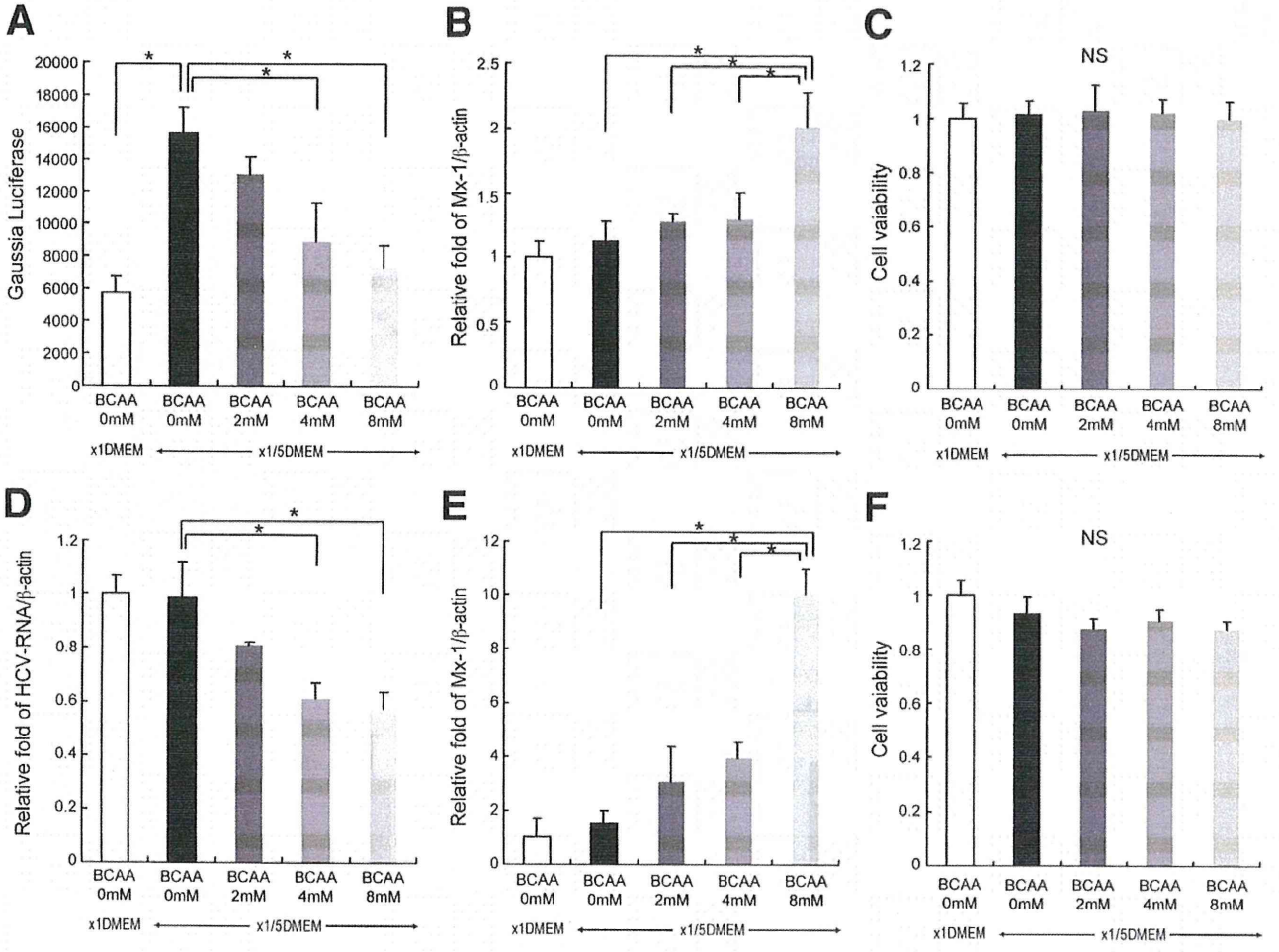

Figure 5. Effect of BCAA on HCV replication in cells in low-amino-acid medium. (*A*) Effect of BCAA on H77S.3/GLuc2A replication in Huh-7.5 cells. (*B*) Mx-1 expression in H77S.3/GLuc2A-transfected Huh-7.5 cells supplemented with BCAA. (*C*) Viability of Huh-7.5 cells. (*D*) Effect of BCAA on JFH-1 replication continuously infecting Huh-7 cells. (*E*) Mx-1 expression in continuously JFH-1–infecting Huh-7 cells supplemented with BCAA. (*F*) Viability of Huh-7 cells.

to these metabolic aspects, recent reports have shown that mTORC1 participates in IFN signaling and antiviral defense responses,[9,10] although the precise signaling pathway has not yet been clarified. In the present study, we evaluated mTORC1 signaling in CH-C livers using gene expression profiling of 91 patients (Figure 1, Supplementary Table 1). We observed a significant negative correlation between plasma Fischer's ratio and hepatic expression of BCAT1, an important catalytic enzyme of BCAA (Figure 1*A*). Moreover, BCAT1 expression was correlated positively with PDCD4 expression, which in turn is regulated negatively by pS6K at the transcriptional level (Figure 1*D*).[16] Thus, the expression of BCAT1 appears to be a negative indicator of mTORC1 signaling in the liver, and the plasma Fischer's ratio is partially reflected by mTORC1 signaling in the liver and muscle.

Interestingly, the expression of c-myc was correlated significantly with BCAT1 (Figure 1*C*) as reported previously.[15] Several studies observed up-regulated c-myc expression in advanced stages of CH-C[19] but, on the other hand, c-myc recently was shown to be a target of mTORC1 in hepatic cells.[17] The existence of a feedback mechanism between c-myc and mTORC1 signaling to maintain liver homeostasis (Figure 1*E*) is plausible, although the precise mechanisms need to be confirmed.

Impaired mTORC1 signaling is suggested to affect the IFN-alfa–induced signaling pathway. To address this, the relationship between mTORC1 and IFN signaling was assessed using a cell culture system. In low-amino-acid medium (×1/5, ×1/30, and ×1/100 DMEM), expression of pSTAT1 was decreased substantially, correlating with the impaired mTORC1 signaling represented by decreased p-mTOR and pS6K expression in Huh-7 cells (Figure 2*A*).

The relationship between mTORC1 and IFN signaling was confirmed further by the knock-down experiment of Raptor, a specific subunit of mTORC1 (Figure 2*B*), although a more precise analysis should be performed to confirm this relationship. Importantly, when Huh-7 cells were stimulated by IFN-alfa, pSTAT1 induction was repressed significantly in low-amino-acid medium (×1/5 DMEM) or in Raptor knocked-down conditions (Figure 2*C*). It therefore could be speculated that IFN treat-

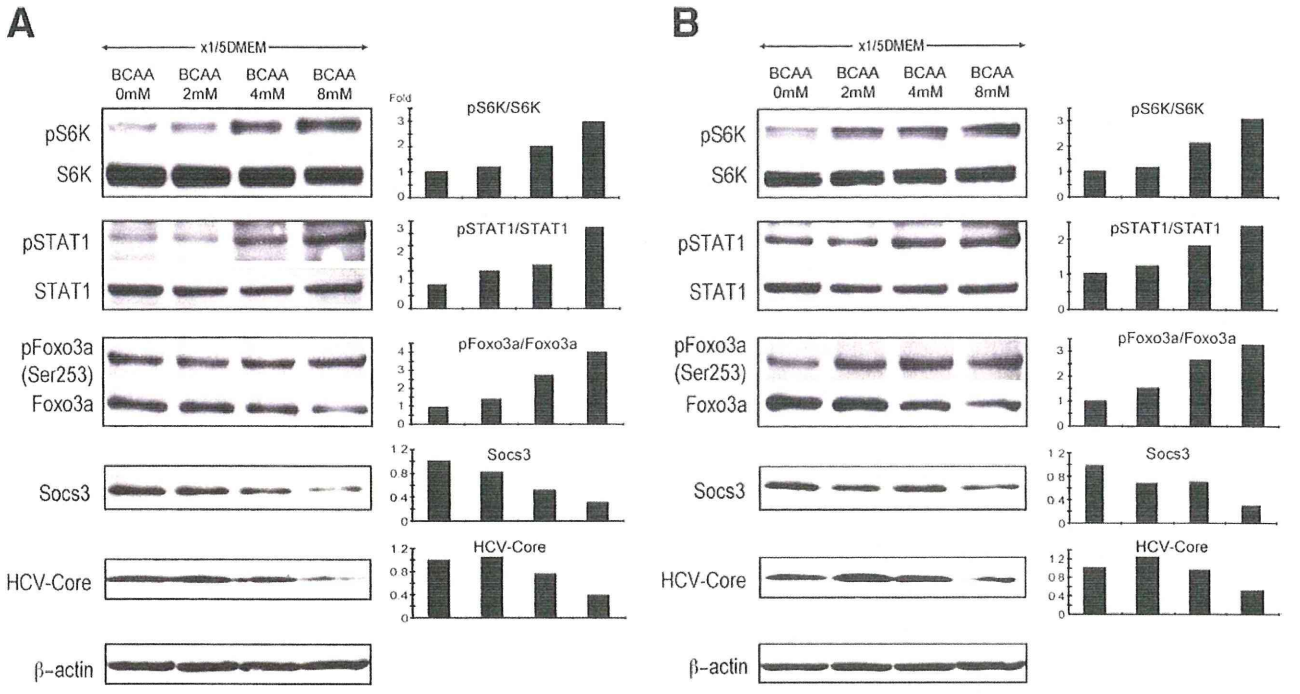

Figure 6. Expression of S6K, STAT1, Foxo3a, Socs3, and HCV core in H77S.3/GLuc2A-transfected Huh-7.5 cells or continuously JFH-1–infecting Huh-7 cells supplemented with BCAA.

ment of patients with liver malnutrition and impaired mTORC1 signaling would lead to reduced induction of ISGs. Importantly, BCAA was able to restore impaired IFN signaling through increased binding of ISGF3γ to its targets (Figure 2*D–F*).

Besides cross-talk of mTORC1 and IFN signaling, we revealed that Foxo3a also is involved in the IFN inhibitory pathway. In low-amino-acid medium, expression of pFoxo3a (ser253) was decreased substantially whereas that of Socs3 was increased. A decreased pFoxo3a/Foxo3a ratio indicates nuclear accumulation of Foxo3a before activation of its target genes, and this was confirmed by immunofluorescent staining (Figure 3*C*). The expression of Foxo3a was significantly positively correlated with that of Socs3 in CH-C liver (Figure 3*F*). These findings prompted us to identify a putative FBE in the Socs3 promoter region (Figure 4*A*). In fact, Socs3 promoter reporter activity was activated by overexpression of Foxo3a, and mutation of FBE impaired Foxo3a-dependent Socs3 promoter activation. Conversely, induction of Socs3 was not observed when expression of Foxo3a was knocked down by siRNA in low-amino-acid medium. Socs3 induction in low-amino-acid medium was owing to increased binding of Foxo3a to the FBE, which was confirmed by ChIP (Figure 4*D*). Therefore, in addition to impaired mTORC1 signaling, the Foxo3a-mediated Socs3 IFN inhibitory pathway might be involved in impaired IFN signaling in patients with liver malnutrition (Figure 4*E*).

Finally, we examined whether BCAA could restore impaired IFN signaling and inhibit HCV replication in cells under conditions of malnutrition. Importantly, BCAA could repress replication of the recombinant genotype 1a-derived HCV, H77S.3/GLuc2A, in a dose-dependent manner (Figure 5*A*). H77S.3/GLuc2A RNA produces infectious virus[14] and, therefore, the results indicate that BCAA might act on a naive HCV infection. Moreover, BCAA inhibited JFH-1–infected Huh-7 cells in which JFH-1 continuously was infecting in a dose-dependent manner. These results indicate that BCAA had an inhibitory effect on either naive or persistent HCV infection irrespective of genotypes (1a and 2a). Consistent with these results, BCAA induced the expression of pSTAT1 and Mx protein in a dose-dependent manner, and repressed Socs3 expression through increasing the ratio of pFoxo3a (ser243) to Foxo3a in a dose-dependent manner (Figures 5 and 6). Therefore, BCAA potentially could restore impaired IFN signaling and inhibit HCV replication in a CH-C state of malnutrition.

In conclusion, we addressed the clinical significance of the nutritional state of the liver on the treatment response of Peg-IFN and RBV combination therapy for CH-C. Although further studies are required to fully define the precise mechanisms underlying mTOR and IFN signaling, we showed that plasma values of Fischer's ratio are a useful nutritional parameter associated with treatment response. Fischer's ratio reflects mTORC1 signaling in the liver, which is correlated with IFN signaling and related to Socs3 IFN inhibitory signaling through Foxo3a. The potential usefulness of BCAA for the augmentation of IFN signaling could suggest a new therapeutic application for advanced-stage CH-C.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.03.051.

Appendix A

The Hokuriku Liver Study Group is composed of the following members: Drs Takashi Kagaya, Kuniaki Arai, Kaheita Kakinoki, Kazunori Kawaguchi, Hajime Takatori, and Hajime Sunakosaka (Department of Gastroenterology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan); Drs Touru Nakahama and Shinji Kamiyamamoto (Kurobe City Hospital, Kurobe, Toyama, Japan); Dr Yasuhiro Takemori (Toyama Rosai Hospital, Uozu, Toyama, Japan); Dr Hikaru Oguri (Koseiren Namerikawa Hospital, Namerikawa, Toyama, Japan); Drs Yatsugi Noda and Hidero Ogino (Toyama Prefectural Central Hospital, Toyama, Japan); Drs Yoshinobu Hinoue and Keiji Minouchi (Toyama City Hospital, Toyama, Japan); Dr Nobuyuki Hirai (Koseiren Takaoka Hospital, Takaoka, Toyama, Japan); Drs Tatsuho Sugimoto and Koji Adachi (Tonami General Hospital, Tonam, Toyama, Japan); Dr Yuichi Nakamura (Noto General Hospital, Nanao, Ishikawa, Japan); Drs Masashi Unoura and Ryuhei Nishino (Public Hakui Hospital, Hakui, Ishikawa, Japan); Drs Hideo Morimoto and Hajime Ohta (National Hospital Organization Kanazawa Medical Center, Kanazawa, Ishikawa, Japan); Dr Hirokazu Tsuji (Kanazawa Municipal Hospital, Kanazawa, Ishikawa, Japan); Drs Akira Iwata and Shuichi Terasaki (Kanazawa Red Cross Hospital, Kanazawa, Ishikawa, Japan); Drs Tokio Wakabayashi and Yukihiro Shirota (Saiseikai Kanazawa Hospital, Kanazawa, Ishikawa, Japan); Drs Takeshi Urabe and Hiroshi Kawai (Public Central Hospital of Matto Ishikawa, Hakusan, Ishikawa, Japan); Dr Yasutsugu Mizuno (Nomi Municipal Hospital, Nomi, Ishikawa, Japan); Dr Shoni Kameda (Komatsu Municipal Hospital, Komatsu Ishikawa, Japan); Drs Hirotoshi Miyamori and Uichiro Fuchizaki (Keiju Medical Center, Nanao, Ishikawa, Japan); Dr Haruhiko Shyugo (Kanazawa Arimatsu Hospital, Kanazawa, Ishikawa, Japan); Dr Hideki Osaka (Yawata Medical Center, Komatsu, Ishikawa, Japan); Dr Eiki Matsushita (Kahoku Central Hospital, Tsubata, Ishikawa, Japan); Dr Yasuhiro Katou (Katou Hospital, Komatsu, Ishikawa, Japan); Drs Nobuyoshi Tanaka and Kazuo Notsumata (Fukuiken Saiseikai Hospital, Fukui, Japan); Dr Mikio Kumagai (Kumagai Clinic, Tsuruga, Fukui, Japan); and Dr Manabu Yoneshima (Municipal Tsuruga Hospital, Tsuruga, Fukui, Japan).

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Reprint requests

Address requests for reprints to: Shuchi Kaneko, MD, PhD, Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Takara-Machi 13-1, Kanazawa 920-8641, Japan. e-mail: skaneko@m-kanazawa.jp; e-mail: fax: (81) 76-234-4250.

Acknowledgments

Participating investigators from the Hokuriku Liver Study Group are listed in Appendix A.

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Conflicts of interest

The authors disclose no conflicts.

Supplementary Materials and Methods

Plasma Amino Acid Analysis

Plasma sample amino acid concentrations were measured by high-performance liquid chromatography-electrospray ionization-mass spectrometry followed by derivatization.[1] An MSQ Plus LC/MS system (Thermo Fischer Scientific, Waltham, MA) equipped with an electrospray ionization source was used in positive ionization mode for selected ion monitoring. Xcalibur version 1.4 SR1 software (Thermo Fischer Scientific, Yokohama, Japan) was used for data collection and processing. The high-performance liquid chromatography separation system consisted of an L-2100 pump, L-2200 autosampler, and L-2300 column oven (Hitachi High-Technologies Corporation, Tokyo, Japan). A Wakosil-II 3C8-100HG column (100, 2.1, 3 mm; Wako Pure Chemical Industries, Osaka, Japan) was used for the separation, and the mobile phase consisted of eluent A (25-mmol/L ammonium formate in water, pH 6.0) and eluent B (water:acetonitrile = 40:60).

Western Blotting

The expression of HCV core protein, Socs3, Foxo3a, phospho-Foxo3a (Ser253) (pFoxo3a), STAT1, pSTAT1 (Tyr701), S6K, pS6K, p-mTOR (Ser2448), Raptor, and β-actin were evaluated with mouse anti-core (Affinity BioReagents, Golden, CO), mouse anti-Socs3 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Foxo3a, rabbit anti-β-actin (Sigma-Aldrich, St Louis, MO), rabbit anti-phospho-Foxo3a (Ser253), rabbit anti-STAT1, rabbit anti-p-STAT1 (Tyr701), rabbit anti-p70 S6K, rabbit anti-pS6K, rabbit anti-p-mTOR (Ser2448), and rabbit anti-Raptor (Cell Signaling Technology, Beverly, MA), respectively. Densitometric analysis was conducted directly on the blotted membrane using a charge coupled device camera system (LAS-3000 Mini; Fujifilm, Tokyo, Japan) and Scion Image software (Frederick, MD).

Primer Sequences for PCR and siRNA

Primer sequences for PCR and siRNA were as follows: 2′5′OAS: forward 5′- CTC AGA AAT ACC CCA GCC AAA TC-3′, reverse 5′-GTG GTG AGA GGA CTG AGG AA-3′; Socs3: forward 5′-TAC CAC CTG AGT CTC CAG CTT CTC-3′, reverse 5′-CCT GGC AGT TCT CAT TAG TTC AGC ATT C-3′; Foxo3a: forward 5′-TGC TGT ATG CAA GAA CTT TCC AGT AGC AG-3′, reverse 5′-ACT CTA GCC CCC ATG CTA CTA GTG-3′; glyceraldehyde-3-phosphate dehydrogenase: forward 5′-GAA GGT GAA GGT CGG AGT-3′, reverse 5′-GAA GAT GGT GAT GGG ATT TC-3′, siFoxo3a (SASI_Hs01_00119127; Sigma) sense: 5′-GAA UGA UGG GCU GAC UGA AdTdT-3′, antisense: 5′-UUC AGU CAG CCC AUC AUU CdTdT-3′. Small interfering Raptor was purchased as part of KIAA1303 siGENOME SMART pool siRNA reagents from Dharmacon, Inc (Lafayette, CO).

Construction of ISRE-Luc Reporter and FBEmut-luc Reporter Plasmids

Oligonucleotides containing the ISRE tandem repeat sequence (sense 5′-TCG AGA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA A-3′, antisense 5′-AGC TTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT C-3′, consensus 5′-GAA Ann GAA ACT-3′) were annealed, and integrated into Xho I and Hind III sites of the pGL4.23 luciferase vector (Promega). The human Socs3 promoter region (-109/+217) was amplified by genomic PCR using specific primers (forward, 5′-TGC TGC GAG TAG TGA CTA AAC ATT ACA AG-3′ and reverse, 5′-CCG TGA AGT CCA CAA AGG AGC CTT C-3′) and cloned into the EcoR V site of the pGL4.10-luc2 reporter vector (Promega). The Socs3 FBE mutant reporter vector was created by substituting 2 adenines in the putative FBE with guanines (wild-type sequence 5′-CTAAACA-3′, mutated sequence 5′-CTGAGCA-3′).

ChIP Assay

For the ChIP assay using the anti-ISGF3γ antibody, 1×10^6 Huh-7 cells were treated with IFN-alfa (0 or 100 U/mL) and BCAA (2 mmol/L) in low-amino-acid medium for 6 hours. For ChIP using the anti-Foxo3a antibody, 1×10^6 Huh-7 cells were cultured in low-amino-acid medium for 24 hours.

Cells were cross-linked with 1% formaldehyde in PBS for 10 minutes at 37°C, and the reaction was stopped with 250 mmol/L glycine for 10 minutes. Cells were suspended in sodium dodecyl sulfate-lysis buffer (1% sodium dodecyl sulfate, 10 mmol/L ethylenediaminetetraacetic acid [EDTA], 50 mmol/L Tris-HCl [pH 8.1]), complete protease inhibitor cocktail (Roche Applied Science), and incubated for 30 minutes at 10°C. Cell lysate was sonicated with Bioruptor (Cosmo Bio, Tokyo, Japan) to obtain chromatin fragments and diluted 10-fold in ChIP dilution buffer (0.01% sodium dodecyl sulfate, 1.1% Triton-X 100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris-HCl [pH 8.1], 150 mmol/L NaCl, complete protease inhibitor cocktail). Chromatin fragments were incubated with 2 μg ISGF3γ antibody (Santa Cruz Biotechnology), 2 μg Foxo3a antibody (H-144; Santa Cruz Biotechnology), or normal rabbit immunoglobulin G for 18 hours at 4°C. Dynabeads (30 μL) protein G (Invitrogen) was added and incubated for 1 hour at 4°C. The beads were washed with low-salt-wash buffer (0.1% sodium dodecyl sulfate, 1% Triton-X 100, 2.0 mmol/L EDTA, 20 mmol/L Tris-HCl [pH 8.1], 150 mmol/L NaCl), high-salt-wash buffer (0.1% sodium dodecyl sulfate, 1% Triton-X 100, 2.0 mmol/L EDTA, 20 mmol/L Tris-HCl [pH 8.1], 500 mmol/L NaCl), LiCl wash buffer (250 mmol/L LiCl, 1% NP-40, 1% de-

oxycholate, 1.0 mmol/L EDTA, 1.0 mmol/L Tris-HCl [pH 8.1]) and Tris-EDTA buffer. Immunoprecipitated chromatin fragments were eluted with elution buffer (1% sodium dodecyl sulfate, 100 mmol/L NaHCO3, 10 mmol/L dithiothreitol), and reverse cross-linked by incubating for 6 hours at 65°C in elution buffer containing 200 mmol/L NaCl. DNA fragments were purified and quantified by real-time detection PCR with primers for putative ISRE in the 2′5′OAS promoter region (forward, 5′-AAA TGC ATT TCC AGA GCA GAG TTC AGA G-3′, reverse, 5′-GGG TAT TTC TGA GAT CCA TCA TTG ACA GG-3′) or putative FBE in the Socs3 promoter region (forward, 5′-TGC TGC GAG TAG TGA CTA AAC ATT ACA AG -3′, reverse, 5′-AGC GGA GCA GGG AGT CCA AGT C -3′). Values were normalized by the measurement of input DNA.

pH77S.3/GLuc2A

pH77S.2 is a modification of pH77S[2] containing an additional mutation within the E2 protein (N476D in the polyprotein) that promotes infectious virus yields from RNA-transfected cells (Yi et al, unpublished data). To monitor replication, the GLuc sequence, fused at its C terminus to the foot-and-mouth disease virus 2A autoprotease, was inserted between p7 and NS2 of pH77S.2 (Supplementary Figure 4). To insert the GLuc-coding sequence between p7 and NS2 in pH77S.2, followed by the foot-and-mouth disease virus 2A protein-coding sequence, Mlu I, EcoR V, and Spe I restriction sites were created between the p7 and NS2 coding sequences by site-directed mutagenesis. DNA coding for GLuc was subcloned into the Mlu I and EcoR V sites of the modified plasmid after PCR amplification using the primers: 5′- ATA ATA TTA CGC GTA *TGG GAG TCA AAG TTC TGT TTG CC*-3′ (sequence corresponding to the N-terminal GLuc is italicized and that corresponding to Mlu I is underlined) and 5′-ATA AAT AGAT ATC *GTC ACC ACC GGC CCC CTT GAT CTT*-3′ (C terminal GLuc is italicized and EcoR V is underlined). A DNA fragment encoding the 17 amino acids of the foot-and-mouth disease virus 2A protein was generated by annealing the following complementary oligonucleotides: 5′- ATA TGA TAT CAA CTT TGA CCT TCT CAA GTT GGC CGG CGA CGT CGA GTC CAA CCC AGG GCC CAC TAG CAT AT-3′ and 5′-ATA TGC TAG TGG GCC CTG GGT TGG ACT CGA CGT CGC CGG CCA ACT TGA GAA GGT CAA AGT TGA TAT CAT AT-3′ (underlined sequences indicate EcoR V and Spe I sites). The annealed oligonucleotides were digested by both restriction enzymes and the product inserted into the corresponding sites of pH77S.2 containing GLuc to generate pH77S.2/GLuc2A. Q41R is a cell-culture adaptive mutation within the NS3 protease domain of pH77S. Because it is not essential for production of infectious virus (Yi et al, unpublished data), pH77S.2 and pH77S.2/GLuc2A constructs underwent this mutation by site-directed mutagenesis of a PCR fragment spanning the Afe I and BsrG I sites to replace Gln_{41} with wild-type Arg. The resulting plasmids (pH77S.2/R41Q and pH77S.2/GLuc2A/R41Q) were redesignated pH77S.3 and pH77S.3/GLuc2A, respectively.[3,4] GLuc has several advantages over other luciferase reporter enzymes in that it is smaller and allows more sensitive detection than either firefly or Renilla luciferase.[3,4] In addition, a signal sequence directs its secretion into cell-culture media, allowing real-time dynamic measurements of GLuc expression without the need for cell lysis. H77S.3/GLuc2A RNA produces infectious virus, although with lower efficiency than H77S.3 RNA (10-fold less).

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MECHANISMS OF GASTROINTESTINAL, PANCREATIC AND LIVER DISEASES

Molecular mechanisms of hepatocarcinogenesis in chronic hepatitis C virus infection

Taro Yamashita, Masao Honda and Shuichi Kaneko

Department of Gastroenterology, Kanazawa University Graduate School of Medical Science, Kanazawa, Ishikawa, Japan

Key words
hepatitis C virus, hepatocellular carcinoma, transcriptome.

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Correspondence
Professor Shuichi Kaneko, Department of Gastroenterology, Kanazawa University Graduate School of Medical Science, 13-1 Takara-Machi, Kanazawa, Ishikawa 920-8641, Japan. Email: skaneko@m-kanazawa.jp

Abstract

Hepatitis C virus (HCV) infection is a major cause of hepatocellular carcinoma (HCC) and chronic liver disease worldwide. Recent developments and advances in HCV replication systems *in vitro* and *in vivo*, transgenic animal models, and gene expression profiling approaches have provided novel insights into the mechanisms of HCV replication. They have also helped elucidate host cellular responses, including activated/inactivated signaling pathways, and the relationship between innate immune responses by HCV infection and host genetic traits. However, the mechanisms of hepatocyte malignant transformation induced by HCV infection are still largely unclear, most likely due to the heterogeneity of molecular paths leading to HCC development in each individual. In this review, we summarize recent advances in knowledge about the mechanisms of hepatocarcinogenesis induced by HCV infection.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third leading cause of cancer death worldwide.[1] The majority of HCCs arise from a background of chronic liver diseases caused by infection with hepatitis B virus (HBV) or hepatitis C virus (HCV).[2] Although both viruses are hepatotropic and regarded as causative agents of HCC, the underlying mechanisms of hepatocarcinogenesis are considered to be largely different, partly due to differences in the nature of DNA viruses (with an integration capacity for the host genome) and RNA viruses (with no genome integration capacity).

Hepatitis C virus is an RNA virus that is unable to integrate into the host genome but, instead, its proteins interact with various host proteins and induce host responses that potentially contribute to the malignant transformation of cells. In addition, HCC usually develops in the setting of liver cirrhosis after long-term continuous inflammation/regeneration processes; these accelerate the turnover of hepatocytes with increased risk of replication errors and DNA damage. Furthermore, recent genome-wide association studies have suggested that the natural course of HCV infection might be modified by the genetic background of the host.[3,4] Thus, both host and virus factors are considered to affect the process of hepatocarcinogenesis in a complex manner.

In this review, we summarize the current knowledge of the mechanisms of hepatocarcinogenesis induced by HCV infection. We also focus on recent findings of transcriptomic characteristics of HCV-related HCC and summarize the potential signaling pathways that are altered in this condition.

Epidemiology

Chronic HCV infection is a major risk factor for the development of HCC worldwide. According to the World Health Organization (WHO), approximately 170 million people are chronically infected with HCV. Although epidemiological evidence has suggested a clear, close relationship between HCV infection and HCC,[5,6] the prevalence of HCV infection in HCC patients differs noticeably between geographical regions. Thus, HCV infection is found in 70–80% of HCC patients in Japan, 70% in Egypt, 40–50% in Italy and Spain, about 20% in the United States (US), and less than 10% in China.[7–9] In industrialized countries including the US, a recent increase in HCC incidence and mortality has been observed, potentially due to the rising incidence of HCV infection transmitted through contaminated blood.[10]

Hepatitis C virus increases the risk of HCC by promoting inflammation and fibrosis of the infected liver that eventually results in liver cirrhosis. Once HCV-related cirrhosis is established, HCC develops at an annual rate of about 4–7%.[11] Other factors including alcohol intake, diabetes, and obesity have also been reported to increase the risk of HCC development by about two- to fourfold, indicating a strong life-style effect on the process of hepatocarcinogenesis.[12,13] Age and male gender are also contributing risk factors for HCV-related HCC, although the detailed mechanisms are still debatable.

Virus proteins and host responses

Hepatitis C virus belongs to the Flaviviridae family. It has a positive-stranded linear RNA genome of about 9.6-kb containing a

single large open reading frame encoding three structural (core, E1, and E2) and seven non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins.[14] The structural proteins form the HCV virions, whereas non-structural proteins are involved in the processes of viral replication, assembly, and maturation. HCV proteins are known to be processed by host and viral proteases. Both structural and non-structural proteins can interact with various host cellular proteins to potentially promote the malignant transformation of hepatocytes (see recent reviews[7,15,16]). In this review, because of space limitations, we focus on the findings of core and NS5A proteins in terms of host responses potentially evoked during the process of HCV-related hepatocarcinogenesis.

Core protein

Hepatitis C virus core is a 21-kDa nucleocapsid protein with an RNA-binding capacity. In addition to its function in regulating HCV-RNA translation and HCV particle assembly, core protein is known to be involved in mediating the alteration of various host cell signaling pathways, transcriptional activation, modulation of immune responses, apoptosis, oxidative stress, and lipid metabolism.[7] Several recent studies have indicated the statistically significant high frequency of mutations in the *core* gene in HCV-infected patients who developed HCC.[17,18] However, the functional relevance of mutant core proteins on the malignant transformation of hepatocytes or the HCV life cycle has yet to be clarified.

Evidence of core protein as a causative agent of HCC was initially obtained from the transgenic mice model in which *core* gene overexpression, under the regulation of the HBV regulatory element used as a promoter, resulted in steatosis of mouse livers in early life, with subsequent development of adenoma and HCC.[19] However, another mouse model using a different promoter and of a different strain background resulted only in steatosis or different phenotypes without HCC development.[20,21] Similar controversial findings were reported in transgenic mice expressing HCV polyprotein or structural protein with regards to the development of HCC.[22,23] Thus, the role of core protein alone in the development of HCC remains unclear in transgenic mouse models.

Although the direct role of core protein in the malignant transformation of hepatocytes is still under investigation, it seems to be related to the development of hepatic steatosis.[19,24] Indeed, steatosis is one of the risk factors for the development of HCV-related HCC,[25,26] and activation of the lipogenic pathway has been reported in a subset of HCC cases.[27] Core protein is associated with the surface of lipid droplets in infected cells and might be directly related to steatosis through several factors responsible for lipid biogenesis and degradation, including peroxisome proliferator-activated receptor alpha and sterol-regulatory element binding protein-1.[21,28–30] Furthermore, core protein is reported to interact with endoplasmic reticulum (ER) or mitochondrial outer membranes and induce ER stress by perturbation of protein folding or by the accumulation of reactive oxygen species (ROS) through mitochondrial dysfunction.[31,32] ROS produced in this way might result in DNA damage to the host genome and accelerate the process of hepatocarcinogenesis. Increased hepatic iron deposition may also induce oxidative stress and lipid peroxidation, thus increasing the risk of HCC development in HCV polyprotein transgenic mice.[33]

Since the discovery of HCV, various studies have investigated the role of core on host cells. Its effects have been demonstrated on signaling pathways responsible for the cell cycle, and apoptosis through interaction with several tumor suppressors including p53, p73, and p21[34–39] as well as apoptosis regulators such as tumor necrosis factor-α (TNF-α) signaling or Bcl-2 members.[40–42] However, the data obtained from these studies are relatively inconsistent with each other and have varied across experimental models. Core protein may influence the growth and proliferation of host cells through activation of signaling pathways such as Raf/mitogen activated protein kinase (MAPK),[43] Wnt/beta catenin,[16] and transforming growth factor-β (TGF-β).[15,44] These pathways are known to be activated in HCC.[45] The findings therefore indicate a potential role for core in cell proliferation or suppression of apoptosis during malignant transformation of hepatocytes in the liver of chronic hepatitis C, where chronic inflammation and regeneration of hepatocytes continuously occurs.

NS5A protein

NS5A is a 56–58-kDa protein phosphorylated at serine residues by serine-threonine kinase[46] and is essential for replication of the HCV genome. NS5A protein forms part of the viral replicase complex and is localized mainly in the cytoplasm of infected cells in association with the ER. NS5A can become a lower molecular weight protein through post-translational modification, after which it can undergo translocation to the nucleus where it acts as a transcriptional activator. High frequencies of wild-type *NS5A* genes were reported to be dominant in liver cirrhosis patients who finally developed HCC compared with those who did not,[47] but the mechanistic significance of the *NS5A* wild/mutant genotypes in the process of HCV-related hepatocarcinogenesis remains uncertain.

NS5A protein has been suggested to interact with various signaling pathways including cell cycle/apoptosis[48] and lipid metabolism[28,49,50] in host cells and shares some signaling targets with core protein. NS5A is recognized as a transcriptional activator for many target genes[51] including p53 and its binding protein, TATA binding protein (TBP). Transcription factor IID activities were reported to be modified by NS5A in the suppression of p53-dependent transcriptional transactivation and apoptosis.[52,53] NS5A may also interact with pathways such as Bcl2,[54] phosphatidylinositol 3-kinase (PI3-K),[55] Wnt/beta catenin signaling,[56] and mTOR[57] to activate cell proliferation signaling and inhibit apoptosis.

Taken together, intriguing data concerning the function of core and NS5A proteins on host cell signaling pathways, transcriptional activation, apoptosis, oxidative stress, and lipid metabolism described above suggest a diverse role for HCV proteins in the pathophysiology of chronic hepatitis C that leads to malignant transformation in infected hepatocytes. Key findings and present concepts are summarized in Figure 1.

Transcriptomic characteristics of HCV-related HCC

As described above, HCV proteins can evoke various host responses in infected cells at transcriptional/translational/post-translational levels. Furthermore, enhanced cell death/regeneration processes are considered to induce DNA damage and accelerate replication errors that cause frequent mutations and genomic alter-

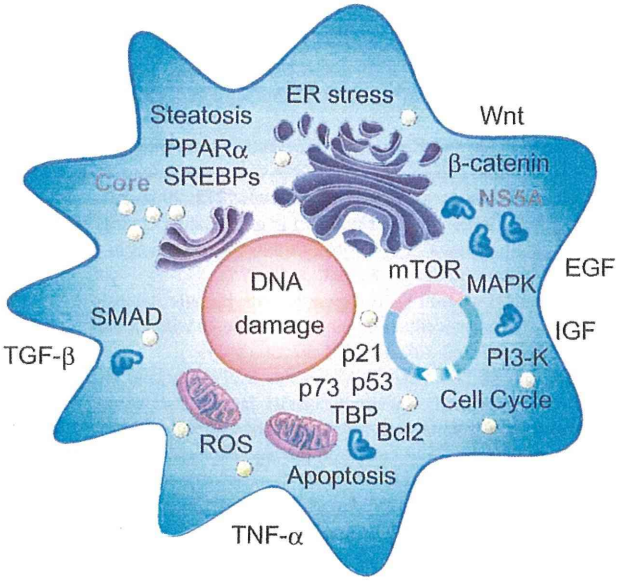

Figure 1 Signaling pathways potentially affected by hepatitis C virus (HCV) proteins. EGF, epidermal growth factor; ER, endoplasmic reticulum; IGF, insulin-like growth factor; MAPK, mitogen activated protein kinase; mTOR, mammalian target of rapamycin; PI3-K, phosphatidylinositol 3-kinase; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; SREBP, sterol-regulatory element binding protein; TBP, TATA binding protein.

ation in the host genome. The central dogma is defined as the flow of genetic information from DNA to mRNA and then to protein, so genetic/genomic alterations and transcriptional/translational modifications are ultimately considered to affect the cellular signaling pathway at the transcriptional level.

Over the past decade, several methods (including differential display, serial analysis of gene expression [SAGE], and microarray) have been developed to allow comparative studies of gene expression between normal and cancer cells on a genome-wide scale,[58] and the analysis of a set of all RNA molecules (mainly indicating mRNAs) is termed as whole transcriptome analysis. Extensive transcriptome analysis of HCC and corresponding noncancerous livers has been performed, and the results have greatly increased our knowledge about the transcriptome characteristics of HCV-related HCC.

Early microarray and SAGE studies investigating the gene expression patterns of chronic hepatitis B and C tissues indicated that these two chronic hepatitis tissues had distinct gene expression profiles; the genes activated in chronic hepatitis C were correlated with signaling pathways associated with apoptosis, oxidative stress responses, and Th1 cytokine signaling.[59,60] An early study comparing genes activated in HCV-related and HBV-related HCCs showed that the genes associated with xenobiotic metabolism were more abundantly expressed in HCV-related HCC,[61] suggesting a detoxification role, which is potentially induced by chronic inflammation and generation of ROS resulting from HCV infection. In contrast, HBV-related HCC might closely correlate with the activation of imprint genes, including insulin-like growth factor-II (IGF-II) as investigated by oligo-DNA microarray,[62] suggesting a role of de-differentiation or epigenetic alteration of the host genome in HBV-related HCC. Activation of genes associated with interferon, oxidative stress, apoptosis, and lipid metabolism signaling was detected in HCV-related HCC and chronic hepatitis C specimens,[27,60,63] consistent with numerous functional studies that have investigated the host response evoked by HCV structural and non-structural proteins.[48]

Transcriptome analysis has also recently shed new light on the transcriptional alteration events occurring in early stages of HCV-related hepatocarcinogenesis. *GPC3* (encoding Glypican 3) was identified as one of the most activated transcripts in the early stage of hepatocarcinogenesis,[60,64] while several recent studies showed that gene signatures including *GPC3* can successfully discriminate HCCs from pre-malignant dysplastic nodules and cirrhosis nodules.[65,66] Close examination of genes differentially expressed among cirrhotic nodules, dysplastic nodules, and early and advanced HCV-related HCC tissues has also suggested roles for Toll-like receptor signaling, Wnt signaling, bone morphogenetic protein (BMP)/TGF-β signaling, JAK-STAT signaling, and DNA repair/cell cycle responses in each step of the malignant transformation processes.[67] These processes might therefore provide candidate molecular targets for the chemoprevention of HCV-related HCC.

Recent advances in transcriptome analysis have also provided detailed information on the status of small noncoding RNAs, microRNAs, that can regulate the expression of target genes and viral replication in normal and cancer tissues. Expression of microRNAs including miR-122 and −199a has been reported to modulate HCV replication,[68–70] and miR-122 expression can be regulated by host interferon signaling and responses.[71] HCV protein expression in turn could induce miRNAs and might affect the tumor suppressor DLC1 and the chemosensitivity of malignantly transformed cells.[72,73] Several microRNAs were also differentially expressed between HCV-related and HBV-related HCCs as well as their corresponding non-cancerous liver tissues. The candidate signaling pathways potentially altered by microRNAs in HCV-related tissues were those associated with antigen presentation, cell cycle, and lipid metabolism,[74] consistent with the mRNA microarray data described above. MicroRNAs have also recently been reported to successfully discriminate between HCC and cirrhotic liver tissues,[75] implicating their role in the early stages of malignant transformation. These data suggest that microRNAs may be good targets for the eradication of HCC as well as hepatocytes infected with HCV.

Conclusion

The heterogeneity of genetic/transcriptomic/proteomic events observed in hepatocytes or cell lines expressing HCV proteins and HCV-related HCCs reported thus far has suggested that complex mechanisms underlie malignant transformation induced by HCV infection. These potentially act through convoluted virus-host interactions including HCV replication with host cell cycles, apoptosis, proliferation, quality control of protein synthesis, lipid metabolism, and DNA damage responses. Indeed, HCC is a heterogeneous disease in terms of drug sensitivity, metastatic capacity, and clinical outcome. The heterogeneity of HCV-related HCC may closely correlate with the origin of malignantly transformed cells where multifaceted cellular reactions including apoptosis and

cell proliferation are induced by HCV infection. An in-depth understanding of these molecular complexities associated with HCV-related HCC may provide the opportunity for effective chemoprevention of HCC among those with HCV-cirrhosis, and to design tailor-made treatment options for HCV-related HCC patients in the future.

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Comparative Analysis of Various Tumor-Associated Antigen-Specific T-Cell Responses in Patients with Hepatocellular Carcinoma

Eishiro Mizukoshi, Yasunari Nakamoto, Kuniaki Arai, Tatsuya Yamashita, Akito Sakai, Yoshio Sakai, Takashi Kagaya, Taro Yamashita, Masao Honda, and Shuichi Kaneko

Many tumor-associated antigens (TAAs) recognized by cytotoxic T cells (CTLs) have been identified during the last two decades and some of them have been used in clinical trials. However, there are very few in the field of immunotherapy for hepatocellular carcinoma (HCC) because there have not been comparative data regarding CTL responses to various TAAs. In the present study, using 27 peptides derived from 14 different TAAs, we performed comparative analysis of various TAA-specific T-cell responses in 31 HCC patients to select useful antigens for immunotherapy and examined the factors that affect the immune responses to determine a strategy for more effective therapy. Twenty-four of 31 (77.4%) HCC patients showed positive responses to at least one TAA-derived peptide in enzyme-linked immunospot assay. The TAAs consisting of cyclophilin B, squamous cell carcinoma antigen recognized by T cells (SART) 2, SART3, p53, multidrug resistance-associated protein (MRP) 3, alpha-fetoprotein (AFP) and human telomerase reverse transcriptase (hTERT) were frequently recognized by T cells and these TAA-derived peptides were capable of generating peptide-specific CTLs in HCC patients, which suggested that these TAAs are immunogenic. HCC treatments enhanced TAA-specific immune responses with an increased number of memory T cells and induced *de novo* T-cell responses to lymphocyte-specific protein tyrosine kinase, human epidermal growth factor receptor type 2, p53, and hTERT. Blocking cytotoxic T-lymphocyte antigen-4 (CTLA-4) resulted in unmasking of TAA-specific immune responses by changing cytokine and chemokine profiles of peripheral blood mononuclear cells stimulated by TAA-derived peptides. *Conclusion:* Cyclophilin B, SART2, SART3, p53, MRP3, AFP, and hTERT were immunogenic targets for HCC immunotherapy. TAA-specific immunotherapy combined with HCC treatments and anti-CTLA-4 antibody has the possibility to produce stronger tumor-specific immune responses. (HEPATOLOGY 2011;53:1206-1216)

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and becoming an important public health concern.[1,2] Although many kinds of treatments have been performed for HCC, their effects are limited because the recurrence rate of HCC is very high; therefore, the development of new therapeutic options to prevent recurrence is necessary.[3,4]

To protect against recurrence, tumor antigen-specific immunotherapy is an attractive strategy. Many tumor-associated antigens (TAAs) and their epitopes recognized by cytotoxic T cells (CTLs) have been identified during the last two decades and some of them have been used in clinical trials for several cancers.[5-21] The epitopes have been under investigation for the treatment of cancer, with major clinical responses in some trials.[22,23] With regard to immunotherapy for HCC, few kinds of TAAs and their epitopes have been used and only clinical data of α-fetoprotein (AFP) have been reported.[24,25] In human trials targeting AFP, it is possible to raise an AFP-specific T-cell response using AFP-derived peptides, but this has shown little

Abbreviations: AFP, alpha-fetoprotein; CTL, cytotoxic T cell; ELISPOT, enzyme-linked immunospot; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HLA, human leukocyte antigen; hTERT, human telomerase reverse transcriptase; IFN, interferon; Lck, lymphocyte-specific protein tyrosine kinase; MRP, multidrug resistance-associated protein; PBMC, peripheral blood mononuclear cell; TAA, tumor-associated antigen.

From the Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Kanazawa, Ishikawa, Japan.

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Address reprint requests to: Shuichi Kaneko, M.D., Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Kanazawa, Ishikawa 920-8641, Japan. E-mail: skaneko@m-kanazawa.jp; fax: 81-76-234-4250.

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antitumor effect. On the other hand, immunotherapy trials using autologous tumor lysate or dendritic cells have shown statistically significant improvements in the risk of HCC recurrence and recurrence-free survival.[26] These reports suggest that tumor antigen-specific immunotherapy is effective to reduce the recurrence rate after HCC treatment; therefore, it is necessary to find immunogenic antigens or their epitopes to develop more effective immunotherapy.

In addition, in the field of molecular targeting therapies, developments of monoclonal antibodies targeting immunomodulatory molecules to enhance antitumor immunity are progressing and some of these are under clinical trial.[27] In particular, clinical data of anti-cytotoxic T-lymphocyte antigen-4 (anti-CTLA-4) antibody have shown durable objective response and stable disease in melanoma patients.[28]

In the present study we performed comparative analysis of various TAA-specific T-cell responses in patients with HCC and examined the factors that affect the immune responses, including anti-CTLA-4 antibody. This approach offers useful information to select immunogenic TAAs and to develop a new strategy for HCC immunotherapy.

Patients and Methods

Patients and Laboratory Testing. In this study we examined 31 human leukocyte antigen (HLA)-A24-positive patients with HCC, 29 chronic hepatitis C patients without HCC, who were diagnosed by liver biopsy, and 11 healthy blood donors who did not have a history of cancer and were negative for hepatitis B surface antigen and anti-hepatitis C virus (HCV) antibody (Ab). The diagnosis of HCC was histologically confirmed in 21 patients. For the remaining 10 patients the diagnosis was based on typical hypervascular tumor staining on angiography in addition to typical findings, which showed hyperattenuated areas in the early phase and hypoattenuation in the late phase on dynamic computed tomography (CT).[29]

HLA-based typing of peripheral blood mononuclear cells (PBMCs) from patients and normal blood donors was performed as described.[19] The pathological grading of tumor cell differentiation was assessed according to the general rules for the clinical and pathological study of primary liver cancer.[30] The severity of liver disease was evaluated according to the criteria of Desmet et al.[31] using biopsy specimens of liver tissue.

All patients gave written informed consent to participate in the study in accordance with the Helsinki Declaration and this study was approved by the regional ethics committee (Medical Ethics Committee of Kanazawa University, No. 829).

Table 1. Peptides

Peptide No.	Peptide Name	Source	Reference	Amino Acid Sequence	Number of Specific Spots In Normal Donors (Mean SD)
1	$ART1_{188}$	ART1	5	EYCLKFTKL	0.9 ± 1.1
2	$ART4_{161}$	ART4	6	AFLRHAAL	0.3 ± 0.5
3	$ART4_{899}$	ART4	6	DYPSLSATDI	0.6 ± 1.0
4	$Cyp\text{-}B_{109}$	Cyp-B	7	KFHRVIKDF	0.5 ± 0.9
5	$Cyp\text{-}B_{315}$	Cyp-B	7	DFMIQGGDF	1.2 ± 1.7
6	Lck_{208}	Lck	8	HYTNASDGL	0.3 ± 0.6
7	Lck_{486}	Lck	8	TFDYLRSVL	0.2 ± 0.8
8	Lck_{488}	Lck	8	DYLRSVLEDF	0.9 ± 1.5
9	$MAGE1_{135}$	MAGE-A1	9	NYKHCFPEI	1.0 ± 0.9
10	$MAGE3_{195}$	MAGE-A3	10	IMPKAGLLI	1.4 ± 1.7
11	$SART1_{1690}$	SART1	11	EYRGFTQDF	0.9 ± 1.3
12	$SART2_{899}$	SART2	12	SYTRLFLIL	1.0 ± 1.4
13	$SART3_{109}$	SART3	13	VYDYNCHVDL	2.1 ± 1.9
14	$Her\text{-}2/neu_8$	Her-2/neu	14	RWGLLLALL	1.4 ± 2.0
15	$p53_{125}$	p53	15	TYSPALNKMF	1.4 ± 1.5
16	$p53_{161}$	p53	16	AIYKQSQHM	0.4 ± 0.6
17	$p53_{204}$	p53	17	EYLDDRNTF	1.1 ± 1.5
18	$p53_{211}$	p53	17	TFRHSVVV	0.9 ± 1.9
19	$p53_{235}$	p53	17	NYMCNSSCM	2.1 ± 2.6
20	$MRP3_{503}$	MRP3	18	LYAWEPSFL	0.2 ± 0.5
21	$MRP3_{692}$	MRP3	18	AYVPQQAWI	1.5 ± 2.1
22	$MRP3_{765}$	MRP3	18	VYSDADIFL	0.9 ± 1.0
23	AFP_{357}	AFP	19	EYSRRHPQL	1.8 ± 2.0
24	AFP_{403}	AFP	19	KYIQESQAL	1.1 ± 1.5
25	AFP_{434}	AFP	19	AYTKKAPQL	0.8 ± 1.1
26	$hTERT_{167}$	hTERT	20	AYQVCGPPL	0.8 ± 1.1
27	$hTERT_{324}$	hTERT	20	VYAETKHFL	0.5 ± 0.7
28	$HIV\ env_{584}$	HIV env	32	RYLRDQQLL	1.3 ± 2.0
29	$HCV\ NS3_{1031}$	HCV NS3	33	AYSQQTRGL	ND
30	$CMV\ pp65_{328}$	CMV pp65	34	QYDPVAALF	13.3 ± 15.7

ND, not determined.

Peptides, Cell Lines, and Preparation of PBMCs. Twenty-seven peptides derived from 14 different TAAs (Table 1), human immunodeficiency virus (HIV) envelope-derived peptide ($HIVenv_{584}$),[32] HCV NS3-derived peptide ($HCVNS3_{1031}$),[33] and cytomegalovirus (CMV) pp65-derived peptide ($CMVpp65_{328}$),[34] which were identified as HLA-A24 restricted CTL epitopes in previous studies, were used. Peptides were synthesized at Mimotope (Melbourne, Australia) and Sumitomo Pharmaceuticals (Osaka, Japan). They were identified using mass spectrometry and their purities were determined to be >80% by analytical high-performance liquid chromatography (HPLC). The HLA-A*2402 gene-transfected C1R cell line (C1R-A24) was cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS) and 500 μg/mL hygromycin B (Sigma, St. Louis, MO), and K562 was cultured in RPMI 1640 medium containing 10% FCS.[35] PBMCs were isolated before HCC treatments as described.[20] In 12 patients their PBMCs were also obtained 4 weeks after treatments.

Table 2. Characteristics of the Patients Studied

Clinical Diagnosis	No. of Patients	Sex M/F	Age (yr) Mean ± SD	ALT (IU/L) Mean ± SD	AFP (ng/ml) Mean ± SD	Child Pugh (A/B/C)	Diff. Degree* (wel/mod/ por/ND)	Tumor Size† (large/small)	Tumor Multiplicity (multiple/solitary)	Vascular Invasion (+/−)	TNM Stage (I/II/IIIA/IIIB/ IIIC/IV)
Normaldonors	11	8/3	35 ± 2	ND	ND	ND	ND	ND	ND	ND	ND
Chronichepatitis	29	16/13	59 ± 10	92 ± 94	31 ± 87	27/2/0	ND	ND	ND	ND	ND
HCC	31	23/8	71 ± 4	74 ± 33	1768 ± 9103	20/10/1	11/10/0/10	22/9	20/11	9/22	10/12/3/1/2/3

*Histological degree of HCC; wel: well differentiated, mod: moderately differentiated, por: poorly differentiated, ND: not determined.
†Tumor size was divided into either "small" (≦2 cm) or "large" (>2 cm).

CTL Induction and Cytotoxicity Assay. CTL induction and cytotoxicity assays were performed as described.[20] Briefly, stimulated PBMCs were added at effector to target ratios of 100:1, 50:1, 25:1, 13:1, 6:1, and 3:1. In cases where the number of CTLs was insufficient, cytotoxicity assays were performed at effector to target ratios less than 100:1.

Interferon Gamma IFN-γ Enzyme-Linked Immunospot (ELISPOT) Assay. IFN-γ ELISPOT assays were performed as reported.[20] Responses to TAA-derived peptides were considered positive if more than 10 specific spots were detected, which is greater than the mean plus 3 standard deviations (SDs) of the baseline response detected in 11 normal blood donors (Table 1), and if the number of spots in the presence of an antigen was at least 2-fold that in its absence. Responses to HIV-, HCV-, and CMV-derived peptides were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of an antigen was at least 2-fold that in its absence. In ELISPOT assay with blocking CTLA-4, anti-human CTLA-4 (eBioscience, Tokyo, Japan) was added at a final concentration of 50 μg/mL, which has been described to have maximum effect in *in vitro* cultures.[36] As a control, functional grade mouse immunoglobulin G (IgG)2a isotype control was used. The assay with blocking CTLA-4 was performed in triplicate and the results were statistically analyzed using the unpaired Student's *t* test.

Cytokine and Chemokine Profiling. The effect of CTLA-4 antibody on TAA-specific T-cell responses was also analyzed by cytokine and chemokine profiling. Cytokine and chemokine levels in the medium of ELISPOT assay were measured using the Bio-plex assay (Bio-Rad, Hercules, CA). These included interleukin (IL)-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, basic fibroblast growth factor (FGF), eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor (PDGF)-BB, RANTES, tumor necrosis factor alpha (TNF-α), and vascular endothelial growth factor (VEGF). Eight standards (ranging from 2 to 32,000 pg/mL) were used to generate calibration curves for each cytokine. Data acquisition and analysis were carried out using Bio-plex Manager software v. 4.1.1.

Cytokine Secretion Assay. TAA-specific IFN-γ-producing T cells were also analyzed by cytokine secretion assay. The assay was performed with the MACS cytokine secretion assay (Miltenyi Biotec K.K., Tokyo, Japan), in accordance with the manufacturer's instructions. Briefly, 5,000,000 PBMCs were pulsed with TAA-derived peptides for 16 hours and then incubated with 20 μL of IFN-γ detection antibody, 10 μL of anti-CD8-APC Ab (Becton Dickinson, Tokyo, Japan), 10 μL of anti-CCR7-FITC Ab (eBioscience, Tokyo, Japan), and 10 μL of anti-CD45RA-PerCP-Cy5.5 Ab (eBioscience, Tokyo, Japan) for 10 minutes at 4°C. After washing with a cold buffer (phosphate-buffered saline/0.5% bovine serum albumin with 2 mM EDTA), the cells were resuspended with 500 μL of cold buffer and analyzed using FACSCalibur (Becton Dickinson, Tokyo, Japan). As a positive control, CMVpp65$_{328}$-specific IFN-γ-producing T cells were also analyzed by the same methods. The number of IFN-γ-producing T cells was calculated from the results of FACS analysis and is shown as a number per 300,000 PBMCs.

Results

Patient Profile. The clinical profiles of the 11 healthy blood donors, 29 patients with chronic hepatitis C, and 31 patients with HCV-related HCC analyzed in the present study are shown in Table 2 and Fig. 1. Using TNM staging of the Union Internationale Contre Le Cancer (UICC) system (6th v.), 10, 12, 3, 1, 2, and 3 patients were classified as having stage I, II, IIIA, IIIB, IIIC, and IV tumors, respectively.

Detection of TAA-Specific T Cells in HCC Patients. First we examined the frequency of cells that specifically reacted with TAA-derived and control peptides in HCC patients. Fifty-one responses in total were observed against TAA-derived peptides. Twenty-

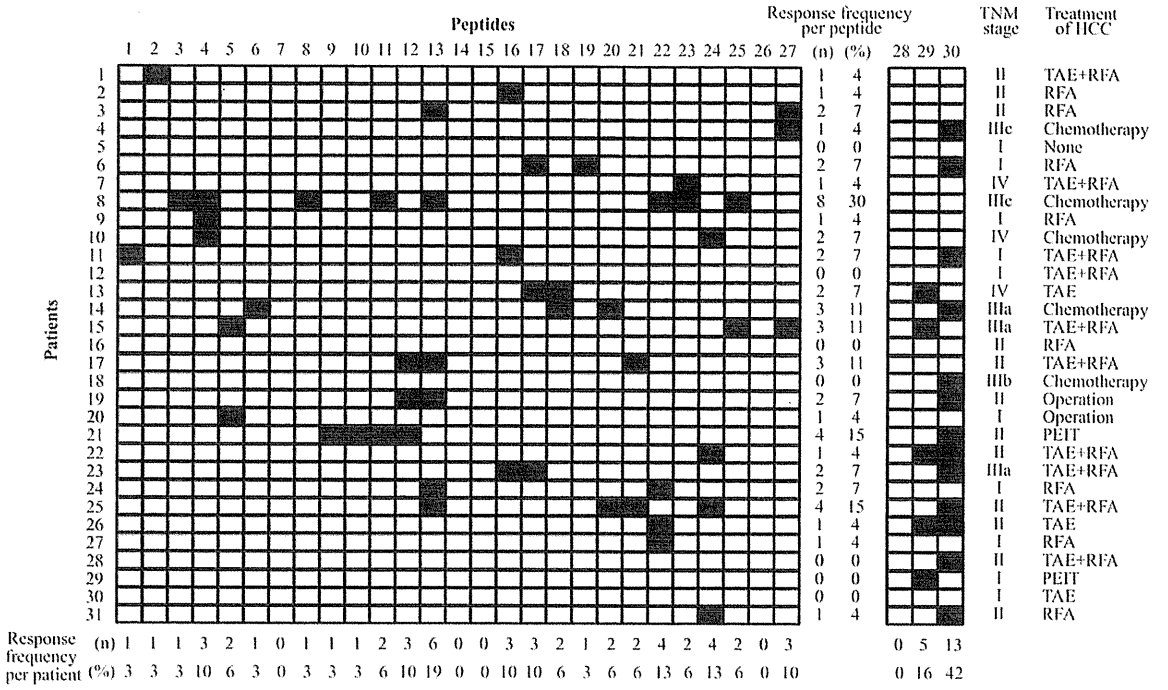

Fig. 1. TAA-, HIV-, HCV-, and CMV-derived peptide-specific T-cell responses. Results of all HCC patients examined are shown. The T-cell responses were examined by IFN-γ ELISPOT assay. Responses to peptides were considered positive if more than 10 specific spots per 300,000 PBMCs were detected and if the number of spots in the presence of an antigen was at least 2-fold that in its absence. Black boxes indicate the presence of a significant IFN-γ T-cell response to peptides. Peptide sequences are described in Table 1 and characteristics of patients in Table 2.

four of 31 (77.4%) patients showed positive responses to at least one TAA-derived peptide and most of them showed responses to 1 to 4 kinds of TAA-derived peptide. Twenty-three of 27 (85.2%) TAA-derived peptides were recognized by T cells of at least one patient. Peptides 4, 12, 13, 16, 17, 22, 24, and 27 were recognized in more than two patients, suggesting that these peptides were immunogenic. Peptides 28 (HIV env_{584}), 29 (HCV $_{1031}$), and 30 (CMV $pp65_{328}$) were recognized by 0 (0%), 5 (16%), and 13 (42%) patients, respectively.

The magnitude of TAA-specific T-cell responses was assessed by the frequency of peptide-specific IFN-γ-producing T cells in the PBMC population (Fig. 2A). The range of TAA-derived peptide-specific T-cell frequency was 10-60.5 cells/300,000 PBMCs. Those specific to peptides 13 and 16 numbered more than 30 cells/300,000 PBMCs, suggesting that these peptides were immunogenic. The frequencies of T cells specific to HCV- and CMV-derived peptides were 12-22 cells and 12-92/300,000 PBMCs, respectively.

Whether these TAA-derived peptides were capable of generating peptide-specific CTLs from PBMCs was investigated in HCC patients. The seven peptides were selected according to the magnitude of TAA-specific T-cell responses determined by the frequency of T cells with a positive response. The CTLs generated with these peptides were cytotoxic to C1RA24 cells pulsed with the corresponding peptides (Fig. 2B).

Comparison of TAA-Specific T-Cell Responses Between the Patient Groups With and Without HCC. To characterize the immunogenicity and specificity of TAA-derived peptides, we compared T-cell responses to the peptides derived from TAA, HIV, HCV, and CMV among three groups consisting of normal blood donors, patients with chronic hepatitis C, and patients with HCV-related HCC. A significant TAA-specific T-cell response was not detected in normal blood donors (Fig. 3A). A response was detected in both chronic hepatitis C and HCC patient groups, but it was more frequently observed in HCC patients. HIV-specific T-cell response was not detected in any group. HCV-specific T-cell response rate was not different between the groups with chronic hepatitis C and HCC. CMV-specific T-cell response rates were similar among the three groups. Similar tendencies were observed in the analysis of individual peptides (Fig. 3B). We also examined the frequency of T cells responsive to peptides among the three groups. The mean frequency of TAA-specific T cells without *in vitro* expansion was higher in HCC patients than in

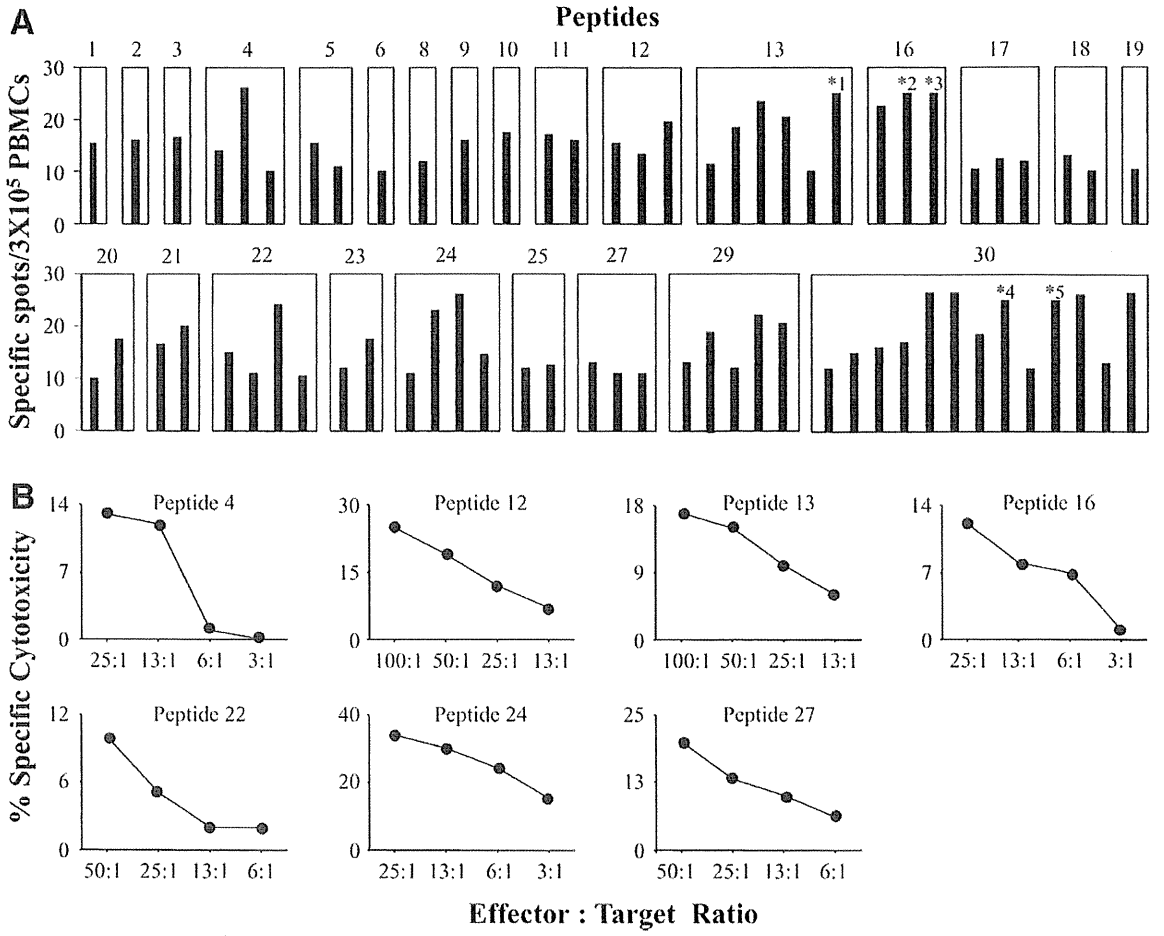

Fig. 2. Vigor of TAA-, HCV-, and CMV-derived peptide-specific T-cell responses. (A) The frequency of TAA-specific IFN-γ-producing T cells was analyzed by ELISPOT assay. Only positive responses are shown. Black bars indicate the response of one patient. *1, *2, *3, *4, and *5 denote 33, 60.5, 44, 92, and 67.5 specific spots, respectively. (B) Representative TAA-specific T-cell responses were also analyzed by CTL assay. T cell lines were generated from PBMC of the HLA-A24-positive HCC patients by stimulation with TAA-derived peptides (peptides 4, 12, 13, 16, 22, 24, and 27) (see Table 1). Expanded T cell lines were then tested for specific cytotoxicity against the corresponding peptides in a standard ^{51}Cr release assay at the indicated E:T ratios.

patients with chronic hepatitis C for 14 of 27 TAA-derived peptides (peptides 1, 2, 3, 4, 12, 16, 18, 19, 20, 21, 22, 24, 25 and 27) (Fig. 3C).

Enhancement of TAA-Specific T-Cell Responses After HCC Treatments. Several studies including our own have clarified that HCC treatments enhanced HCC-specific immune responses (19, 37, 38). In this study, we examined whether the enhancement was observed equally in all kinds of TAAs or specifically in some TAAs. For this purpose we measured the frequency of TAA-specific T cells before and after HCC treatment by ELISPOT assay in 12 cases who received transcatheter arterial embolization (TAE), radiofrequency ablation (RFA), or chemotherapy. The frequency of TAA-specific T cells increased in all patients and it was observed for 23 of 27 TAA-derived peptides (Fig. 4A). The enhancement was observed in the patients who received TAE, RFA, or chemotherapy and even in the patients without an increase in the frequency of CMV-specific T cells. Peptides 7, 14, 15, and 26, which were not recognized by T cells in all HCC patients before treatments (Fig. 1), were recognized by T cells in 1, 4, 1, and 5, respectively, of 12 patients after treatments. Representative results of enhancement of TAA-specific immune responses are shown in Fig. 4B. The frequency of TAA-specific T cells increased to 11-80 cells/300,000 PBMCs after treatments.

The enhancement of TAA-specific immune responses was also confirmed by cytokine secretion assay. Representative results are shown in Fig. 4C. In this patient (patient 25) the frequency of TAA-specific IFN-γ-producing $CD8^+$ T cells was increased from 0.4% to 1.4% of $CD8^+$ T cells after HCC treatment.

A

	TAA	HIV antigen	HCV antigen	CMV antigen
Normal donors	0/11(0%)	0/11(0%)	ND	4/11(36%)
Patients with HCV	10/29(34%)	0/29(0%)	8/29(28%)	15/29(52%)
Patients with HCC	24/31(77%)	0/31(0%)	5/31(16%)	13/31(42%)

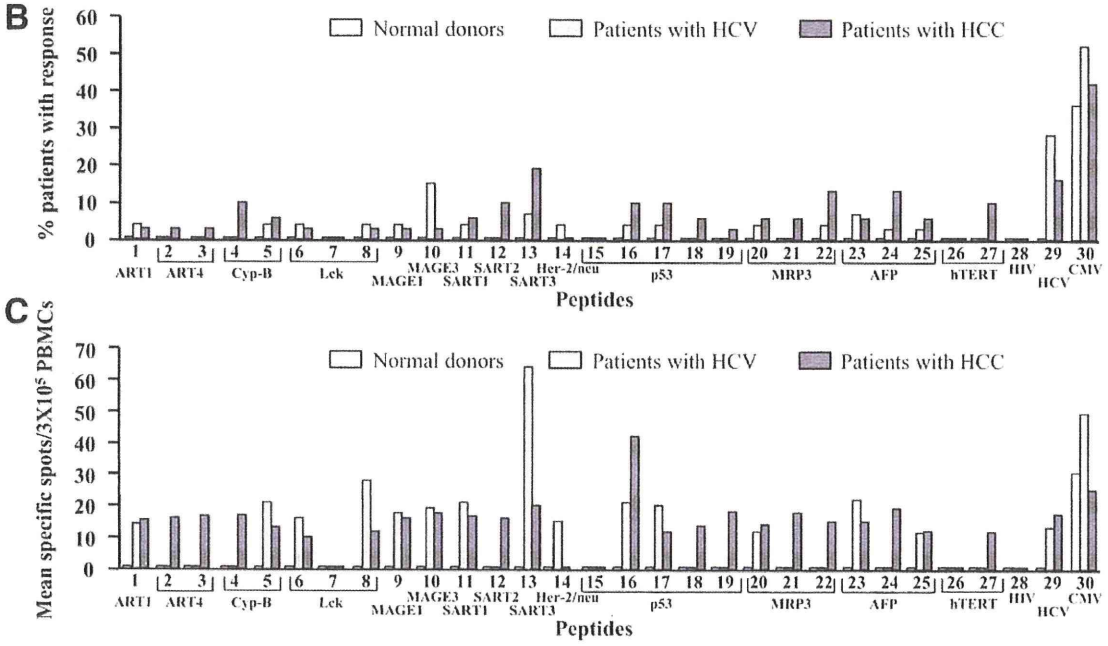

Fig. 3. Comparative analysis of TAA-, HIV-, HCV-, and CMV-derived peptide-specific T-cell responses among three groups of subjects: normal donors, patients with chronic hepatitis C not complicated by HCC, and HCC patients. (A) Summary of the number of patients with a significant IFN-γ T-cell response to tumor-associated, HIV, HCV, and CMV antigens in each group. (B) Graph shows the percentage of patients in each group who showed a significant IFN-γ T-cell response to individual peptides. Peptide sequences are described in Table 1. (C) Mean frequency of peptide-specific IFN-γ-producing T cells in each group. The frequency of IFN-γ-producing T cells was analyzed by ELISPOT assay.

In this assay we also examined the naïve/effector/memory phenotype of these cells by the criterion of CD45RA/CCR7 expression.[39] Phenotypic analysis of TAA-specific, IFN-γ-producing memory $CD8^+$ T cells before and after treatment showed that the frequency of $CD45RA^-/CCR7^+$ central memory T cells was the highest, indicating that the posttherapeutic increase in these T cells is due to the increase in cells with this phenotype (Fig. 4D). In this patient the number of T cells with the $CD45RA^-/CCR7^+$ phenotype increased from 73 cells/300,000 PBMCs before treatment to 316 cells/300,000 PBMCs after treatment. Similar results were noted in five patients.

Blocking CTLA-4 Restores TAA-Specific T-Cell Responses. In previous studies including our own,[19,20,24] the CTL epitopes that correlate with the prevention of tumor progression or prognosis of HCC patients have not been identified. One of the reasons for this is considered to be that the naturally occurring T-cell responses to the epitopes are weak; therefore, recent tumor immunotherapeutic studies are moving toward modulation of T-cell responses.

CTLA-4 is recognized as a critical negative regulator of immune response; therefore, its blockade has been considered to contribute to antitumor activity.[27] In a recent study it was reported that blocking of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of CTLA-4 antibodies.[40] To examine whether similar occurs for immune response in HCC patients, we analyzed 32 separate TAA-specific T-cell responses in 15 HCC patients using 13 TAA-derived peptides. Incubation of T cells with CTLA-4 antibodies resulted in an increase of the number of TAA-specific T cells in 18 of 32 (56%) responses and in 9 of 15 (60%) patients (Fig. 5A). Fourteen and four patients showed increases of 1-10 and more than 10 TAA-specific T cells, respectively. Representative results of six patients are shown

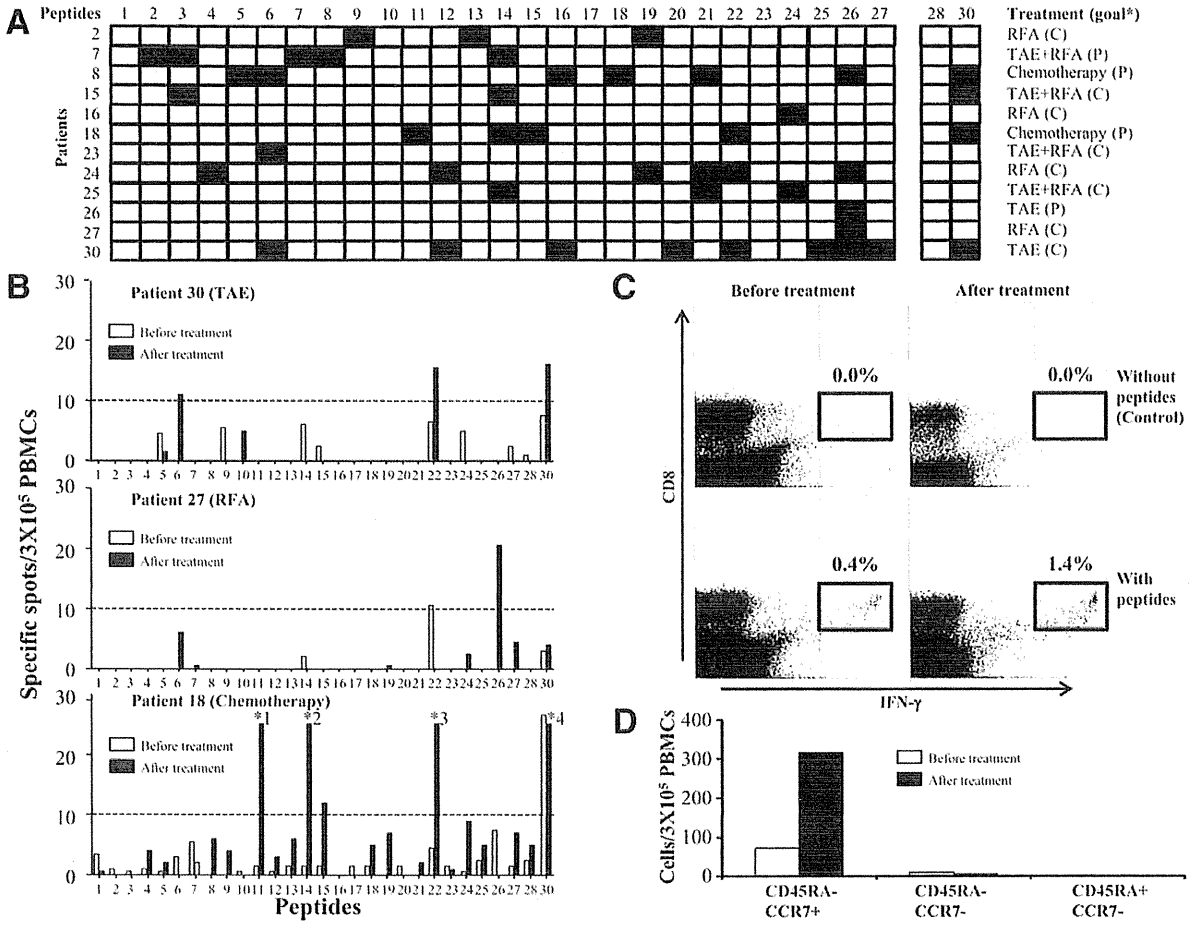

Fig. 4. Enhancement of TAA-specific T-cell responses in HCC patients after treatments. (A) Summary of patients and peptides with a significant increase of the number of IFN-γ-producing T cells (black boxes). A significant change in the IFN-γ response was defined as a more than 2-fold increase and the presence of more than 10 specific spots in ELISPOT assay after HCC treatments. The assays were performed in 12 HCC patients using 27 TAA-, HIV-, and CMV-derived peptides. Goal* shows the goal of HCC treatment. C and P denote "curative intention" and "palliative intention," respectively. (B) Representative results of ELISPOT assay are shown. White and black bars indicate the frequency of T cells before and after HCC treatments, respectively. *1, *2, *3, and *4 denote 53, 60, 80, and 121 specific spots, respectively. (C) Enhancement of TAA-specific T-cell responses was also analyzed by cytokine secretion assay. Representative results are shown (patient 25). PBMCs were pulsed with TAA-derived peptides (peptides 14, 21, and 24) for 16 hours and then analyzed for IFN-γ production. (D) IFN-γ-producing T cells were also examined for naïve/effector/memory phenotype by the criterion of CD45RA/CCR7 expression. The number of cells was calculated from the results of FACS analysis and is shown as a number per 300,000 PBMCs. White and black bars indicate the frequency of TAA-specific IFN-γ-producing T cells before and after HCC treatments, respectively. The experiments were performed in five patients and similar results were observed.

in Fig. 5B. The magnitude of TAA-specific T-cell increase was statistically significant in four patients.

To examine the effect of CTLA-4 antibodies for production of other cytokines by T cells, we measured 27 kinds of human cytokines and chemokines in the medium of ELISPOT assay. Figure 5C shows the results of cytokine production in the well with positive T-cell responses against TAA-derived peptides. The various cytokines consisting of IL-1β, IL-4, IL-6, IL-10, IL-17, eotaxin, G-CSF, GM-CSF, IFN-γ, MIP-1α, MIP-1β, RANTES, and TNF-α were increased in the medium with CTLA-4 antibodies compared with that without CTLA-4 antibodies. In contrast, increased production of these cytokines in the well without positive T-cell responses against TAA-derived peptides was not observed in medium either with or without CTLA-4 antibodies (Fig. 5D).

Discussion

In recent years, specific TAAs and their CTL epitopes have been identified in many tumors.[21] Several TAAs and their CTL epitopes, such as AFP, MAGE, and human telomerase reverse transcriptase (hTERT) have also been reported in HCC.[19,20,24,41] Although AFP-targeting immunotherapy could induce TAA-

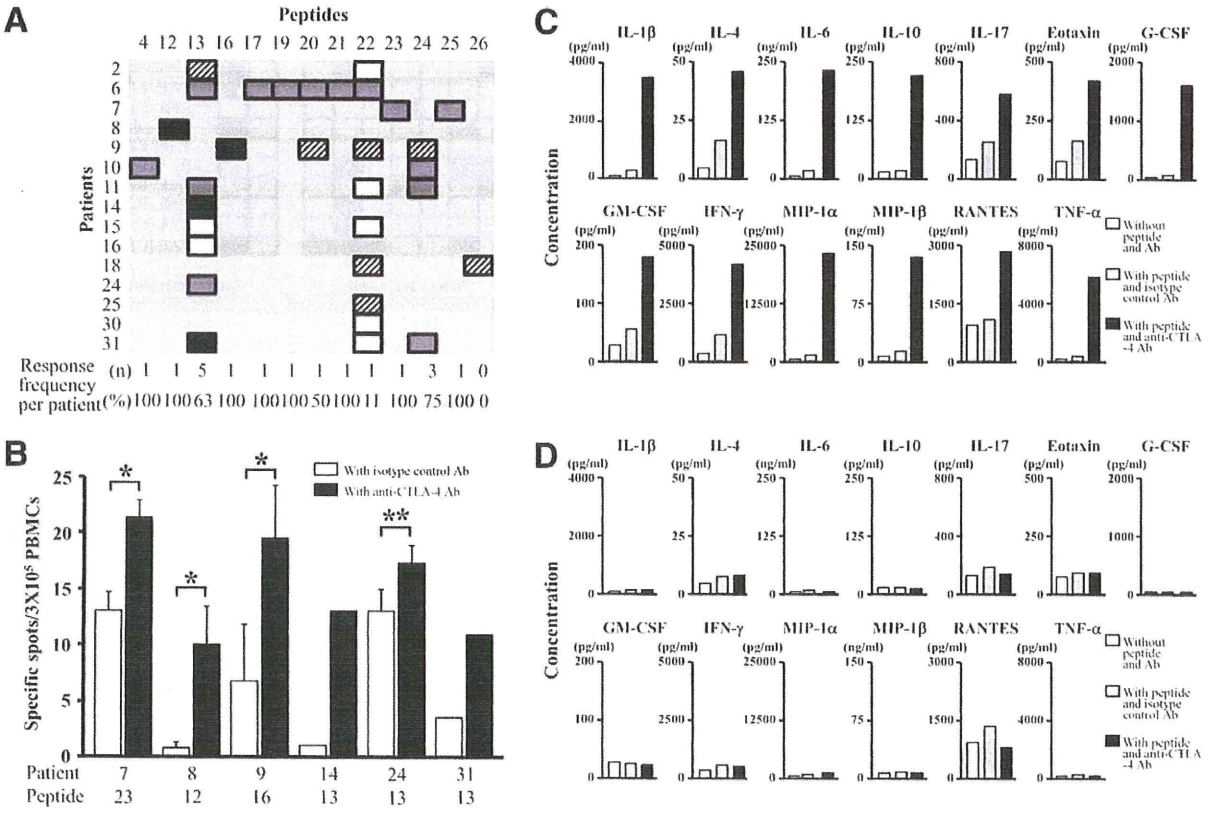

Fig. 5. Enhancement of TAA-specific T-cell responses in HCC patients by CTLA-4 antibodies. (A) Summary of patients and peptides with an increase of the number of IFN-γ-producing T cells. Black, gray, white, and hatched boxes indicate the immune responses with an increase of more than 10 specific spots, an increase of 1-10 specific spots, without change and a decrease of 1-10 specific spots, respectively. (B) Representative results of six patients are shown. Black and white bars indicate the results of assays incubated with CTLA-4 antibodies and mouse IgG2a isotype control, respectively. Data are expressed as the mean ± SD of specific spots, except for patients 14 and 31. (C) Effects of CTLA-4 antibodies on production of cytokine and chemokine. Cytokine and chemokine levels in the medium of ELISPOT assay were measured using the Bio-plex assay. The graphs indicate the concentrations of cytokine and chemokine in the medium of ELISPOT assay using PBMCs of patient 31 and peptide 13 (medium in ELISPOT assay with enhancement of T-cell response) (see A,B). The increase of cytokines and chemokines after incubation with anti-CTLA-4 antibodies was confirmed in another three experiments using PBMCs of three other patients. (D) The graphs indicate the concentrations of cytokine and chemokine in the medium of ELISPOT assay using PBMCs of patient 31 and peptide 22 (medium in ELISPOT assay without enhancement of T-cell response) (see A).

specific CTLs, no patients achieved an objective tumor response; therefore, the search for TAAs as suitable targets for HCC immunotherapy and identification of their epitopes are important issues in therapy development. However, to date, T-cell responses to previously identified TAAs or their epitopes have been measured simultaneously and comparatively in only one study involving several patients with HBV-related HCC,[42] but no T-cell responses to the many other TAAs or their epitopes have been evaluated.

In this study we performed a simultaneous, comparative analysis of immune responses to 27 different CTL epitopes derived from 14 previously reported TAAs in the peripheral blood lymphocytes of 31 HCV-related HCC patients. We noted immune responses to epitopes (peptides 4, 12, 13, 16, 17, 22, 24, and 27) derived from CypB, SART2, SART3, p53, MRP3, AFP, and hTERT in more than two patients (Fig. 1). These findings suggest the immunogenicity of these TAAs and their epitopes. In addition, the frequencies of peripheral blood CTLs specific to epitopes (peptides 4, 13, 16, 22, and 24) derived from CypB, SART3, p53, MRP3, and AFP, as detected by the ELISPOT assay, were high (≥20 specific spots/300,000 PBMCs), suggesting the high immunogenicity of these TAAs and their epitopes.

Among these immunogenic antigens the expression of p53, MRP3, AFP, and hTERT was reported in HCC.[18,19,43,44] We also previously confirmed that the expression of SART2 and SART3 was observed in 100% of human HCC tissue (data not shown). As for CypB, this protein is well known to be widely expressed in normal and malignant tissue[7]; therefore, it is considered to be expressed in HCC.

Regarding tumor immunotherapy, it has recently been reported that strong immune responses can be induced at an earlier postvaccination time using, as peptide vaccines, epitopes that frequently occur in peripheral blood CTL precursors.[23] The epitopes (peptides 4, 12, 13, 16, 22, 24, and 27) that were derived from CypB, SART2, SART3, p53, MRP3, AFP, and hTERT and considered to be highly immunogenic in this study were capable of inducing epitope-specific CTLs from the PBMCs of HCC patients, suggesting that these epitopes can be candidates for peptide vaccines.

Next, TAA-specific immune responses were compared among three groups of subjects: HCC patients, normal blood donors, and patients with chronic hepatitis C not complicated by HCC. The results showed that there were no differences in the positive rate of immune responses to CMV among the three groups and no difference in the positive rate of immune responses to HCV between chronic hepatitis C patients with and without HCC. However, TAA-specific immune responses were observed frequently only in HCC patients, indicating that these immune responses are specific to HCC.

In the present study we also analyzed factors influencing host immune responses to these TAA-derived epitopes. Previous studies have reported that treatments, such as RFA and TAE, enhance HCC-specific T-cell responses.[19,37,38] However, TAAs and their epitopes, to which these enhanced immune responses occur, have not been identified. Thus, we simultaneously measured immune responses to 27 different epitopes derived from 14 TAAs in 12 patients who were available for analysis before and after treatment. The results showed that the antigens and their epitopes to which treatment-enhanced T-cell responses occur were diverse and some of them were newly induced after HCC treatment, suggesting that HCC treatments could induce *de novo* T-cell responses and these TAAs and their epitopes can be candidates as targets for HCC immunotherapy.

Furthermore, it became clear that enhanced immune responses to TAAs were induced not only by previously reported RFA and TAE, but also by cytotoxic drug chemotherapy. The patients who received chemotherapy showed partial responses after the treatment; therefore, we considered that it induced release of TAA into the tumor environment by tumor necrosis and/or apoptosis such as the mechanism reported in RFA or TAE.[19,37,38] Thus, our findings suggest that combined cancer chemotherapy and immunotherapy is useful as a treatment for HCC.

Analysis of the memory phenotypes of the T cells thus induced showed that the phenotypes of T cells whose frequency increased were mostly $CD45RA^-/CCR7^+$ T cells (central memory T cells). Previous studies have reported that T cells with this phenotype differentiate into effector memory T cells and effector T cells, and that they require secondary stimulation by antigen to exert stronger antitumor effects.[39] Therefore, our findings suggest that the antitumor effect of tumor-specific T cells induced by HCC treatment is insufficient, and a booster with TAAs or epitope-containing peptides is a suitable method to further enhance antitumor effects.

Finally, we investigated the effect of anti-CTLA-4 antibodies, which have recently been in clinical trials as drugs enhancing antitumor immunity, on the host immune response to HCC. Regarding the mechanism of the antitumor activity of anti-CTLA-4 antibodies, it has been reported that they maximize the antitumor effect by blocking CTLA-4 on the surface of effector and regulatory T cells.[40] Because the number of peripheral blood regulatory T cells has been reported to increase in HCC patients,[45] TAA-specific CTLs that should be present but may not be detected by the ELISPOT assay. Therefore, in this study anti-CTLA-4 antibodies were added along with peptides to examine their effect on the ELISPOT assay.

The addition of anti-CTLA-4 antibodies resulted in an increase in the frequency of TAA-specific T cells in 60% of HCC patients. Although most patients showed an increase of only 1-10 TAA-specific T cells, the increased number of T cells was statistically significant. In addition, an increase of more than 10 TAA-specific T cells and a conversion from a negative to a positive response were observed in four patients. These results suggested that the anti-CTLA-4 antibodies unmasked IFN-γ production by CTLs. However, the function might be limited because the number of TAA-specific T cells was not changed and even decreased in some patients.

The cytokine and chemokine profiling showed that the addition of anti-CTLA-4 antibodies increased the production of not only IFN-γ but also cytokines, such as TNF-α, IL-1, and IL-6, and chemokines such as MIP-1; therefore, we speculate that the increased production of these antitumor immunity substances also plays a role in the unmasking of TAA-specific CTLs by anti-CTLA-4 antibodies. These results suggest that anti-CTLA-4 antibody is promising as a drug to enhance antitumor immunity, and that the ELISPOT assay with this antibody may serve as a more appropriate test tool to detect more HCC-specific TAAs or their epitopes.