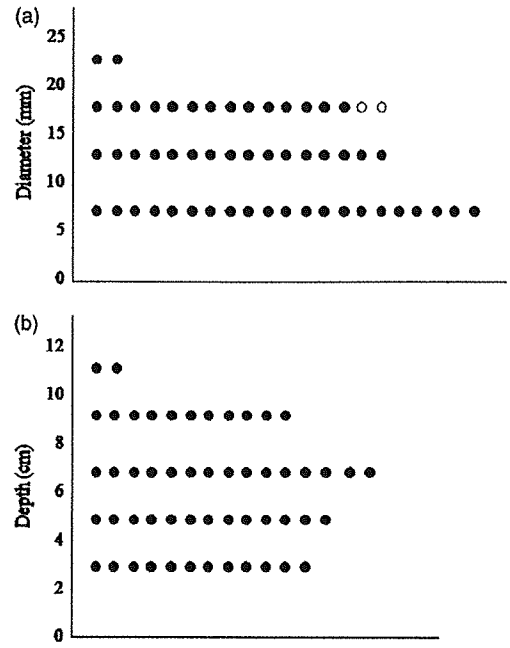

Fig. 3. (a) Distribution of hepatic lesions in relation to the diameter of the tumour. Twenty-one of the 55 lesions (38.2%) were < 10 mm. Closed circles: detected lesions [diameter measured by ultrasound (US)]. Open circles: undetected lesions (diameter measured by computed tomography). (b) Distribution of hepatic lesions in relation to the depth of the tumour location. Thirteen of the 55 lesions (23.6%) were located deeper than 8 cm from the skin surface. Two lesions undetected by contrast-enhanced US were not included because their depth was not measured on sonograms.

lesions may be the difficulty of sonographical demonstration because of the location and/or the pathological property of the vascular abnormality. Although our technique achieved quite a high detection rate for malignant hepatic lesions, improvement in diagnostic ability for such vascular abnormalities would be a worthwhile mission for contrast-enhanced US development in the near future.

There was one benign hepatic lesion histologically proven in one patient with alcoholic liver disease. It is known that hypervascular hepatic lesions do not always reflect the fact that the final diagnosis of the nodule is HCC in heavy drinkers (24). The ring-shaped appearance on liver-specific contrast-enhanced sonograms with Levovist is reported to be a useful sign for the diagnosis of a benign nodule in heavy drinkers (25). However, the benign lesion in the present study did not show this sign in the late phase. Although this might be explained by the very small size of the lesion in which the ring-shaped appearance was hard to recognize and/or the difference of microbubble property between Levovist and Sonazoid™, further studies would be needed to investigate this issue.

CT-guided needle puncture is also an effective method for hepatic tumours not recognized by US examination (26–29). However, it is not an easy procedure, and it requires radiation exposure and is time consuming. As contrast-enhanced US with Sonazoid™ could provide quite sufficient detectability of ultrasonically unrecognizable hepatic lesions, the application of CT-guided treatment may be confined to cases not demonstrated by contrast-enhanced US.

Cost, time and manpower are not negligible aspects to discuss the value of physical examination. As for the cost of contrast agent, one vial of Sonazoid™ (about US$100) is usually enough for one patient, because 2.0 ml of Sonazoid™

solution is available by one vial and each injection was applied at a dose of 0.0075ml/kg in our method. Next, contrast-enhanced US examination is not a costless work, because it needs an assistant for the preparation and injection of a contrast agent, in addition to the US operator. Furthermore, as about 10-min observation was required for both the early and the late phase in our study, contrast-enhanced US may not be as brief as conventional US. However, the benefit and safety of contrast-enhanced US with Sonazoid™ would outweigh the cost manpower and time required in this procedure.

The present study has the limitation that the diagnosis of all hepatic lesions was not proven histologically. The European Association for the Study of the Liver has documented that nodules larger than 2 cm with an arterial hypervascular pattern by two imaging techniques are diagnosed as HCC without pathological findings, and sampling error could not be denied in the needle biopsy for small hepatic lesions (3, 30). Therefore, histological proof for all hepatic lesions may not be indispensable in our study. However, various kinds of hypervascular hepatic tumours such as haemangioma or focal nodular hyperplasia might have been included, although their diagnosis on contrast-enhanced CT was HCC. A second limitation was that there were no additional lesions detected by contrast-enhanced US, because the observation of contrast enhancement was limited to the tumour area estimated by contrast-enhanced CT. In fact, we had six patients treated by TACE alone because CT angiography following contrast-enhanced US showed more than one lesion that was presented on contrast-enhanced CT in the liver. Although the early-phase observation for multiple hepatic lesions may be difficult, scanning for other areas of the liver in the late phase might be helpful for detecting additional lesions as hypo-enhancement lesions. As these six patients were, after all, treated not by US-guided treatment but by TACE, demonstration of additional lesions might not affect the therapeutic strategy for them. However, this point may be improved with an increase in the detectability of hepatic lesions by contrast-enhanced US.

In conclusion, the detection rate of ultrasonically unrecognized hypervascular HCC was improved by contrast-enhanced US with Sonazoid™. This technique may allow the wider application of percutaneous US-guided treatments, which are minimally invasive procedures, in patients with HCC.

References

1. Bosch FX, Ribes J, Borras J. Epidemiology of primary liver cancer. *Semin Liver Dis* 1999; **19**: 271–85.
2. Okuda K. Hepatocellular carcinoma. *J Hepatol* 2000; **32**: 225–37.
3. Bruix J, Sherman M, Llovet JM, *et al.* Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. *J Hepatol* 2001; **35**: 421–30.
4. Ryu M, Shimamura Y, Kinoshita T, *et al.* Therapeutic results of resection, transcatheter arterial embolization and percutaneous ethanol injection in 3225 patients with hepatocellular carcinoma: a retrospective multicenter study. *Jpn J Clin Oncol* 1997; **27**: 251–7.
5. Livraghi T, Bolondi L, Lazzaroni S, *et al.* Percutaneous ethanol injection in the treatment of hepatocellular carcinoma in cirrhosis. A study on 207 patients. *Cancer* 1992; **69**: 925–9.
6. Goldberg SN, Gazelle GS, Solbiati L, Rittman WJ, Mueller PR. Radiofrequency tissue ablation: increased lesion diameter with a perfusion electrode. *Acad Radiol* 1996; **3**: 636–44.
7. Giorgio A, Tarantino L, de Stefano G, *et al.* Percutaneous sonographically guided saline-enhanced radiofrequency ablation of hepatocellular carcinoma. *Am J Roentgenol* 2003; **181**: 479–84.
8. Lencioni RA, Allgaier HP, Cioni D, *et al.* Small hepatocellular carcinoma in cirrhosis: randomized comparison of radio-frequency thermal ablation versus percutaneous ethanol injection. *Radiology* 2003; **228**: 235–40.
9. Ebara M, Okabe S, Kita K, *et al.* Percutaneous ethanol injection for small hepatocellular carcinoma: therapeutic efficacy based on 20-year observation. *J Hepatol* 2005; **43**: 458–64.
10. Takayasu K, Muramatsu Y, Asai S, *et al.* CT fluoroscopy-assisted needle puncture and ethanol injection for hepatocellular carcinoma: a preliminary study. *Am J Roentgenol* 1999; **173**: 1219–24.
11. Sato M, Watanabe Y, Tokui K, *et al.* CT-guided treatment of ultrasonically invisible hepatocellular carcinoma. *Am J Gastroenterol* 2000; **95**: 2102–6.
12. Maruyama H, Kobayashi S, Yoshizumi H, *et al.* Application of percutaneous ultrasound-guided treatment for ultrasonically invisible hypervascular hepatocellular carcinoma using microbubble contrast agent. *Clin Radiol* 2007; **62**: 668–75.
13. Goldberg BB. *Ultrasound Contrast Agents*. London: Martin Dunitz Ltd, 1997; 169.
14. Morel DR, Schwieger I, Hohn L, *et al.* Human pharmacokinetics and safety evaluation of SonoVue, a new contrast agent for ultrasound imaging. *Invest Radiol* 2000; **35**: 80–5.
15. Maruyama H, Matsutani S, Saisho H, Mine Y, Yuki H, Miyata K. Extra-low acoustic power harmonic images of the liver with Perflutren: novel imaging for real-time observation of liver perfusion. *J Ultrasound Med* 2003; **22**: 931–8.
16. von Herbay A, Vogt C, Willers R, Haussinger D. Real-time imaging with the sonographic contrast agent SonoVue: differentiation between benign and malignant hepatic lesions. *J Ultrasound Med* 2004; **23**: 1557–68.
17. Marelli C. Preliminary experience with NC100100, a new ultrasound contrast agent for intravenous injection. *Eur Radiol* 1999; **9**(Suppl. 3): S343–6.
18. Watanabe R, Matsumura M, Munemasa T, Fujimaki M, Suematsu M. Mechanism of hepatic parenchyma-specific contrast of microbubble-based contrast agent for ultrasonography: microscopic studies in rat liver. *Invest Radiol* 2007; **42**: 643–51.
19. Numata K, Morimoto M, Ogura T, *et al.* Ablation therapy guided by contrast-enhanced sonography with sonazoid for hepatocellular carcinoma lesions not detected by conventional sonography. *J Ultrasound Med* 2008; **27**: 395–406.
20. Maruyama H, Matsutani S, Okugawa H, *et al.* Microbubble disappearance-time is the appropriate timing for liver-specific imaging after injection of Levovist. *Ultrasound Med Biol* 2006; **32**: 1809–15.
21. Yu JS, Kim KW, Sung KB, *et al.* Small arterial-portal venous shunts: cause of pseudolesions at hepatic imaging. *Radiology* 1997; **203**: 737–42.
22. Kim TK, Choi BI, Han JK, *et al.* Nontumorous arterioportal shunt mimicking hypervascular tumor in cirrhotic liver: two-phase spiral CT findings. *Radiology* 1998; **208**: 597–603.
23. Brancatelli G, Baron RL, Peterson MS, *et al.* Helical CT screening for hepatocellular carcinoma in patients with cirrhosis: frequency and causes of false-positive interpretation. *Am J Roentgenol* 2003; **180**: 1007–14.
24. Kim SR, Maekawa Y, Ninomiya T, *et al.* Multiple hypervascular liver nodules in a heavy drinker of alcohol. *J Gastroenterol Hepatol* 2005; **20**: 795–9.
25. Maruyama H, Matsutani S, Kondo F, *et al.* Ring-shaped appearance on liver-specific image with Levovist: a characteristic enhancement

pattern for hypervascular benign nodule in the liver of heavy drinkers. *Liver Int* 2006; **26**: 688–94.
26. Schweiger GD, Brown BP, Pelsang RE, *et al.* CT fluoroscopy: technique and utility in guiding biopsies of transiently enhancing hepatic masses. *Abdom Imaging* 2000; **25**: 81–5.
27. Shibata T, Iimuro Y, Yamamoto Y, *et al.* CT-guided transthoracic percutaneous ethanol injection for hepatocellular carcinoma not detected with US. *Radiology* 2002; **223**: 115–20.
28. Kirchner J, Kickuth R, Laufer U, *et al.* CT fluoroscopy-assisted puncture of thoracic and abdominal masses: a randomized trial. *Clin Radiol* 2002; **57**: 188–92.
29. Adam A, Hatzidakis A, Hamady M, *et al.* Percutaneous coil placement prior to radiofrequency ablation of poorly visible hepatic tumors. *Eur Radiol* 2004; **14**: 1688–91.
30. Nakashima T, Kojiro M. *Hepatocellular Carcinoma*. Tokyo: Springer-Verlag, 1987.

Scandinavian Journal of Gastroenterology, 2009; 44: 872–877

informa
healthcare

ORIGINAL ARTICLE

Quantification of hepatitis C amino acid substitutions 70 and 91 in the core coding region by real-time amplification refractory mutation system reverse transcription-polymerase chain reaction

SHINGO NAKAMOTO, TATSUO KANDA, YUTAKA YONEMITSU, MAKOTO ARAI, KEIICHI FUJIWARA, KENICHI FUKAI, FUMIHIKO KANAI, FUMIO IMAZEKI & OSAMU YOKOSUKA

Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Chiba, Japan

Abstract

Objective. The effects of hepatitis C virus (HCV) sequence variations on the success of antiviral therapy or the development of hepatocellular carcinoma (HCC) are complex for many reasons. Recently, there have been several reports on the effects of genotype 1b HCV core amino acid substitutions 70 and/or 91 on the outcome of antiviral therapies and the clinical course. The purpose of this study was to establish real-time amplification refractory mutation system (ARMS) reverse transcription (RT)-polymerase chain reaction (PCR) assays for easy detection of these HCV mutations. ***Material and methods.*** Plasmids p-core-W, including the wild-type HCV core coding region (70R and 91L), and p-core-M, including the mutant-type HCV core (70Q and 91M), were constructed by cloning and PCR-based mutagenesis for control vector of the wild-type core and that of the mutant core, respectively. Using serially diluted forms of these vectors, SyBr Green-based real-time ARMS RT-PCR detection with each of the specific primer pairs was performed. ***Results.*** Each primer could clearly distinguish the difference between p-core-W and p-core-M at the same copy numbers. Concerning substitution 70, the ratios 100:1, 10:1, 1:1, 1:10, and 1:100 of p-core-W versus p-core-M could be distinguished, while for substitution 91, the ratios 100:1, 10:1, 1:1, 1:10, 1:100, and 1:1000 could be distinguished, confirming the sensitivity and specificity of the assay. ***Conclusions.*** This method could be a useful alternative for the detection of genotype 1b HCV core amino acid substitutions 70 and 91 and be reliably applied for rapid screening.

Key Words: *ARMS, core, HCV, interferon response, real-time PCR*

Introduction

More than 170 million people world-wide are chronically infected with hepatitis C virus (HCV), which can lead to hepatic cirrhosis and hepatocellular carcinoma (HCC) [1]. Treatment with peginterferon and ribavirin for 24–48 weeks can result in a sustained loss of serum HCV-RNA (termed a sustained virological response (SVR)), with resolution of chronic hepatitis in approximately half of the patients [2]. Several new, potent HCV protease and polymerase inhibitors have been described recently, but none of them are available for therapeutic use.

The genomic region encoding the HCV core protein is located between amino acids 1 and 191 and is likely to be the first gene product synthesized due to its localization at the 5′ end of the HCV polyprotein transcript [3]. The core protein has an ability to interact with the viral genomic region to form nucleocapsids [4], and the presence of a putative DNA-binding motif, nuclear localization signals, phosphorylation sites, and a nucleocytoplasmic localization of the core protein suggest its possible function as a gene regulatory protein [3,5]. In many previous studies it has been suggested that the HCV core protein may be important in hepatocarcinogenesis and interferon signaling [3,6–8].

HCV genotype 1b is a major genotype (~70%) in Japan. HCV genotype 1 is one of the most refractory to interferon treatment with or without ribavirin. It has been reported that its response to interferon

Correspondence: Tatsuo Kanda, MD, PhD, Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8677, Japan. Tel: +81 43 2262 086. Fax: +81 43 2262 088. E-mail: kandat-cib@umin.ac.jp

(Received 5 February 2009; accepted 29 March 2009)

ISSN 0036-5521 print/ISSN 1502-7708 online
DOI: 10.1080/00365520902937362

monotherapy is affected by HCV NS5A gene diversity [9]. Thus, sequence diversity may predict the response to the combination therapy of peginterferon and ribavirin. Furthermore, ribavirin has different antiviral effects from those of interferon [10]. An approach to the prediction of treatment against hepatitis C in patients who do not have SVR is urgently needed. Several reports suggest that HCV amino acid substitutions 70 and 91 in the core coding region affect the results of combination therapies of interferon and ribavirin [11–13], but most of these studies were retrospective, and we do not know whether these substitutions already existed before treatment or were selected by the treatment. A sensitive, real-time polymerase chain reaction (PCR)-based assay for the detection of these mutations in the presence of high levels of wild-type virus is described here. The method is based on the amplification refractory mutation system (ARMS) reverse transcription (RT)-PCR for detection of single base mutations [14,15].

Material and methods

Plasmid DNA controls

Plasmids carrying HCV genotype 1 b core wild-type and mutant clones were made as described previously [16,17] and are summarized in Table I. Plasmid DNA was purified using the QIAprep spin miniprep kit (Qiagen, Hilden, Germany). Plasmids were serially diluted 1:10 in EASY dilution (for real-time PCR) (Takara, Ohtsu, Shiga, Japan) to give a dilution range of 1–1 $\times 10^9$ copies for controls of real-time PCR.

Extraction of HCV-RNA from serum

Serum samples (100 µl) were extracted using the high pure viral RNA kit (Roche Diagnostics, Indianapolis, Ind., USA) according to the manufacturer's protocol. The RNA was eluted in RNase-free water. Written informed consent was obtained from each patient included in this study.

cDNA synthesis and SyBr Green real-time PCR

Reverse transcription was carried out using random hexamers to make HCV cDNA by superscript cDNA synthesis kit (Invitrogen, Carlsbad, Calif., USA). ARMS primers were designed so that the 3′ base matched either the wild-type or mutant sequence [18] (Table II). Each 25-µl reaction contained 2× Power SYBR Green PCR Master Mix (Applied Biosystems, Tokyo, Japan), 2.5 pmol of each primer (Table II). Reactions were run on the Step One real-time PCR system (Applied Biosystems). Cycling conditions were: denaturation at 95°C for 10 min, then 40 cycles at 95°C for 15 s and 60°C for 1 min, followed by a melting curve analysis, confirming their specificity. A plasmid DNA standard was included in each run.

Cloning of clinical HCV sequences and site-directed mutagenesis

To make the plasmid p-core-mutant, PCR products were cloned into pCR-TOPO2.1 vector (Invitrogen). To make the plasmid p-core-wild, PCR-based *in vitro* site-directed mutagenesis was performed using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, Calif., USA). DNA sequences of clones were confirmed by direct sequencing.

Results

Optimization of real-time PCR

For this study, real-time PCR using the SYBR Green I detection system (Applied Biosystems) was implemented to detect the HCV amplicon. ARMS PCR specificity is conferred by direct placement of the 3′ end of one of the primers (Figure 1). Cross-reactivity was tested to ensure that the primer sets specifically bound their targets.

When 10^8 copies of the CAA (codon c70) template were amplified using the primer with a base mismatch, approximately 15 cycles were required before the crossing threshold was reached. This compares with 8 cycles for the matching primer. On the other hand, when 10^8 copies of the CGA (codon c70) template were amplified using the primer with a base mismatch, approximately 16 cycles were required before the crossing threshold was reached. This compares with 6 cycles for the matching primer (Figure 1A and B).

When 10^8 copies of the ATG (codon c91) template were amplified using the primer with a base mismatch, approximately 25 cycles were required before the crossing threshold was reached. This compares

Table I. Plasmid DNA used as standard in this study.

Plasmid	Amino acid c70	Codon c70	Amino acid c91	Codon c91
p-core-wild	Arginine	CGA	Leucine	CTG
p-core-mutant	Glutamine	CAA	Methionine	ATG

Table II. Primers used for detection of substitutions at residues c70 (A) and c91 (B).

A.

Primers for detection of substitution at c70		
Primer common to all reactions		
c70 sense primers HCV-c-reverse: 5′-CGGGGTGACAGGAGCCATCC-3′	Codon	Amino acids
HCV 70W: 5′-TATCCCCAAGGCTCGCCG-3′	CGN	Arg
HCV 71M: 5′-TATCCCCAAGGCTCGCCA-3′	CAN	Gln, His

N = A, G, T, or C; Arg = arginine; Gln = glutamine; His = histidine.

B.

Primers for detection of substitution at c91		
Primer common to all reactions		
c91 reverse primers HCV-c-sense: 5′-TCGCAACCTCGTGGAAGGC-3′	Codon	Amino acids
HCV 91W: 5′-CATCCTGCCCACCCCAR-3′	TTG or CTG	Leu
HCV 91M: 5′-CATCCTGCCCACCCCAT-3′	ATG	Met

R = A, G; Met = methionine; Leu = leucine.
HCV sequences are identical to AJ238799. Ref. [11].

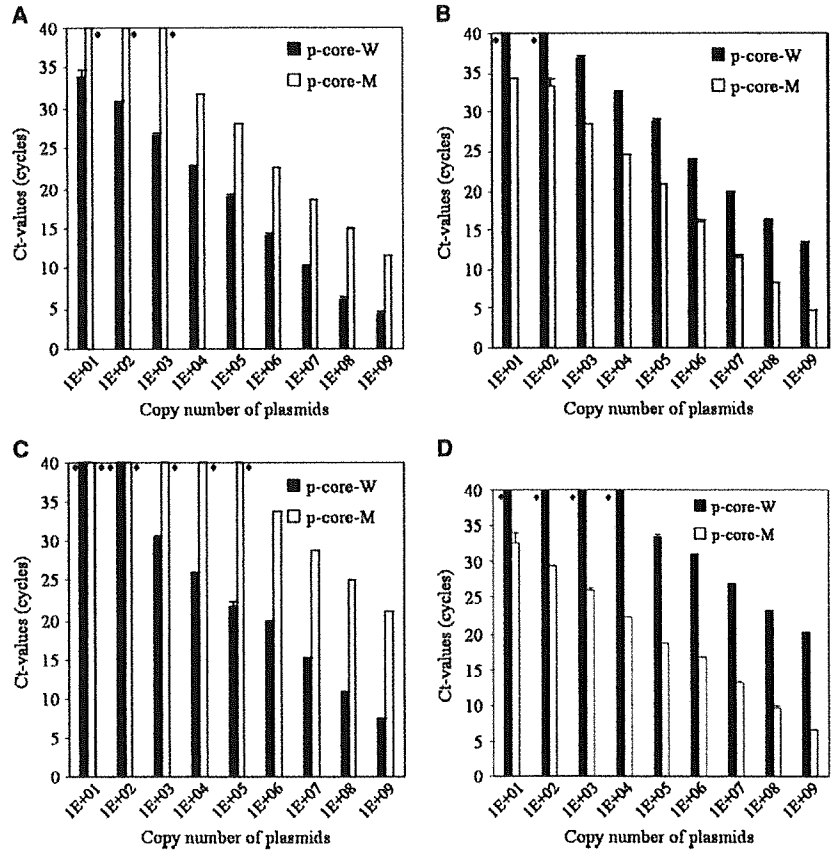

Figure 1. Quantitation of a 10-fold dilution of plasmid p-core-W or p-core-M with wild- or mutant-type primers. Cycle numbers were plotted against the logarithmic concentration of serial dilutions. A. c70-wild primer sets (HCV-70W and HCV-c-reverse). B. c70-mutant primer sets (HCV-70M and HCV-c-reverse). C. c91-wild primer sets (HCV-c-sense and HCV-91W). D. c91-mutant primer sets (HCV-c-sense and HCV-91M). *Unable to detect any signals by 40 cycles.

with 10 cycles for the matching primer. On the other hand, when 10^8 copies of the CTG (codon c91) template were amplified using the primer with a base mismatch, approximately 23 cycles were required before the crossing threshold was reached. This compares with 10 cycles for the matching primer (Figure 1C and D).

The detection limits of these methods were at least 10 copies, 10 copies, 1000 copies, and 10 copies of c70-wild primer sets (HCV-70W and HCV-c-reverse), c70-mutant primer sets (HCV-70M and HCV-c-reverse), c91-wild primer sets (HCV-c-sense and HCV-91W), and c91-mutant primer sets (HCV-c-sense and HCV-91M), respectively (Figure 1).

Selectivity of ARMS assay

Using the plasmid mixture containing the wild-type (p-core-W) and the mutant-type (p-core-M) as a template, real-time ARMS PCR was performed to establish the concentration at which the c70-wild primer sets (HCV-70W and HCV-c-reverse) would detect the wild-type DNA (codon c70). In Table IIIA we present the results of these primer sets showing that, when the wild DNA was 10^9 copies/tube, from 10^5 to 10^9 copies of mutant templates did not affect the results. When the mutant DNA was 10^9 copies/tube, from 10^9 to 10^7 copies of the wild templates could be detected. Similarly, each primer could clearly distinguish the difference between p-core-W and p-core-M at the same copy numbers. Concerning substitution 70, the ratios 100:1, 10:1, 1:1, 1:10, and 1:100 of p-core-W versus p-core-M could be distinguished (Table IIIA and B). However, for substitution 91, the ratios 100:1, 10:1, 1:1, 1:10, 1:100, and 1:1000 could be distinguished, confirming the sensitivity and specificity of the assay [16] (Table IIIC and D).

Hepatitis C core substitutions in serum by real-time ARMS RT-PCR

Quantitative ARMS assays were carried out in parallel reactions, one with a primer matching the variant at the 3′ end, and the other with the primer matching the wild-type variant. We measured the HCV core substitutions at residues c70 and c91 in two patients who did not respond to combination peginterferon and ribavirin therapy after 12 weeks and finally did not become SVRs (Table IV). In patient no. 1, we could detect the minority, wild-type at c70 (4% at 4 weeks). This became diminished at 12 weeks after treatment. In both patients, we could not detect any wild-type template at 12 weeks after treatment.

Comparison of real-time ARMS RT-PCR and conventional sequencing

The real-time ARMS RT-PCR method was compared to direct sequencing in patients treated with peginterferon and ribavirin. In patient no. 1, the minority, wild-type at c70 at 4 weeks could not be detected by direct sequencing (Table IV). In patient no. 2, there were some discrepancies between the results of direct sequencing and those of real-time ARMS RT-PCR (Table IV).

Table III. A mixture of the dilution series of mutants with fixed concentration of wild-type DNA or mutant-type DNA was assayed with each primer to establish the concentration at which the primers would detect each DNA by real-time ARMS PCR. Copy number: copies/tube; template W: p-core-W; template M: p-core-M.

Copy number of template (W:M)	Ct (cycle number)
A. c70-wild primer sets (HCV-70W and HCV-c-reverse).	
10^5:10^9	12.66±0.050
10^6:10^9	12.47±0.099
10^7:10^9	11.46±0.036
10^8:10^9	8.87±0.279
10^9:10^9	5.29±0.018
10^9:10^8	5.24±0.075
10^9:10^7	5.24±0.070
10^9:10^6	5.15±0.091
10^9:10^5	5.13±0.014
B. c70-mutant primer sets (HCV-70M and HCV-c-reverse).	
10^9:10^5	14.44±0.026
10^9:10^6	14.18±0.017
10^9:10^7	12.66±0.044
10^9:10^8	9.68±0.041
10^9:10^9	6.00±0.126
10^8:10^9	5.72±0.10
10^7:10^9	5.57±0.028
10^6:10^9	5.90±0.072
10^5:10^9	5.77±0.063
C. c91-wild primer sets (HCV-c-sense and HCV-91W).	
10^5:10^9	22.77±0.197
10^6:10^9	20.99±0.182
10^7:10^9	17.46±0.0457
10^8:10^9	13.36±0.10
10^9:10^9	9.30±0.053
10^9:10^8	9.29±0.12
10^9:10^7	9.19±0.043
10^9:10^6	9.14±0.060
10^9:10^5	9.23±0.0011
D. c91-mutant primer sets (HCV-c-sense and HCV-91M).	
10^9:10^5	20.89±0.056
10^9:10^6	18.52±0.351
10^9:10^7	14.89±0.016
10^9:10^8	11.53±0.033
10^9:10^9	7.99±0.023
10^8:10^9	7.82±0.0040
10^7:10^9	7.80±0.0098
10^6:10^9	7.86±0.044
10^5:10^9	7.82±0.0025

Table IV. HCV core substitutions at residues c70 and c91 detected by real-time ARMS RT-PCR and direct sequencing.

Patients No.	Study Week	ALT (IU/L)	HCV-RNA (log copies/ml)	c70 W:M	c91 W:M	Direct sequencing c-70/c-91
1.	0	31	6.6	0:100	0:100	M/M
	4	26	6.3	4:96	0:100	M/M
	12	24	5.8	0:100	0:100	M/M
2.	0	53	6.3	0:100	ND	Mix/M
	4	25	6.0	0:100	0:100	M/M
	12	14	5.3	0:100	0:100	M/M

Abbreviations: ARMS = amplification refractory mutation system; ALT = alanine aminotransferase; W = wild-type; M = mutant-type; Mix = mixed-type; ND = not determined.
"Study Week" = weeks after administration of peginterferon and ribavirin.

Discussion

In this article we describe a rapid and sensitive method for the quantitative detection and monitoring of the core amino acid substitutions of HCV genotype 1b. SyBr Green real-time PCR and specific ARMS primers were used to quantify viral RNAs carrying particular sequences, HCV amino acid substitutions 70 and 91 in the core coding region. The specificity of the ARMS primers results in large differences in PCR crossing thresholds being observed between matching and mismatched targets.

For the current standard treatment with peginterferon alpha and ribavirin in patients with chronic hepatitis C, infection with HCV genotypes 2 and 3, lower baseline viral load, Asian and Caucasian ethnicity, younger age, low γ-GTP levels, absence of advanced fibrosis/cirrhosis, and absence of steatosis in the liver have been identified as independent pretreatment predictors of SVR [19]. Early virological response (EVR), defined as a ≥2-log reduction in HCV-RNA or undetectable HCV-RNA at 12 weeks, is associated with a favorable virological response. EVR is reached in only ~70% of patients infected with genotype 1 treated with combination therapy [20,21].

Recently, it was reported that core residues Arg70 and Leu91 were associated with response therapy in Japanese genotype 1b patients [11,13]. Donlin et al. [12] reported a similar association of Arg70 with a marked response for genotype 1b but not 1a; however, Met91 was highly dominant in both the marked- and poor-responder sequences, but few other studies have examined the role of diversity in the core in the outcome of therapy. Concerning hepatocarcinogenesis associated with HCV genotype 1b, Akuta et al. [22] reported that cumulative hepatocarcinogenesis rates in double wild-type (Arg70 and Leu91) of the HCV core region were significantly lower than those in non-double wild-type. Direct sequencing [11,13] and nested-RT-PCR using ARMS primers with gel electrophoresis [12,22] were performed in these studies. Higher sensitivity assays may be more useful for predicting the outcomes of therapy and hepatocarcinogenesis [23]. The real-time ARMS RT-PCR described here does not require restriction enzyme digestion, gel-electrophoresis or sequence analysis of PCR products, and it can quantify the core substitution proportions more quickly.

Hepatitis C core substitutions in serum detected by real-time ARMS RT-PCR showed mutant c70 and mutant c91 at 12 weeks in two non-EVRs (Table IV). Most non-SVR rates result from non-EVR. It was reported that the 72-week regimen significantly improved the SVR rates in non-EVRs with Arg70 and/or Leu91 of core [24]. Peginterferon plus ribavirin treatment is costly and has several side effects, possibly reducing its attractiveness for patients. If we were able to identify these HCV core substitutions at 12 weeks, we would know whether to stop or continue treating patients. This could prevent patients from serious side effects or bring about a better treatment outcome by the resulting shorter regimens. Moreover, if direct viral enzyme inhibitors such as protease inhibitor and polymerase inhibitor, which potently suppress viral replication, could be used, the predictability of outcome would be even more important. Recently, it was also reported that maintenance or prolonged peginterferon did not reduce the incidence of HCC in advanced chronic hepatitis C patients [1,25]. We are now focusing on a larger study, and real-time ARMS RT-PCR is expected to be useful for the important prediction of peginterferon plus ribavirin treatment outcomes or that of hepatocarcinogenesis in hepatitis C patients.

Acknowledgements

We thank Professor M. Omata, Department of Gastroenterology, University of Tokyo, for invaluable discussions. This work was supported by the Ministry of Education, Culture, Sports, Science and Technology, Japan (TK, OY).

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Di Bisceglie AM, Shiffman ML, Everson GT, Lindsay KL, Everhart JE, Wright EC, et al. Prolonged therapy of advanced chronic hepatitis C with low-dose peginterferon. N Engl J Med 2008;359:2429–41.

[2] George SL, Bacon BR, Brunt EM, Mihindukulasuriya KL, Hoffmann J, Di Bisceglie AM. Clinical, virologic, histologic, and biochemical outcomes after successful HCV therapy: a 5-year follow-up of 150 patients. Hepatology 2008; Epub ahead of print.

[3] Basu A, Meyer K, Ray RB, Ray R. Hepatitis C virus core protein modulates the interferon-induced transacting factors of Jak/Stat signaling pathway but does not affect the activation of downstream IRF-1 or 561 gene. Virology 2001;288:379–90.

[4] Shimoike T, Mimori S, Tani H, Matsuura Y, Miyamura T. Interaction of hepatitis C virus core protein with viral sense RNA and suppression of its translation. J Virol 1999;73: 9718–25.

[5] Otsuka M, Kato N, Lan K-H, Yoshida H, Kato J, Goto T, et al. Hepatitis C core protein enhances p53 function through augmentation of DNA binding affinity and transcriptional ability. J Biol Chem 2000;275:34122–30.

[6] Kanda T, Steele R, Ray R, Ray RB. Hepatitis C virus core protein augments androgen receptor-mediated signaling. J Virol 2008;82:11066–72.

[7] Pavio N, Battaglia S, Boucreux D, Arnulf B, Sobesky R, Hermine O, et al. Hepatitis C virus core variants isolated from liver tumor but not from adjacent non-tumor tissue interact with Smad3 and inhibit the TGF-beta pathway. Oncogene 2005;24:6119–32.

[8] Miller K, McArdle S, Gale M Jr, Geller DA, Tenoever B, Hiscott J, et al. Effects of the hepatitis C virus core protein on innate cellular defense pathways. J Interferon Cytokine Res 2004;24:391–402.

[9] Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 1996; 334:77–81.

[10] Kanda T, Yokosuka O, Imazeki F, Tanaka M, Shino Y, Shimada H, et al. Inhibition of subgenomic hepatitis C virus RNA in Huh-7 cells: ribavirin induces mutagenesis in HCV RNA. J Viral Hepat 2004;11:479–87.

[11] Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 2005;48:372–80.

[12] Donlin MJ, Cannon NA, Yao E, Li J, Wahed A, Taylor MW, et al. Pretreatment sequence diversity differences in the full-length hepatitis C virus open reading frame correlate with early response to therapy. J Virol 2007;81:8211–24.

[13] Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol 2007;46: 403–10.

[14] Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, et al. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res 1989;17:2503–16.

[15] Tada M, Omata M, Kawai S, Saisho H, Ohto M, Saiki R, et al. Detection of ras gene mutations in pancreatic juice and peripheral blood of patients with pancreatic adenocarcinoma. Cancer Res 1993;53:2472–4.

[16] Kanda T, Yokosuka O, Tagawa M, Kawai S, Imazeki F, Saisho H. Quantitative analysis of GBV-C RNA in liver and serum by strand-specific reverse transcription-polymerase chain reaction. J Hepatol 1998;29:707–14.

[17] Kanda T, Yokosuka O, Nagao K, Saisho H. State of hepatitis C viral replication enhances activation of NF-kB- and AP-1-signaling induced by hepatitis B virus X. Cancer Lett 2006;234:143–8.

[18] Okamoto K, Akuta N, Kumada H, Kobayashi M, Matsuo Y, Tazawa H. A nucleotide sequence variation detection system for the core region of hepatitis C virus-1b. J Virol Methods 2007;141:1–6.

[19] Kau A, Vermehren J, Sarrazin C. Treatment predictors of a sustained virologic response in hepatitis B and C. J Hepatol 2008;49:634–51.

[20] Herrmann E, Zeuzem S. Ribavirin plus either peginterferon alpha-2a or peginterferon alpha-2b for patients with chronic HCV infection? Nat Clin Pract Gastroenterol Hepat 2008;5: 362–3.

[21] Di Bisceglie AM, Ghalib RH, Hamzeh FM, Rustgi VK. Early virologic response after peginterferon alpha-2a plus ribavirin or peginterferon alpha-2b plus ribavirin treatment in patients with chronic hepatitis C. J Viral Hepat 2007;14: 721–9.

[22] Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Amino acid substitutions in the hepatitis C virus core region are the important predictor of hepatocarcinogenesis. Hepatology 2007;46:1357–64.

[23] Kawai S, Yokosuka O, Kanda T, Imazeki F, Maru Y, Saisho H. Quantification of hepatitis C virus by TaqMan PCR: comparison with HCV Amplicor Monitor assay. J Med Virol 1999;58:121–6.

[24] Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, et al. A matched case-controlled study of 48 and 72 weeks of peginterferon plus ribavirin combination therapy in patients infected with HCV genotype 1b in Japan: amino acid substitutions in HCV core region as predictor of sustained virological response. J Med Virol 2009;81:452–8.

[25] 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.

Cancer Science
The official journal of the Japanese Cancer Association

Phase I clinical study of a peptide vaccination for hepatitis C virus-infected patients with different human leukocyte antigen-class I-A alleles

Shigeru Yutani,[1,2] Nobukazu Komatsu,[1,3] Shigeki Shichijo,[1] Kazumi Yoshida,[1] Hiroko Takedatsu,[1] Minoru Itou,[2] Ryoko Kuromatu,[2] Tatsuya Ide,[2] Masatoshi Tanaka,[2] Michio Sata,[2] Akira Yamada[1,3,4] and Kyogo Itoh[1,3]

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

(Received April 17, 2009/Revised June 12, 2009/Accepted June 14, 2009/Online publication July 13, 2009)

Hepatitis C virus (HCV) infection has a high risk of liver cirrhosis and hepatocellular carcinoma at later stages. We recently identified a peptide derived from the HCV core protein capable of inducing both cellular and humoral responses to nearly all HCV-positive patients in Japan with different human leukocyte antigen (HLA)-class I-A alleles. To assess the safety and immune responses to this novel peptide, we conducted a phase I dose-escalation study of the vaccination for 26 HCV-positive patients who were either non-responders to the interferon-based therapy ($n = 23$) or refused it ($n = 3$). The regimen was well tolerated, with no severe vaccine-related toxicity. Twenty-five and 22 patients completed the first and second cycle vaccination (6 and 12 vaccine injections), respectively. After a series of six vaccine injections, peptide-specific CTL activity was augmented in peripheral blood mononuclear cells from 15 of 25 patient samples, with an expected optimal dose of 1 mg peptide. After 12 vaccine injections, peptide-specific IgG production was augmented in plasma from the majority of patients (15 of 22 patients) tested, but not in a dose-dependent fashion. There were two HCV RNA responders with >1 log declines. Among patients whose pre-vaccination levels of alanine aminotransferase and alpha feto-protein exceeded the normal ranges, a <30% decrease was found in 7 of 24 and three of six patients, respectively. Because of its tolerability and higher rate of immune boosting, this protocol is recommended for a phase II study to investigate its clinical efficacy. (*Cancer Sci* 2009; 100: 1935–1942)

Hepatitis C virus (HCV) is prevalent worldwide, with nearly 180 million individuals infected.[1,2] Interferon (IFN)-based therapies, although effective in 80% of patients infected with the HCV2 and HCV3 genotypes and also 50% of patients with the HCV1b genotype, have several limitations, including medical or physical contraindications, adverse events, and high cost.[1–4] HCV1b is the most frequently observed genotype in Japan (70%) and is also frequently observed in the USA.[3,4] HCV-infected patients tend to develop liver cirrhosis (LC) and ultimately hepatocellular carcinoma (HCC). From this point of view, therapeutic HCV vaccines are also prophylactic cancer vaccines for HCV-related HCC. Thus, there have been a variety of efforts to develop a HCV vaccine, including clinical trials of HCV vaccines with peptides capable of inducing human leukocyte antigen (HLA)-A2- or HLA-A24-restricted CTL responses.[5–7] However, no promising clinical outcome in those trials have been reported at the present time from the view of sustained viral responses. This might be in part due to insufficient activity to boost CTL activity. Alternatively, this might be in part due to an inability to induce humoral responses.

We recently identified a peptide derived from the HCV core protein capable of inducing both cellular and humoral responses to nearly all HCV-positive patients in Japan with various HLA-class I-A alleles (Niu Y, Komatsu N, Komohara Y, Matsueda S, Yutani S, Ishihara Y, Itou M, Yamada A, Itoh K, Shichijo S unpubl. data). This peptide is well known as a HLA-A2-restricted CTL epitope,[8,9] and its sequence is shared in the HCV1a, HCV1b, HCV2a, and HCV3a major genotypes found worldwide. In the present paper, we have reported the results of a phase I dose-escalation study of this peptide vaccination.

Materials and Methods

Patient eligibility. This was a phase I dose-escalation study. All laboratory tests required in order to assess eligibility had to be completed within 7 days prior to the start of treatment. The following inclusion criteria were mandatory:

(1) Patients were required to have persistent HCV infection confirmed by HCV RNA test using serum.
(2) All patients were diagnosed with chronic hepatitis (CH) or LC by laboratory tests (hepatic enzymes and platelet count) and ultrasonography.
(3) Patients were either non-responders to the previously conducted IFN-based treatments ($n = 23$) or refused to receive them ($n = 3$).
(4) Patients had no detectable levels of HCC at the time of entry.
(5) Patients were required to be positive for one of the following alleles: HLA-A2, HLA-A11, HLA-A24, HLA-A26, HLA-A31, or HLA-A33.
(6) Patients were required to have an Eastern Cooperative Oncology Group performance status of 0–1, age between 20 and 75 years, and adequate hematological (white blood cell count ≥2400/μL, hemoglobin level ≥8.0 g/dL, platelet counts ≥50 000/μL), renal (serum creatinine ≤1.4 g/dL), and hepatic (total bilirubin <2.5 mg/dL) functions.
(7) Patients were required to be negative for hepatitis B antigens.
(8) Patients were required to have had at least 4 weeks of recovery from the toxic effects of any previous therapy before trial entry.

Pregnant patients and patients with an autoimmune disease, an active infection, cancer, or hepatic encephalopathy were excluded. Patients with ascites were also excluded. The study protocol was approved by the Ethical Committee of Kurume University, and complete written informed consent was obtained from all patients at the time of enrollment. A total of 26 patients who were seen at our institution between October 2004 and June 2008 were included in this study.

Peptide and vaccination. The vaccinated peptide was originated from HCV core protein at positions 35–44 (C35-44,

[4]To whom correspondence should be addressed. E-mail: aklymd@med.kurume-u.ac.jp

doi: 10.1111/j.1349-7006.2009.01256.x

YLLPRRGPRL), a well-known HLA-A2-restricted CTL epitope[8,9] that is conserved in various HCV genotypes (3, 4, 8, and 9). Our manuscript currently under submission elsewhere can be summarized as follows. This peptide demonstrated binding activity to HLA-A*2402, HLA-A*2601, HLA-A*3101, and HLA-A*3303 molecules, but showed no binding to HLA-A*1101. With regard to HLA-A2 subtypes, the peptide demonstrated binding activity to HLA-A*0201 and HLA-A*0206 molecules, but not to HLA-A*0207 molecules. The peptide induced CTL activity in both patients and healthy donors with all the HLA-class I-A molecules mentioned above including HLA-A*1101 and HLA-A*0207, as far as tested by the IFN-γ production assay.

The peptide was prepared under the conditions of Good Manufacturing Practices by the American Peptide Company (San Diego, CA, USA), and was dissolved and stored at −80°C. Stock solutions were diluted with saline just before use. For injection of 0.3, 1, and 3 mg of peptide (levels 1, 2, and 3), 1 mL of the peptide, which was supplied in vials containing 1, 2, or 4 mg/mL sterile solution, was mixed with 1 mL of incomplete Freund's adjuvant (Montanide ISA51VG; Seppic, Paris, France) and emulsified in 5-mL sterilized syringes followed by 0.6, 1, and 1.5 mL injection, respectively. The peptide emulsion was injected biweekly into the subcutaneous region of the side abdomen. All patients were treated in an outpatient clinic. Blood for immunological studies was obtained before vaccine injections, after the sixth and 12th injections. The first cycle consisted of six vaccinations, and second or later cycles of six vaccinations were conducted when the patients agreed and in the absence of severe toxicity. Twenty-five and 22 patients completed the first and second cycle vaccination (six and 12 vaccine injections), respectively.

Cellular and humoral responses to peptides. Thirty milliliters of peripheral blood was obtained before and after vaccinations for the measurement of CTL precursors in peripheral blood mononuclear cells (PBMC) and IgG specific to C35-44 peptide in plasma, according to previously reported methods.[10–13] The positive control peptides used in this study were Epstein–Bar virus (EBV)- and influenza virus (Flu)-derived peptides capable of inducing CTL activity restricted to HLA-A2, HLA-A3 supertype (A11, A31, A33), and HLA-A24 alleles, as reported previously.[10,11,14] The negative control peptides were human immunodeficiency virus (HIV)-derived peptides capable of inducing CTL activity restricted to HLA-A2, HLA-A3 supertype, and HLA-A24 alleles as reported previously,[10,11,14] whereas the sequence of the peptide to the HLA-A26 allele is EVIPMFSAL.[15] In brief, for the CTL precursor assay, the peptide-stimulated PBMC were harvested and tested for their ability to produce IFN-γ in response to T2 cells for HLA-A2$^+$ (A0201, A0206, A0207) cases or CIR cells expressing HLA-A1101, HLA-A2401, HLA-A2601, HLA-A3101, or HLA-A3303 molecules for the corresponding cases. These cells were preloaded with either a corresponding peptide or with a HIV peptide as a negative control. The level of IFN-γ was determined by an ELISA carried out in quadruplicate. A two-tailed Student's *t*-test was used for the statistical analysis. A well was considered positive when the level of IFN-γ production in response to a corresponding peptide was significantly higher ($P < 0.05$) than that in response to a HIV peptide, and when the amount of IFN-γ produced in response to the peptide was more than 50 ng/mL greater than the amount produced in response to a HIV peptide.

The plasma levels of peptide-specific IgG were measured by an ELISA system, and the results were shown as optical density as reported previously.[12] An increment of peptide-specific IgG was judged to have occurred if the IgG amount showed a more than 1.5-fold increase.

The peptide-specific antibody levels were also measured by microsuspension array technique as reported previously.[7] Briefly, diluted plasma samples were incubated with peptide-coated microspheres. After washing, the microspheres were incubated with antihuman Ig isotype-specific antibodies. After washing, the microspheres bound with each antibody were reacted with the biotin-labeled detection antibody and R-phycoerythrin corresponding antibody, and the antibody levels were detected using a Luminex system, FLEXMAP3D, Luminex Corp., Austin, TX. As a control, IgG to HCV core protein was also measured by radioimmunoassay at the commercial level (SRL, Tokyo, Japan), and the levels were shown in international units (IU). All of the pre- and post-vaccination samples were measured simultaneously to avoid any possible biases associated with *in vitro* assays.

Adverse events. Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 (http://ctep.cancer.gov).

Clinical laboratory data. Clinical laboratory values, such as serum levels of alanine aminotransferase (ALT), alpha fetoprotein (AFP), and blood platelet numbers, were measured by the clinical laboratory division of the Kurume University Hospital. Quantitation of HCV RNA was carried out by a clinical laboratory company (SRL).

Results

Patient demographics. Seven, seven, and 14 patients were initially planned for level 1 (0.3 mg per peptide), level 2 (1 mg per peptide), and level 3 (3 mg per peptide), respectively, in this phase I dose-escalation study. As a result, a total of 26 patients infected with HCV (25 HCV1b and 1 HCV2a: patient 5) were enrolled (Table 1). Among them, three patients under level 1 setting (patients 2, 3, and 6) received at first 0.3 mg per peptide followed by 1 mg per peptide because of both no severe toxicity and stable disease with a new informed consent. Therefore, only five patients were invited for level 2 setting. One patient (patient 14) dropped out of the study after the second vaccination with vaccine-unrelated death. Twenty-five and 22 patients completed the first and second cycle vaccination (six and 12 vaccine injections), respectively, and thus were evaluable for toxicity and immunological evaluations. The patients' characteristics are shown in Table 1. Twenty-one patients were diagnosed with CH, and the remaining five were diagnosed with LC. Of the five LC patients, three had HCC that had been removed prior to their enrollment in the study. Twenty-three patients were non-responders to the IFN-based treatments, whereas the remaining three had no history of IFN therapy.

Toxicity. One LC patient (patient 14) had acute intestinal infection and pneumococcal infection with acidosis, disseminated intravascular coagulation, and renal insufficiency 11 days after the second vaccination, and died of sepsis 21 days after the vaccination. Any vaccination-related symptomatic alterations were not observed after the last vaccination. The institutional safety evaluation committee concluded that the death of patient 14 was not a vaccine-related event. It should be noted that patient 14 had a history of splenectomy 11 months earlier, which might have affected host immunity against the infection. Except for this case, no severe toxicity was observed during the study. Grade 1 or 2 local inflammation at the injection site was observed in 13 or 11 patients during the vaccinations, respectively. The local events disappeared within 1 week after the vaccination in most cases. Grade 1 fatigue and headache were observed in 11 and four patients, whereas grade 1 or 2 flu-like symptoms were observed in three or one patients, respectively. No correlation was observed between the inoculated doses of vaccine peptides and the onset or duration of symptoms. These results indicated that this protocol was well tolerated.

Cellular immune responses. Peptide (C35-44)-specific cellular immune activities were measured using the PBMC before vaccination and after the sixth and 12th vaccinations (Table 2). Each of the two HLA allele-restricted CTL activities was independently measured in the patients with heterozygous HLA-class I-A alleles. Augmentation of peptide-specific CTL responses was judged to have occurred either if the number of positive wells increased or

doi: 10.1111/j.1349-7006.2009.01256.x

Table 1. Patient's characteristics

Patient	HLA type†	Disease‡	Age/Sex	Previous treatment	Peptide dose (mg)	Total vaccination (times)
1	A*3303	CH	50/F	IFN + RBV	0.3	15
2	A*1101/3101	CH	61/F	IFN, IFN + RBV	0.3, 1	33
3	A*2602/A3101	CH	51/M	IFN	0.3, 1	19
4	A*0201/A3303	LC	50/M	IFN + RBV	0.3	38
5	A*0207/A2402	CH	71/M	IFN + RBV	0.3	64
6	A*0206/A2402	CH	55/M	IFN	0.3, 1	57
7	A*0206/A2402	CH	62/M	IFN + RBV	0.3	8
8	A*0206/A3303	CH	49/F	IFNβ, IFN + RBV	1	26
9	A*0206/A3303	CH	38/M	IFN (3 times), p-IFN	1	39
10	A*0201	LC	61/M	IFN + RBV	1	10
11	A*3101	CH	58/M	–	1	13
12	A*0207/A2402	CH	60M	IFN + RBV, p-IFN	1	6
13	A*0206/A2402	LC	61/F	IFN + RBV, IFN	3	22
14	A*0206	LC	69/F	IFN + RBV, p-IFN	3	2
15	A*0201/A3303	LC	58/M	IFN, p-IFN	3	27
16	A*1101/A3101	CH	70/M	IFN + RBV	3	33
17	A*0207/A2402	CH	68/M	IFNβ, IFN + RBV	3	15
18	A*2402	CH	64/M	IFNβ, IFN + RBV	3	19
19	A*2603/A3303	CH	70/M	IFNα, IFNβ	3	29
20	A*2402	CH	62/F	–	3	25
21	A*0201/A3303	CH	53/F	IFN	3	22
22	A*1101/A2402	CH	63/F	–	3	26
23	A*0201/A2603	CH	58/F	IFN + RBV	3	25
24	A*1101/A2402	CH	63/F	IFN + RBV (2 times)	3	24
25	A*2402	CH	57/F	IFN + RBV	3	17
26	A*0201/A2402	CH	58/F	IFN	3	17

†HLA, human leukocyte antigen. ‡CH, chronic hepatitis; IFN, interferon; LC, liver cirrhosis; p-IFN, peg interferon; RBV, ribavirin.

if the amounts of INF-γ increased more than twofold in the case of equal numbers of positive wells in the quadruplicate assays. Under these circumstances, the peptide-specific CTL responses at least by one of the HLA-I-A alleles in PBMC after the sixth vaccination were augmented in one of seven, five of five, and 9 of 13 of patients at levels 1, 2, and 3, respectively. The augmentation also occurred in PBMC after the 12th vaccination. CTL augmentation in more than two wells among four wells occurred mostly in the post-vaccination samples from patients with levels 2 and 3. These results indicated that level 1 (0.3 mg peptide per injection) is not sufficient to induce peptide-specific CTL activity, and level 2 (1 mg peptide) seemed to be sufficient to induce CTL activity under this biweekly protocol.

We also measured HLA-restricted CTL activity to EBV-derived peptide in 15 patients and Flu-derived peptide in nine patients, taken as positive peptides, to determine whether or not the vaccination of C35-44 peptide influences cellular responses to the other viruses. Pre-injection, post-sixth injection, and post-12th injection PBMC from 15 patients were incubated with each of C35-44, EBV, or Flu peptide relevant to the patients' HLA alleles, after which their CTL activity was measured in quadruplicate assays. CTL precursors were considered to be present when the level of IFN-γ production in response to a corresponding peptide was significantly higher ($P < 0.05$) than that in response to a HIV peptide, and if the amount of IFN-γ produced in response to the peptide was more than 50 ng/mL greater than the amount produced in response to a HIV peptide. Under these circumstances, the CTL precursors for C35-44, EBV, and Flu were detectable in the pre-vaccination PBMC from 6 of 15, 2 of 15, and one of nine patients tested; in the post-sixth vaccination, PBMC from 9 of 15, 4 of 14, and six of nine patients; and in the post-12th vaccination PBMC from 11 of 13, 4 of 13, and six of nine patients, respectively (data not shown). Representative results of four cases are shown in Figure 1. These results indicate that the C35-44 peptide vaccination did not suppress, but rather had a trend to facilitate CTL activity to both EBV- and Flu-derived peptides.

Humoral immune responses. Peptide (C35-44)-specific IgG responses were measured using plasma collected before vaccination and after the sixth and 12th vaccinations (Table 2). The increment was rarely observed in the samples collected after the sixth injections (5 of 26 patients, 19%), but was observed in the majority (15 of 22 patients, 68%) of the post-12th vaccination samples without a dose-dependent manner.

We further measured all the other Ig isotypes and all IgG subclasses reactive to the vaccinated peptide to examine a dominant type of vaccine-induced immune reaction (Th1- or Th2-type reactions), if any. IgG against HCV core protein was also measured as a control. As a result, augmentation in all the other Ig isotypes and all IgG subclasses (IgG1–IgG4) was observed in most patients whose plasma showed the increased IgG response shown in Table 2. Detailed results are given in Tables 3 and 4. The results suggest that both Th1 and Th2 cells are involved in the vaccination-induced humoral responses. The vaccination, however, did not augment IgG reactive to a recombinant HCV core protein (Table 3).

Clinical evaluation. Clinical evaluation was not the objective of this phase 1 study. However, the available information, though very limited, might be important for developing further clinical studies of peptide-based vaccination to HCV-infected patients. During the vaccination period for up to the 12th vaccination, no patient received any treatment other than the vaccination; notably, none of them received injection of glycyrrhizin, a standard drug for patients unresponsive to IFN-based therapies. Detailed results of the clinical evaluation are given in Table 5. A more than one log difference in HCV RNA was considered significant, whereas a more than 30% difference with a consistent trend throughout the vaccination period (1st to 12th) in ALT, platelet counts, and AFP was considered significant. Under these circumstances, no patient showed an increase in HCV RNA, whereas two patients showed a decrease. Two patients at level 1 showed an increase in ALT after the vaccination, whereas seven patients showed a significant decrease. No patient showed either a significant decrease or increase in platelet count. Three patients showed a significant decrease in

Table 2. Immune responses during vaccination[†]

Patient	CTL response (IFN-γ, pg/mL)				IgG response (OD)[§]		
	HLA-restriction[‡]	Pre	Post-sixth	Post-12th	Pre	Post-6th	Post-12th
1	A*3303	0	0	61/66[¶]	0.403	0.441	1.238
2	A*1101	0	0	0	0.637	0.743	1.624
	A*3101	0	0	0			
3	A*2602	0	179	50	0.526	1.786	2.335
	A*3101	0	56	0			
4	A*0201	0	0	111	0.24	0.24	0.326
	A*3303	0	0	0			
5	A*0207	61	0	130	0.8	2	2
	A*2402	0	0	0			
6	A*0206	0	0	0	0.805	0.878	0.959
	A*2402	0	0	0			
7	A*0206	0	0	NA	0.94	1	2.639
	A*2402	0	0	616			
8	A*0206	182	60	94	0.512	0.65	2.735
	A*3303	0	55	0			
9	A*0206	0	0	82	0.867	0.912	2.82
	A*3303	0	272	0			
10	A*0201	0	52/107/116	NA	0.556	0.456	NA
11	A*3101	82	168/213/571	NA	0.677	0.488	NA
12	A*0207	0	94	NA	0.853	0.631	NA
	A*2402	0	467				
13	A*0206	0	0	405/1094/1534	1.588	1.595	2.568
	A*2402	0	50	0			
15	A*0201	340	277/278	0	0.764	0.66	1.164
	A*3303	0	0	0			
16	A*1101	0	0	0	0.1	0.2	0.4
	A*3101	0	0	0			
17	A*0207	0	0	0	1.296	1	1.631
	A*2402	0	153/2339	780			
18	A*2402	0	0	0	0.5	0.5	0.8
19	A*2603	0	0	152/1147	2	2	2.5
	A*3303	0	66	0			
20	A*2402	0	69/343	276	2.538	2.33	2.761
21	A*0201	169	1670	290/380/1520	0.514	0.922	2.703
	A*3303	161	92/643	163/650/678/5288			
22	A*1101	86/332/395	0	0	0.824	0.685	0.985
	A*2402	151	513	88			
23	A*0201	0	199	0	1.128	1.317	1.768
	A*2603	76	104	50			
24	A*1101	134	0	0	1.087	2.084	2.757
	A*2402	90	0	111/250			
25	A*2402	0	0	0	0.886	0.556	0.597
26	A*0201	0	74/171	54/56/79	1.296	0.801	2.368
	A*2402	0	0	166			

[†]CTL activity was measured by interferon (IFN)-γ production assay, whereas IgG response was measured by ELISA; [‡]HLA alle-restricted CTL responses were measured; [§]optical density at 450 nm; [¶]IFN-γ production levels in positive wells of quadruplicate culture are shown. Background levels of values (<50 pg/mL) are indicated as 0. NA, not available.

AFP, a biomarker for liver cancer, among six patients whose pre-vaccination AFP levels exceeded the normal range (>8.7 ng/mL). In addition, one patient at level 1 showed a sharp but transient decline in AFP after the sixth vaccination.

Discussion

Liver damage may be induced by the boosted CTL-directed destruction of HCV-infected liver cells. However, there was no such liver damage throughout the vaccination period despite the fact that peptide vaccination induced both cellular and humoral responses in the majority (>60%) of patients. Rather, decreases in ALT and AFP were seen in a substantial numbers of patients. The other concern was the difficulty of inducing immune responses by the peptide vaccination for non-responders to IFN-based therapy, primarily because of the heterogeneity of HCV, and also because the immune system was suppressed in HCV-positive patients.[16,17] However, this concern also did not arise, and the vaccination successfully induced immune responses in the post-vaccination samples of the majority (>60%) of patients in both the CTL and IgG assays. Our present results, along with the recent increased demand for development of a HCV vaccine,[18] keep alive our hope of developing a clinically effective HCV vaccine.

Level 1 (0.3 mg per peptide) was considered too low to induce CTL activity. Level 2 (1 mg per peptide) was considered an optimal dose to induce CTL activity under this biweekly injection protocol, although level 3 (3 mg per peptide) was also recommended

doi: 10.1111/j.1349-7006.2009.01256.x

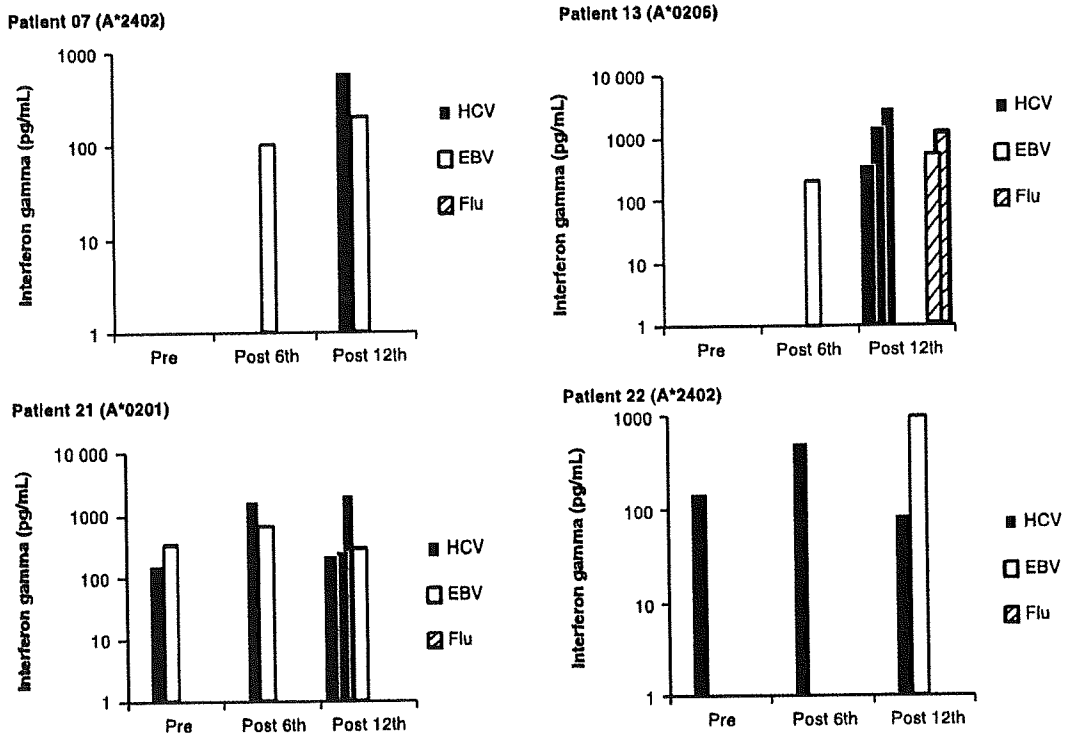

Fig. 1. CTL activity to C35-44, Epstein–Barr virus (EBV), and influenza virus (Flu) peptides. Peripheral blood mononuclear cells from pre-, post-6th, and post-12th vaccination were incubated with each of C35-44, EBV, or Flu peptide relevant to the patients' human leukocyte antigen (HLA) alleles, after which their CTL activity was measured in quadruplicate assays. Representative results of four cases (patients 7, 13, 21, and 22) are shown. Each bar indicates the interferon-γ value of positive wells of quadruplicate culture.

Table 3. Isotypes of antipeptide Ig during vaccination†

Patient	Anti-C35 peptide antibody								Anti-HCV core IgG	
	IgG		IgA		IgM		IgE			
	Pre	Post-12th	Pre	Post-12th	Pre	Post-12th	Pre	Post-12th	Pre	Post-12th
1	1721	2379	387	590	144	338	48	62	130	130
2	823	1071	174	227	88	103	16	17	210	180
5	13 803	12 571	6064	5479	393	276	651	596	350	330
6	432	507	85	123	94	75	10	15	47	49
8	4781	12 158	1668	5732	154	286	158	560	120	130
9	10 531	11 807	4541	4693	32	83	375	398	220	220
10	514	645	191	220	354	412	13	20	460	440
11	13 023	NA†	417	NA	70	NA	536	NA	NA	NA
12	2054	NA	417	NA	37	NA	58	NA	NA	NA
13	740	5070	378	1067	<5	<5	121	151	340	420
15	635	1052	33	426	127	916	19	39	86	100
16	407	1115	117	378	<5	24	8	29	120	120
17	14 537	13 154	6502	6196	70	68	874	822	260	190
18	2357	2615	610	833	50	202	60	61	260	240
19	2449	6720	677	2244	<5	626	43	175	230	250
20	8107	13 318	2990	6058	75	321	339	690	260	330
21	271	5743	68	2224	<5	383	8	154	46	77
22	2987	2134	768	534	177	88	90	68	170	79
23	3445	2925	1148	966	82	72	99	88	170	130
24	477	5404	160	2489	415	441	15	185	49	32
25	13 490	11 111	5865	4168	96	242	474	369	430	330
26	1370	2943	417	1238	169	233	31	100	130	99

†Isotypes of antipeptide (C35-44) Ig in the pre- and post-vaccination (12th) plasma were measured using a Luminex system, and the levels were shown by fluorescence intensity units (FIU). As a control, IgG to hepatitis C virus (HCV) core protein was also measured by radioimmunoassay, and the levels were shown by international units (IU). NA, not available.

Table 4. Subclasses of antipeptide IgG during vaccination†

Patient	Anti-C35 IgG subclass							
	IgG1		IgG2		IgG3		IgG4	
	Pre	Post-12th	Pre	Post-12th	Pre	Post-12th	Pre	Post-12th
1	1002	1735	462	641	309	487	596	783
2	301	410	241	294	265	209	335	404
5	17 950	17 095	9868	8498	6214	5432	8038	7302
6	291	372	117	124	57	73	119	166
8	4994	15 594	2681	9217	1341	5395	2228	7098
9	10 401	13 220	6351	7872	3821	4672	5303	5927
10	803	916	237	250	122	185	386	422
11	17 640	NA	6552	NA	5887	NA	5490	NA
12	2853	NA	495	NA	484	NA	363	NA
13	5541	6750	1927	2269	1550	1635	1881	2148
15	567	1123	148	373	57	227	184	604
16	557	1268	131	501	89	559	172	395
17	17 578	16 423	9124	8877	6977	6276	7796	7797
18	2151	3192	899	1104	600	760	928	1160
19	557	1268	131	501	611	2057	820	2761
20	9753	16 953	4718	9452	2915	6610	4423	8552
21	196	5939	122	3071	57	2240	66	2546
22	2249	1660	878	528	594	334	938	603
23	3795	3297	1368	1166	843	680	1436	1320
24	583	7089	131	3553	102	2323	98	2218
25	14 979	11 654	4428	2760	5166	3928	4647	3396
26	1979	3278	366	1182	403	2318	305	940

†Subclasses of antipeptide (C35-44) IgG in the pre- and post-vaccination (12th) plasma were measured using a Luminex system, and the levels were shown by fluorescence intensity units (FIU). NA, not available.

Table 5. Data for clinical outcomes after vaccination

Patient	HCV RNA (kIU/mL)			ALT (IU/L)			Plt. (× 10^4/mL)			AFP (μg/mL)		
	Pre	Post-6th	Post-12th	Pre	Post-6th	Post-12th	Pre	Post-6th	Post-12th	Pre	Post-6th	Post-12th
1	1730	1830	2120	62	59	83	13.7	13.4	12.4	10.1	11.9	12.6
2	91	27	NA	77	51	NA	11.4	11.7	NA	32.6	51.1	NA
3	3420	4140	3710	41	51	64	22.4	25.4	24.0	3.2	3.5	3.7
4	500	96	6	206	157	105	11.5	8.7	10.6	74.3	32.2	67.5
5	114	102	328	114	154	186	9.3	10.2	8.7	NA	7.8	10.0
6	892	648	840	60	29	48	13.8	14.4	15.0	5.4	6.1	6.2
7	2100	1680	NA	95	100	NA	7.8	8.0	NA	7.0	NA	NA
8	2480	2660	2290	129	112	108	15.8	17.3	15.2	44.4	29.2	28.2
9	2870	2950	2330	358	233	245	5.8	7.4	6.8	28.7	19.5	19.7
10	32 000	16 000	NA	104	95	NA	11.2	10.3	NA	26.9	24.2	NA
11	20	NA	NA	83	NA	NA	13.9	NA	NA	5.6	NA	NA
12	25 000	25 000	NA	144	155	NA	14.9	14.6	NA	5.6	8.1	NA
13	1670	2130	2660	53	47	49	9.4	7.9	7.8	131	86.9	74.0
15	2130	2810	2600	45	34	25	8.0	8.4	9.2	6.8	7.6	7.9
16	2470	3510	2440	47	52	45	25.2	26.0	24.0	4.7	NA	5.0
17	2260	2140	1540	37	31	32	17.3	19.7	19.1	4	3.4	NA
18	3630	3300	2972	60	50	63	13.2	15.0	16.3	22.7	24.4	27.5
19	591	489	443	51	52	53	23.7	24.4	23.8	4.2	NA	3.3
20	583	591	346	24	22	22	19.2	17.3	20.4	4.9	NA	NA
21	4370	3575	3940	34	37	33	23.1	21.2	23.1	NA	NA	3.6
22	59	<5	5	73	25	40	12.4	12.1	12.1	3.1	NA	1.5
23	3045	2380	2730	66	64	65	10.1	10.0	11.1	8.2	9.4	7.6
24	2230	2095	4510	52	36	27	11.7	12.2	11.4	2.9	NA	NA
25	2340	3160	5000	55	55	49	11.1	10.2	12.0	8.5	NA	NA
26	2150	3025	NA	80	58	56	12.4	14.4	14.9	6.8	NA	NA

AFP, α-fetroprotein; ALT, alanine aminotransferase; NA, not assessed; Plt., platelet number.

doi: 10.1111/j.1349-7006.2009.01256.x

because of the higher rate of CTL induction. In contrast, the boosting of IgG production specific to this peptide seemed not to be dependent on the dose, and even level 1 was associated with an increase (>1.5 fold) in IgG in the post-vaccination (12th) plasma from five of seven patients.

We previously reported that a personalized peptide vaccination protocol is superior to a designated protocol from the standpoints of immune responses and clinical responses in patients with advanced stages of cancer.[19–21] This superiority might be due in part to the fact that the pre-designated peptide vaccination stimulates naive or resting T cells and suppresses memory or activated T cells in certain cases, whereas the personalized peptide vaccination stimulates the latter types of T cells.[19–21] In the present study, significant levels of peptide-specific IgG were detected in pre-vaccination plasma of all 26 patients, indicating that memory B cells at least exist in all 26 patients. The vaccination did not suppress CTL activity against Flu or EBV, but rather had a trend to facilitate CTL activity to EVB- and Flu-derived peptides. Based on these results, we considered that this peptide stimulated memory or activated T cells in those HCV-infected patients, which in turn resulted in the higher rates of immune boosting without suppression of the CTL activity to other viruses in HCV-infected patients who failed the IFN-based therapies.

This peptide boosted the CTL activity restricted to each of the HLA-A2 (A0201, A0206, A0207), HLA-A24 (A2402), HLA-A26 (A2602, A2603), HLA-A31 (A3101), and HLA-A33 (A3303) molecules in the post-vaccination samples as far as tested. This is consistent with the results of an *in vitro* analysis (Niu Y, Komatsu N, Komohara Y, Matsueda S, Yutani S, Ishihara Y, Itou M, Yamada A, Itoh K, Shichijo S unpubl. data). Although the CTL boosting was observed in none of the four HLA-A1101$^+$ patients, further studies with additional cases should be conducted to determine whether or not this peptide can boost CTL activity by *in vivo* vaccination for HLA-A1101$^+$ patients.

The sequence of this peptide is well conserved in the different genotypes of HCV, and thus could be applicable not only to HCV1b patients but also to HCV2a patients. Indeed, this peptide boosted both CTL and IgG responses in one HCV2a patient (patient 05) who received 0.3 mg vaccination, although further studies with additional HCV2a cases should be conducted to confirm this possibility.

Although the clinical benefit of this peptide vaccination will be addressed in a future phase II clinical study, it is of note that a decrease in ALT was observed in 7 of 24 patients (29%) during the vaccination. Th1-type immune responses are suggested to be involved in liver damage at chronic phase.[22,23] Therefore, the vaccination-induced CTL boosting is expected to be associated with increased ALT. Indeed, Klade *et al.* reported two such cases who had a transient decrease in HCV RNA concomitant with an ALT increase during the HCV peptide vaccination.[6] In contrast, our two cases showed declines of both HCV RNA and ALT. Although a reason for this discrepancy is unclear at the present time, the different protocols would be at least responsible for this discrepancy. We used only one CTL epitope (C35-44) emulsified with ISA51 adjuvant, whereas they used five peptides containing four CTL epitopes and three helper epitopes with poly-L-arginine as an adjuvant. Augmentation of CTL or IgG responses was observed in six of seven patients showing a decline in ALT in this trial. Nelson *et al.* reported that interleukin-10 treatment results in normalization of ALT in the majority of CH patients who are non-responders to IFN-based treatments.[24] Interleukin-10 promotes the production of IgA, IgG1, and IgG2.[25,26] We showed that all three of these Ig were increased in the post-vaccination samples, suggesting that Th2-type immune responses are at least boosted by the vaccination. Therefore, an increment in Th2-type reactions might be in part responsible for the decline of ALT in these seven patients, although the biological function of these antibodies specific to C35-44 peptide is unknown at the present time.

In addition to ALT, a decrease in AFP level was also observed in three of six evaluable patients. It is well known that HCV core protein plays a pivotal role in the development of HCC.[27,28] Therefore, the vaccination-induced CTL boosting might eliminate precancerous liver cells expressing the HLA-class I-A–C35-44 peptide complex, which may in turn result in decreased AFP. If this is the case, this vaccine may be effective as a cancer prophylactic in CH or LC patients who are resistant to IFN-based therapies and have a high risk of HCC. One LC patient (patient 4) who had a history of HCC showed a decrease in HCV RNA by the vaccination alone. This patient had received IFN-based therapy combined with the vaccination with the result of sustained viral responses. These results suggest the possible benefit of vaccination and IFN therapy for certain HCV patients who fail the standard IFN-based treatment.

HCV is known as a highly variable virus, but the amino acid sequence of this peptide is well conserved in the entire HCV genotype. HLA-A2, HLA-A11, HLA-A24, HLA-A26, HLA-A31, and HLA-A33 types constitute >99% of Japanese, 98% of Asian, 74% of Caucasian, and 50–67% of Black people.[29] This peptide can induce both cellular and humoral responses in patients with these HLA-class I-A alleles. Therefore, this peptide might be useful as a therapeutic HCV vaccine, as well as a prophylactic cancer vaccine for HCV-related HCC, for the majority of people in the world.

Acknowledgments

This study was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, by the 21st Century COE Program for Medical Science, and by Toshi-area Program from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Disclosure Statement

Yamada, Itoh, and Shichijo received a research grant from the Green Peptide Co.; Yamada, Itoh, and Shichijo have stock ownership of the Green Peptide; Yamada is an executive of the Green Peptide.

References

1 Initiative for Vaccine Research: Hepatitis C. (Website on the internet). 2006 Available from URL: http://www.who.int/vaccine_research/diseases/viral_cancers/en/index2.html#disease%20burden. World Health Organization, Geneve, Switzerland, cited at July 6, 2009.
2 Chander G, Sulkowski MS, Jencken MW *et al.* Treatment of chronic hepatitis C: a systematic review. *Hepatol* 2002; **36**: S135–4.
3 Kato N, Hijikata M, Ootsuyama Y *et al.* Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc Natl Acad Sci USA* 1990; **87**: 9524–8.
4 Blatt LM, Mutchnick MG, Tong MJ *et al.* Assessment of hepatitis C virus RNA and genotype from 6807 patients with chronic hepatitis C in the United States. *J Viral Hepat* 2000; **7**: 196–202.
5 Schlaphoff V, Klade CS, Jilma B *et al.* Functional and phenotypic characterization of peptide-vaccine-induced HCV-specific CD8+ T cells in healthy individuals and chronic hepatitis C patients. *Vaccine* 2007; **25**: 6793–806.
6 Klade CS, Wedemeyer H, Berg T *et al.* Therapeutic vaccination of chronic hepatitis C nonresponder patients with the peptide vaccine IC41. *Gastroenterology* 2008; **134**: 1385–95.
7 Yutani S, Yamada A, Yoshida K *et al.* Phase I clinical study of a personalized peptide vaccination for patients infected with hepatitis C virus (HCV) 1b who failed to respond to interferon-based therapy. *Vaccine* 2007; **25**: 7429–35.
8 Battegay M, Fikes J, Di Bisceglie AM *et al.* Patients with chronic hepatitis C have circulating cytotoxic T cells which recognize hepatitis C virus-encoded peptides binding to HLA-A2.1 molecules. *J Virol* 1995; **69**: 2462–70.

9 Cerny A, McHutchison JG, Pasquinelli C *et al.* Cytotoxic T lymphocyte response to hepatitis C virus-derived peptides containing the HLA A2.1 binding motif. *J Clin Invest* 1995; **95**: 521–30.
10 Matsueda S, Tukedatsu H, Susuda T *et al.* New peptide vaccine candidates for epithelial cancer patients with HLA-A3 supertype alleles. *J Immunother* 2007; **30**: 274–81.
11 Wang Y, Takao Y, Harada M *et al.* New epitope peptides derived from hepatitis C virus (HCV) 2a which have the capacity to induce cytotoxic t lymphocytes in HLA-A2$^+$ HCV-infected patients. *Microbiol Immunol* 2006; **50**: 857–65.
12 Takao Y, Yamada A, Yutani S, Sata M, Itoh K. Antibody reactive a hepatitis C virus (HCV)-derived peptide capable of inducing HLA-A2 restricted cytotoxic T lymphocytes is detectable in a majority of HCV-infected individuals without HLA-A2 restriction. *Microbiol Immunol* 2004; **48**: 507–17.
13 Matsueda S, Yamada A, Takao Y *et al.* A new epitope peptide derived from hepatitis C virus 1b possessing the capacity to induce cytotoxic T-lymphocytes in HCV1b-infected patients with HLA-A11-A31 and -A33. *Cancer Immunol Immunother* 2007; **56**: 1359–66.
14 Ogata R, Matsueda S, Yao A, Noguchi M, Itoh K, Harada M. Identification of polycomb group protein enhancer of zeste homolog 2 (EZH2)-derived peptides immunogenic in HLA-A24+ prostate cancer patients. *Prostate* 2004; **60**: 273–81.
15 Yamada N, Ishikawa Y, Dumrese T *et al.* Role of anchor residues in peptide binding to three HLA-A26 molecules. *Tissue Antigens* 1999; **54**: 325–32.
16 Bowen DG, Walker CM. Mutational escape from CD8+ T cell immunity: HCV evolution, from chimpanzees to man. *J Exp Med* 2005; **201**: 1709–14.
17 Rushbrook SM, Ward SM, Unitt E *et al.* Regulatory T cells suppress *in vitro* proliferation of virus-specific CD8+ T cells during persistent hepatitis C virus infection. *J Virol* 2005; **79**: 7852–9.
18 Strickland GT, El-Kamary SS, Klenerman P, Nicosia A. Hepatitis C vaccine: supply and demand. *Lancet Infect Dis* 2008; **8**: 379–86.
19 Mine T, Sato Y, Noguchi M *et al.* Humoral responses to peptides correlate with overall survival in advanced cancer patients vaccinated with peptides based on pre-existing, peptide-specific cellular responses. *Clin Cancer Res* 2004; **10**: 929–37.
20 Noguchi M, Yao A, Harada M *et al.* Immunological evaluation of neoadjuvant peptide vaccination before radical prostatectomy for patients with localized prostate cancer. *Prostate* 2007; **67**: 933–42.
21 Yajima N, Yamanaka R, Mine T *et al.* Immunologic evaluation of personalized peptide vaccination for patients with advanced malignant glioma. *Clin Cancer Res* 2005; **11**: 5900–11.
22 Bertoketti A, Bertoletti A, D'Elios MM *et al.* Different cytokine profiles of intraphepatic T cells in chronic hepatitis B and hepatitis C virus infections. *Gastroenterology* 1997; **112**: 193–9.
23 McGuinness PH, McGuinness PH, Painter D, Davies S, McCaughan GW. Increases in intrahepatic CD68 positive cells, MAC387 positive cells, and proinflammatory cytokines (particularly interleukin 18) in chronic hepatitis C infection. *Gut* 2000; **46**: 260–9.
24 Nelson DR, Lauwers GY, Lau JY, Davis GL. Interleukin 10 treatment reduces fibrosis in patients with chronic hepatitis C: a pilot trial of interferon nonresponders. *Gastroenterology* 2000; **118**: 655–60.
25 Brière F, Servet-Delprat C, Bridon JM, Saint-Remy JM, Banchereau J. Human interleukin 10 induces naive surface immunoglobulin D+ (sIgD+) B cells to secrete IgG1 and IgG3. *J Exp Med* 1994; **179**: 757–62.
26 Defrance T, Vanbervliet B, Brière F, Durand I, Rousset F, Banchereau J. Interleukin 10 and transforming growth factor beta cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. *J Exp Med* 1992; **175**: 671–82.
27 Moriya K, Fujie H, Shintani Y *et al.* The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 1998; **4**: 1065–7.
28 Tanaka N, Moriya K, Kiyosawa K, Koike K, Aoyama T. Hepatitis C virus core protein induces spontaneous and persistent activation of peroxisome proliferator-activated receptor alpha in transgenic mice: implications for HCV-associated hepatocarcinogenesis. *Int J Cancer* 2008; **122**: 124–31.
29 Imanishi T, Akaza T, Kimura A, Tokunaga K, Gojobori T. Allele and haplotype frequencies for HLA and complement loci in various ethnic groups. In: *Proceedings of the Eleventh International Histocompatibility Workshop and Conference*. Oxford, UK: Oxford University Press, 1992; 1065–220.

doi: 10.1111/j.1349-7006.2009.01256.x