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	$ART4_{889}$	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-

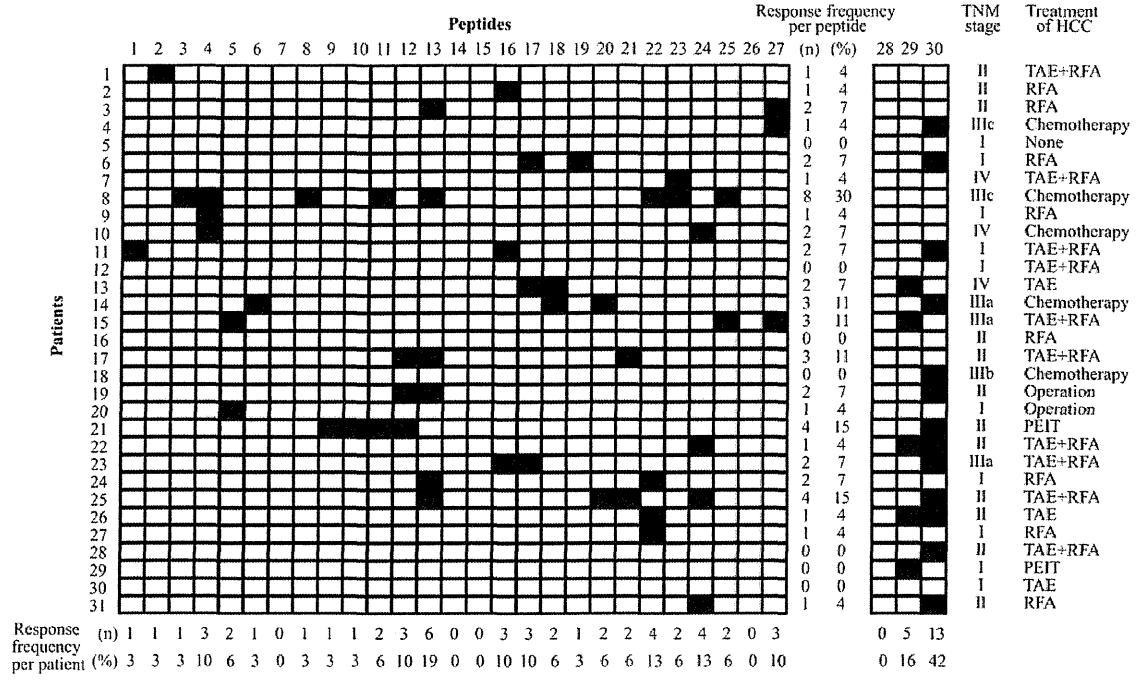

Fig. 1. TAA-, HIV-, HCV-, and CMV-derived peptide-specific T-cell responses. Results of all HCC patients examined are shown. The T-cell responses were examined by IFN-γ ELISPOT assay. Responses to peptides were considered positive if more than 10 specific spots per 300,000 PBMCs were detected and if 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

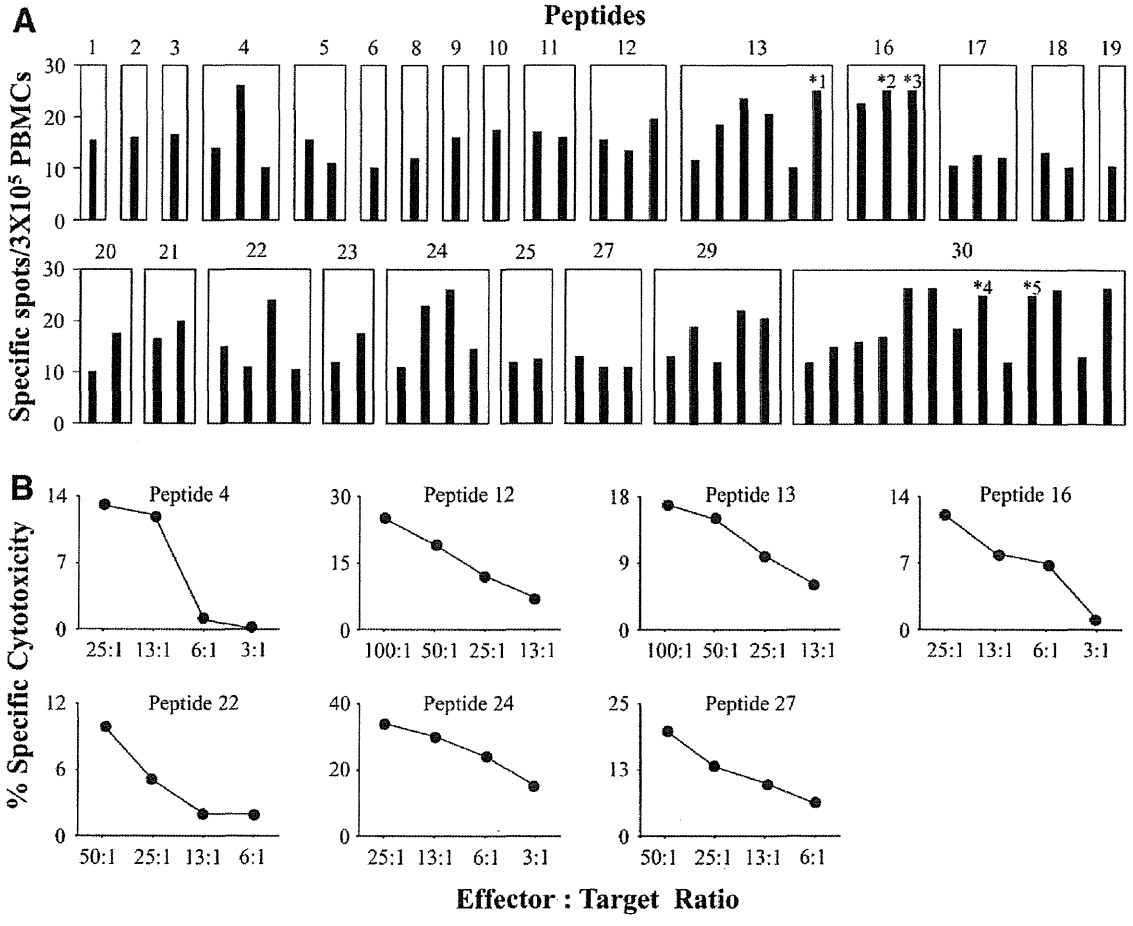

Fig. 2. Vigor of TAA-, HCV-, and CMV-derived peptide-specific T-cell responses. (A) The frequency of TAA-specific IFN-γ-producing T cells was analyzed by ELISPOT assay. Only positive responses are shown. Black bars indicate the response of one patient. *1, *2, *3, *4, and *5 denote 33, 60.5, 44, 92, 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%)

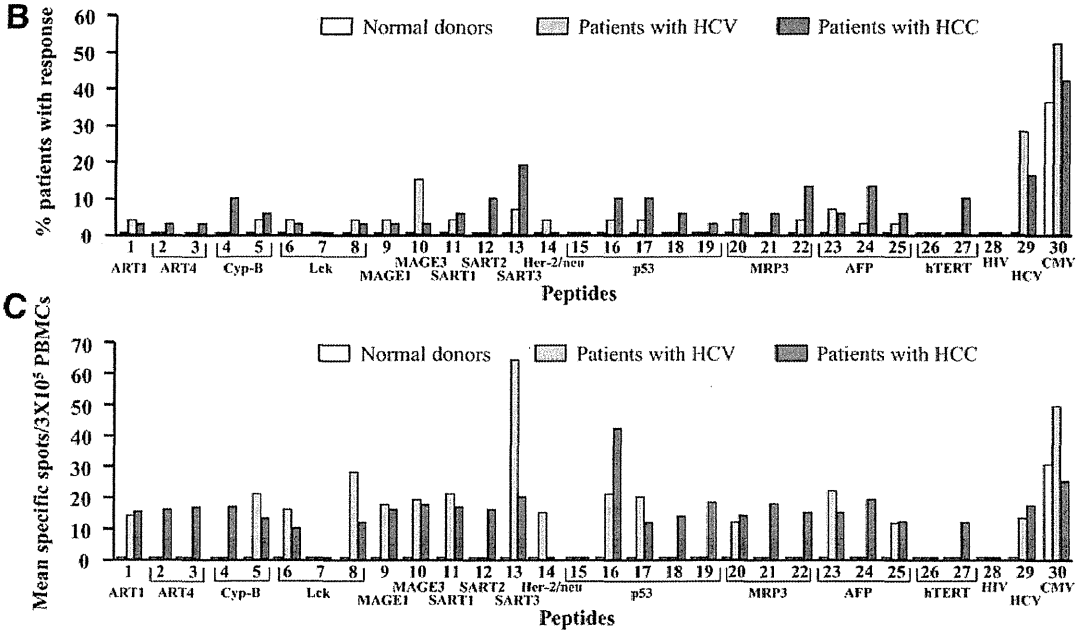

Fig. 3. Comparative analysis of TAA-, HIV-, HCV-, and CMV-derived peptide-specific T-cell responses among three groups of subjects: normal donors, patients with chronic hepatitis C not complicated by HCC, and HCC patients. (A) Summary of the number of patients with a significant IFN-γ T-cell response 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

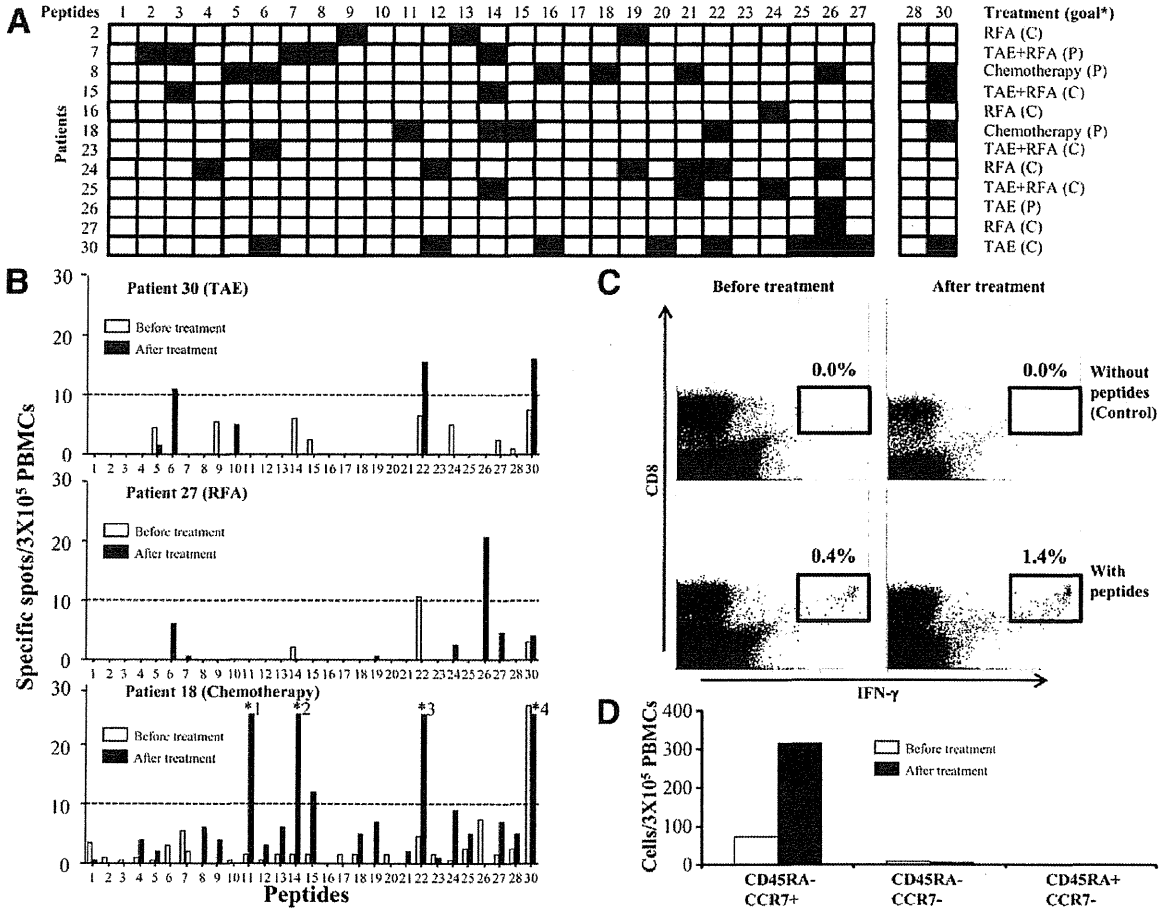

Fig. 4. Enhancement of TAA-specific T-cell responses in HCC patients after treatments. (A) Summary of patients and peptides with a significant increase of the number of IFN-γ-producing T cells (black boxes). A significant change in the IFN-γ response was defined as a more than 2-fold increase and the 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-

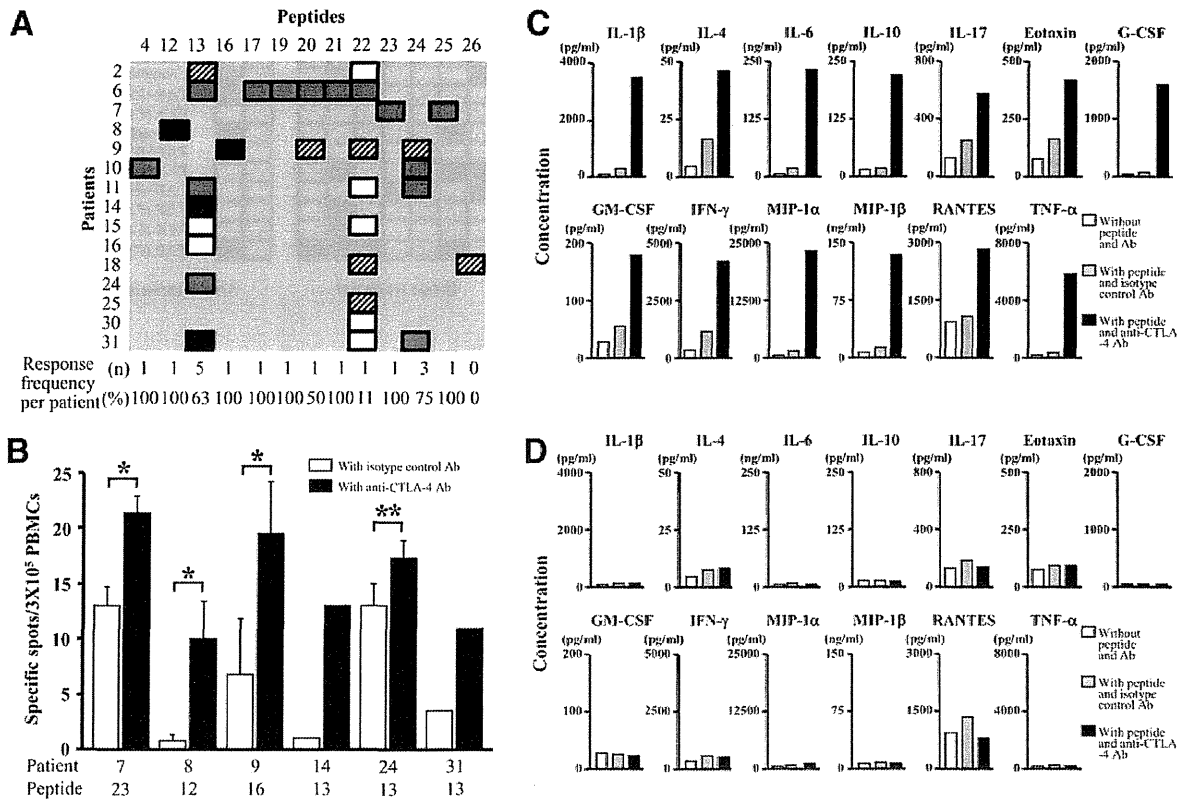

Fig. 5. Enhancement of TAA-specific T-cell responses in HCC patients by CTLA-4 antibodies. (A) Summary of patients and peptides with an increase of the number of IFN-γ-producing T cells. Black, gray, white, and hatched boxes indicate the immune responses with an increase of more than 10 specific spots, 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.

On the other hand, recent studies have shown the important role of $CD4^+$ helper T cells in optimal function and proliferation of $CD8^+$ T cells.[46] Therefore, the lack of $CD4^+$ helper T cells or anergic $CD4^+$ T cells may explain the limited TAA-specific $CD8^+$ T-cell responses in HCC. Further studies using $CD4^+$ T-cell-depleted PBMCs or $CD8^+$ T cells expanded with TAA-derived peptide may enable identification of more immunogenic HCC-specific TAAs and their epitopes.

In conclusion, the results of this study suggest that CypB, SART2, SART3, p53, MRP3, AFP, and hTERT are promising TAAs in HCC immunotherapy, that the administration of these TAAs or peptides containing their epitopes as vaccines after HCC treatment is likely to be effective, and that the concomitant use of anti-CTLA-4 antibodies may further increase antitumor immunity. We believe that the results of this study provide useful information for the development of immunotherapy for HCC.

Acknowledgment: The authors thank Kazumi Fushimi, Maki Kawamura, Masayo Baba and Nami Nishiyama for technical assistance.

References

1. Deuffic S, Poynard T, Buffat L, Valleron AJ. Trends in primary liver cancer. Lancet 1998;351:214-215.
2. Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. Int J Cancer 2001;94:153-156.
3. Lencioni R. Loco-regional treatment of hepatocellular carcinoma. HEPATOLOGY 2010;52:762-773.
4. Okuwaki Y, Nakazawa T, Shibuya A, Ono K, Hidaka H, Watanabe M, et al. Intrahepatic distant recurrence after radiofrequency ablation for a single small hepatocellular carcinoma: risk factors and patterns. J Gastroenterol 2008;43:71-78.
5. Nishizaka S, Gomi S, Harada K, Oizumi K, Itoh K, Shichijo S. A new tumor-rejection antigen recognized by cytotoxic T lymphocytes infiltrating into a lung adenocarcinoma. Cancer Res 2000;60:4830-4837.
6. Kawano K, Gomi S, Tanaka K, Tsuda N, Kamura T, Itoh K, et al. Identification of a new endoplasmic reticulum-resident protein recognized by HLA-A24-restricted tumor-infiltrating lymphocytes of lung cancer. Cancer Res 2000;60:3550-3558.
7. Gomi S, Nakao M, Niiya F, Imamura Y, Kawano K, Nishizaka S, et al. A cyclophilin B gene encodes antigenic epitopes recognized by HLA-A24-restricted and tumor-specific CTLs. J Immunol 1999;163:4994-5004.
8. Harashima N, Tanaka K, Sasatomi T, Shimizu K, Miyagi Y, Yamada A, et al. Recognition of the Lck tyrosine kinase as a tumor antigen by cytotoxic T lymphocytes of cancer patients with distant metastases. Eur J Immunol 2001;31:323-332.
9. Fujie T, Tahara K, Tanaka F, Mori M, Takesako K, Akiyoshi T. A MAGE-1-encoded HLA-A24-binding synthetic peptide induces specific anti-tumor cytotoxic T lymphocytes. Int J Cancer 1999;80:169-172.
10. Nishiyama T, Tachibana M, Horiguchi Y, Nakamura K, Ikeda Y, Takesako K, et al. Immunotherapy of bladder cancer using autologous dendritic cells pulsed with human lymphocyte antigen-A24-specific MAGE-3 peptide. Clin Cancer Res 2001;7:23-31.
11. Kikuchi M, Nakao M, Inoue Y, Matsunaga K, Shichijo S, Yamana H, et al. Identification of a SART-1-derived peptide capable of inducing HLA-A24-restricted and tumor-specific cytotoxic T lymphocytes. Int J Cancer 1999;81:459-466.
12. Nakao M, Shichijo S, Imaizumi T, Inoue Y, Matsunaga K, Yamada A, et al. Identification of a gene coding for a new squamous cell carcinoma antigen recognized by the CTL. J Immunol 2000;164:2565-2574.
13. Yang D, Nakao M, Shichijo S, Sasatomi T, Takasu H, Matsumoto H, et al. Identification of a gene coding for a protein possessing shared tumor epitopes capable of inducing HLA-A24-restricted cytotoxic T lymphocytes in cancer patients. Cancer Res 1999;59:4056-4063.
14. Tanaka H, Tsunoda T, Nukaya I, Sette A, Matsuda K, Umano Y, et al. Mapping the HLA-A24-restricted T-cell epitope peptide from a tumour-associated antigen HER2/neu: possible immunotherapy for colorectal carcinomas. Br J Cancer 2001;84:94-99.
15. Eura M, Chikamatsu K, Katsura F, Obata A, Sobao Y, Takiguchi M, et al. A wild-type sequence p53 peptide presented by HLA-A24 induces cytotoxic T lymphocytes that recognize squamous cell carcinomas of the head and neck. Clin Cancer Res 2000;6:979-986.
16. Umano Y, Tsunoda T, Tanaka H, Matsuda K, Yamaue H, Tanimura H. Generation of cytotoxic T-cell responses to an HLA-A24 restricted epitope peptide derived from wild-type p53. Br J Cancer 2001;84:1052-1057.
17. Ferries E, Connan F, Pages F, Gaston J, Hagnere AM, Vieillefond A, et al. Identification of p53 peptides recognized by CD8(+) T lymphocytes from patients with bladder cancer. Hum Immunol 2001;62:791-798.
18. Yamada A, Kawano K, Koga M, Matsumoto T, Itoh K. Multidrug resistance-associated protein 3 is a tumor rejection antigen recognized by HLA-A2402-restricted cytotoxic T lymphocytes. Cancer Res 2001;61:6459-6466.
19. Mizukoshi E, Nakamoto Y, Tsuji H, Yamashita T, Kaneko S. Identification of alpha-fetoprotein-derived peptides recognized by cytotoxic T lymphocytes in HLA-A24+ patients with hepatocellular carcinoma. Int J Cancer 2006;118:1194-1204.
20. Mizukoshi E, Nakamoto Y, Marukawa Y, Arai K, Yamashita T, Tsuji H, et al. Cytotoxic T-cell responses to human telomerase reverse transcriptase in patients with hepatocellular carcinoma. HEPATOLOGY 2006;43:1284-1294.
21. Ribas A, Butterfield LH, Glaspy JA, Economou JS. Current developments in cancer vaccines and cellular immunotherapy. J Clin Oncol 2003;21:2415-2432.
22. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, et al. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat Med 1998;4:321-327.
23. Itoh K, Yamada A. Personalized peptide vaccines: a new therapeutic modality for cancer. Cancer Sci 2006;97:970-976.
24. Butterfield LH, Ribas A, Meng WS, Dissette VB, Amarnani S, Vu HT, et al. T-cell responses to HLA-A*0201 immunodominant peptides derived from alpha-fetoprotein in patients with hepatocellular cancer. Clin Cancer Res 2003;9:5902-5908.
25. Butterfield LH, Ribas A, Dissette VB, Lee Y, Yang JQ, De la Rocha P, et al. A phase I/II trial testing immunization of hepatocellular carcinoma patients with dendritic cells pulsed with four alpha-fetoprotein peptides. Clin Cancer Res 2006;12:2817-2825.
26. Butterfield LH. Recent advances in immunotherapy for hepatocellular cancer. Swiss Med Wkly 2007;137:83-90.
27. Dougan M, Dranoff G. Immune therapy for cancer. Annu Rev Immunol 2009;27:83-117.
28. O'Day SJ, Maio M, Chiarion-Sileni V, Gajewski TF, Pehamberger H, Bondarenko IN, et al. Efficacy and safety of ipilimumab monotherapy in patients with pretreated advanced melanoma: a multicenter single-arm phase II study. Ann Oncol 2010;21:1712-1717.
29. Araki T, Itai Y, Furui S, Tasaka A. Dynamic CT densitometry of hepatic tumors. AJR Am J Roentgenol 1980;135:1037-1043.

30. Japan. LCSGo. Classification of primary liver cancer. English ed 2. Tokyo: Kanehara; 1997.
31. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. HEPATOLOGY 1994;19:1513-1520.
32. Ikeda-Moore Y, Tomiyama H, Miwa K, Oka S, Iwamoto A, Kaneko Y, et al. Identification and characterization of multiple HLA-A24-restricted HIV-1 CTL epitopes: strong epitopes are derived from V regions of HIV-1. J Immunol 1997;159:6242-6252.
33. Kurokohchi K, Arima K, Nishioka M. A novel cytotoxic T-cell epitope presented by HLA-A24 molecule in hepatitis C virus infection. J Hepatol 2001;34:930-935.
34. Kuzushima K, Hayashi N, Kimura H, Tsurumi T. Efficient identification of HLA-A*2402-restricted cytomegalovirus-specific CD8(+) T-cell epitopes by a computer algorithm and an enzyme-linked immunospot assay. Blood 2001;98:1872-1881.
35. Oiso M, Eura M, Katsura F, Takiguchi M, Sobao Y, Masuyama K, et al. A newly identified MAGE-3-derived epitope recognized by HLA-A24-restricted cytotoxic T lymphocytes. Int J Cancer 1999;81:387-394.
36. Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, et al. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. J Exp Med 2000;192:303-310.
37. Zerbini A, Pilli M, Penna A, Pelosi G, Schianchi C, Molinari A, et al. Radiofrequency thermal ablation of hepatocellular carcinoma liver nodules can activate and enhance tumor-specific T-cell responses. Cancer Res 2006;66:1139-1146.
38. Ayaru L, Pereira SP, Alisa A, Pathan AA, Williams R, Davidson B, et al. Unmasking of alpha-fetoprotein-specific CD4(+) T-cell responses in hepatocellular carcinoma patients undergoing embolization. J Immunol 2007;178:1914-1922.
39. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 1999;401:708-712.
40. Peggs KS, Quezada SA, Chambers CA, Korman AJ, Allison JP. Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. J Exp Med 2009;206:1717-1725.
41. Zerbini A, Pilli M, Soliani P, Ziegler S, Pelosi G, Orlandini A, et al. Ex vivo characterization of tumor-derived melanoma antigen encoding gene-specific CD8+cells in patients with hepatocellular carcinoma. J Hepatol 2004;40:102-109.
42. Gehring AJ, Ho ZZ, Tan AT, Aung MO, Lee KH, Tan KC, et al. Profile of tumor antigen-specific CD8 T cells in patients with hepatitis B virus-related hepatocellular carcinoma. Gastroenterology 2009;137: 682-690.
43. Fujioka M, Nakashima Y, Nakashima O, Kojiro M. Immunohistologic study on the expressions of alpha-fetoprotein and protein induced by vitamin K absence or antagonist II in surgically resected small hepatocellular carcinoma. HEPATOLOGY 2001;34:1128-1134.
44. Hussain SP, Schwank J, Staib F, Wang XW, Harris CC. TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. Oncogene 2007;26:2166-2176.
45. Fu J, Xu D, Liu Z, Shi M, Zhao P, Fu B, et al. Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. Gastroenterology 2007;132:2328-2339.
46. Kennedy R, Celis E. Multiple roles for CD4+ T cells in anti-tumor immune responses. Immunol Rev 2008;222:129-144.

GASTROENTEROLOGY 2011;141:128–140

Malnutrition Impairs Interferon Signaling Through mTOR and FoxO Pathways in Patients With Chronic Hepatitis C

MASAO HONDA,*,‡ KENJI TAKEHANA,§ AKITO SAKAI,* YUSUKE TAGATA,§ TAKAYOSHI SHIRASAKI,‡ SHINOBU NISHITANI,§ TAKAHIKO MURAMATSU,‖ TATSUYA YAMASHITA,* YASUNARI NAKAMOTO,* EISHIRO MIZUKOSHI,* YOSHIO SAKAI,* TARO YAMASHITA,* MIKIKO NAKAMURA,* TETSURO SHIMAKAMI,¶ MINKYUNG YI,# STANLEY M. LEMON,¶ TETSUO SUZUKI,** TAKAJI WAKITA,** SHUICHI KANEKO,* and the Hokuriku Liver Study Group

*Department of Gastroenterology, ‡Department of Advanced Medical Technology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan; §Exploratory Research Laboratories, Research Center, Ajinomoto Pharmaceuticals, Co, Ltd, Kanagawa, Japan; ‖Frontier Research Labs, Institute for Innovation, Ajinomoto, Co, Inc, Kanagawa, Japan; ¶Division of Infectious Diseases, School of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; #Center for Hepatitis Research, Institute for Human Infections and Immunity, and Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas; and **Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan

CLINICAL LIVER

BACKGROUND & AIMS: Patients with advanced chronic hepatitis C (CH-C) often are malnourished, but the effects of malnutrition on interferon (IFN) signaling and response to treatment have not been determined. We assessed the importance of the nutritional state of the liver on IFN signaling and treatment response. **METHODS:** We studied data from 168 patients with CH-C who were treated with the combination of pegylated-IFN and ribavirin. Plasma concentrations of amino acids were measured by mass spectrometry. Liver gene expression profiles were obtained from 91 patients. Huh-7 cells were used to evaluate the IFN signaling pathway, mammalian target of rapamycin complex 1 (mTORC1), and forkhead box O (FoxO). Antiviral signaling induced by branched-chain amino acids (BCAAs) was determined using the in vitro hepatitis C virus replication system. **RESULTS:** Multivariate logistic regression analysis showed that Fischer's ratio was associated significantly with nonresponders, independent of interleukin-28B polymorphisms or the histologic stage of the liver. Fischer's ratio was correlated inversely with the expression of BCAA transaminase 1, and was affected by hepatic mTORC1 signaling. IFN stimulation was impaired substantially in Huh-7 cells grown in medium that was low in amino acid concentration, through repressed mTORC1 signaling, and increased Socs3 expression, which was regulated by Foxo3a. BCAA could restore impaired IFN signaling and inhibit hepatitis C virus replication under conditions of malnutrition. **CONCLUSIONS: Malnutrition impaired IFN signaling by inhibiting mTORC1 and activating Socs3 signaling through Foxo3a. Increasing BCAAs to up-regulate IFN signaling might be used as a new therapeutic approach for patients with advanced CH-C.**

Keywords: HCV; Liver Disease; Therapy; Diet.

Interferon (IFN) and ribavirin (RBV) combination therapy is a popular modality for treating patients with chronic hepatitis C (CH-C), but approximately 50% of patients usually relapse, particularly those with hepatitis C virus (HCV) genotype 1b and a high viral load.[1]

Recent landmark studies of genome-wide associations identified genomic loci associated with treatment responses to pegylated (Peg)-IFN and RBV combination therapy,[2,3] and a polymorphism in the interleukin (IL)-28B gene was found to predict hepatitis C treatment-induced viral clearance. Moreover, we previously showed that expression of hepatic IFN-stimulated genes (ISGs) was associated with the IL-28B polymorphism and might contribute to the treatment response.[4] In addition to the IL-28B polymorphism, host factors such as fibrosis stage and metabolic status of the liver might be associated with the treatment outcome[4,5]; however, the significance of these factors in conjunction with the IL-28B polymorphism has not been evaluated fully.

In CH-C livers, prolonged liver cell damage, fibrosis development, and microcirculation failure can lead to a state of malnutrition in hepatocytes, resulting in the impairment of multiple metabolic pathways. In patients with advanced stage CH-C, hypoalbuminemia and decreased plasma values for the Fischer's ratio of branched-amino acids (BCAA; leucine, isoleucine, and valine) to aromatic amino acids (tyrosine and phenylalanine) commonly are observed. BCAA are the essential amino acids necessary for ammonium metabolism in muscle when the liver is unable to perform this function. Recent reports have shown that BCAA activates albumin synthesis in rat

Abbreviations used in this paper: BCAA, branched-chain amino acid; BCAT1, branched chain amino-acid transaminase 1; CH-C, chronic hepatitis C; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle medium; FBE, Foxo binding element; FBEmut, Foxo binding element mutant; FoxO, forkhead box, subgroup O; GLuc, Gaussia luciferase; IFN, interferon; IL, interleukin; ISG, interferon-stimulated genes; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; NR, no response; PCR, polymerase chain reaction; Peg, pegylated; p-mTOR, phosphorylated form of mammalian target of rapamycin; pS6K, phosphorylated form of p70 S6 protein kinase; pSTAT1, phosphorylated form of signal transducer and activator of transcription 1; Raptor, regulatory associated protein of mTOR; RBV, ribavirin; S6K, p70 S6 protein kinase; siRNA, small interfering RNA; SVR, sustained viral response; TR, transient response.

0016-5085/$36.00
doi:10.1053/j.gastro.2011.03.051

primary hepatocytes[6] and cirrhotic rat liver[7] through mammalian target of rapamycin (mTOR) signaling, a central regulator of protein synthesis, by sensing nutrient conditions.[8] Thus, peripheral amino acid composition is closely related to signaling pathways in the liver.

In addition to metabolic aspects, recent reports have elucidated new functional roles for mTOR in the IFN signaling pathway. Targeted disruptions of tuberous sclerosis 2 and eukaryotic translation initiation factor 4E binding protein 1, which both inhibit mTOR complex 1 (mTORC1) signaling, substantially enhanced IFN-alfa-dependent antiviral responses.[9,10] Therefore, mTORC1 signaling might be involved in the antiviral response as well as in metabolic processes. However, these issues have not yet been addressed in terms of IFN treatment for CH-C. In the present study, therefore, we evaluated the clinical relevance of the nutritional state of the liver, as estimated by the plasma Fischer's ratio, on Peg-IFN and RBV combination therapy. We also evaluated antiviral signaling induced by BCAA using an in vitro HCV replication system.

Materials and Methods

Patients

A total of 168 patients with CH-C at the Graduate School of Medicine at Kanazawa University Hospital (Kanazawa, Japan) and its related hospitals in Japan (Table 1, Supplementary Table 1) were evaluated in the present study. The clinical characteristics of these patients have been described previously.[4] All patients were administered Peg-IFN-alfa 2b (Schering-Plough K.K., Tokyo, Japan) and RBV combination therapy for 48 weeks. The definition of the treatment response was as follows: sustained viral response (SVR), clearance of HCV viremia 24 weeks after the cessation of therapy; transient response (TR), no detectable HCV viremia at the cessation of therapy but relapse during the follow-up period; and no response (NR). Genetic variation of the IL-28B polymorphism at rs8099917 was evaluated in all patients using TaqMan Pre-Designed SNP Genotyping Assays (Applied Biosystems, Carlsbad, CA) as described previously.[4] Gene expression profiling in the liver was performed in 91 patients using the Affymetrix Human 133 Plus 2.0 microarray chip (Affymetrix, Santa Clara, CA) as described previously (Supplementary Table 1).[4]

Plasma Amino Acid Analysis

Amino acid concentrations in plasma samples were measured by high-performance liquid chromatography-electrospray ionization-mass spectrometry, followed by derivatization.[11] Detailed experimental procedures are described in the Supplementary Materials and Methods section.

Culture Medium

Huh-7 and Huh-7.5 cells (kindly provided by Professor C. M. Rice, Rockefeller University, New York, NY) were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Gaithersburg, MD) containing 10% fetal bovine serum and 1% penicillin/streptomycin (normal medium). Amino acid-free medium (ZERO medium) was prepared by mixing 5.81 g nutrition-free DMEM (Nacalai Tesque, Kyoto, Japan), 1.85 g $NaHCO_3$, 1 g glucose, and 0.5 mL 1M (mol/L) sodium pyruvate in 500 mL Milli-Q water, then sterilizing with a 0.22-μm filter (Millipore, Billerica, MA). Low amino acid media (×1/5, ×1/10, ×1/30, and ×1/100 DMEM) were prepared by diluting ×1 DMEM with ZERO medium. Powdered BCAA (leucine-isoleucine-valine, 2:1:1.2) (Ajinomoto Pharma, Tokyo, Japan) was freshly dissolved with distilled water at 100 mmol/L, then applied to cultured medium at 2 mmol/L, 4 mmol/L, or 8 mmol/L.

Western Blotting and Immunofluorescence Staining

A total of 1.5×10^5 Huh-7 cells were seeded in normal medium 24 hours before performing the experiments. The medium was changed to low-amino-acid medium and maintained for up to 24 hours. Western blotting was performed as previously described.[12] Cells were washed in phosphate-buffered saline (PBS) and lysed in RIPA buffer containing complete Protease Inhibitor Cocktail and PhosSTOP (Roche Applied Science, Indianapolis, IN). The membranes were blocked in Blocking One-P (Nacalai Tesque). The antibodies used for Western blotting are summarized in the Supplementary Materials and Methods section.

For immunofluorescence staining, cells were fixed with 4% paraformaldehyde in PBS, then permeabilized with 0.1% Triton-X 100 in PBS. The primary anti-forkhead box O (Foxo)3a antibody (Abcam, Cambridge, MA) was used at a final concentration of 2 μg/mL in PBS containing 2% fetal bovine serum at 4°C for 16 hours. Incubation with the Alexa Fluor 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA) at a 500-fold dilution in PBS containing 3% fetal bovine serum antibody was performed for 4 hours, and cells were stained with Hoechst 33258 to visualize nuclear DNA (Vector Laboratories, Burlingame, CA).

Quantitative Real-Time Detection Polymerase Chain Reaction

A total of 1.5×10^5 Huh-7 cells were seeded in normal medium 24 hours before performing the experiments. The medium was changed to low-amino-acid medium, to which IFN-alfa and/or BCAA was added, and maintained for 24 hours. Rapamycin treatment (100 nmol/L) was performed for 30 minutes in normal medium before a medium change. RNA was isolated using TriPure isolation reagent (Roche Applied Science), and complementary DNA (cDNA) was synthesized using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). Real-time detection polymerase chain reaction (PCR) was performed using the 7500 Real-Time PCR System (Applied Biosystems) and Power SYBR Green PCR Master Mix (Applied Biosystems) containing specific primers according to the manufacturer's

Table 1. Comparison of Clinical Factors Between Patients With and Without NR

Clinical category	SVR+TR	NR	Univariate *P* value	Multivariate odds (95% CI)	Multivariate *P* value
Patients, n	125	43		—	
Age and sex					
Age, y	57 (30–72)	56 (30–73)	.927	—	
Sex, male vs female	68 vs 57	24 vs 19	.872	—	
Liver histology					
F stage (F1–2 vs F3–4)	95 vs 30	20 vs 23	.001	6.35 (2.02–23.7)	.001
A grade (A0–1 vs A2–3)	68 vs 57	19 vs 24	.248	—	
Host gene factors					
IL-28B (TT vs TG/GG)[a]	109 vs 12	12 vs 31	<.001	19.7 (5.74–82.7)	<.001
ISGs (Mx, IFI44, IFIT1), (<3.5 vs ≥3.5)	103 vs 22	12 vs 31	<.001	5.26 (1.65–17.6)	.005
Metabolic factors					
BMI, kg/m^2	23.2 (16.3–34.7)	23.4 (19.5–40.6)	.439	—	
TG, mg/dL	98 (30–323)	116 (45–276)	.058	—	
T-Chol, mg/dL	167 (90–237)	160 (81–214)	.680	—	
LDL-Chol, mg/dL	82 (36–134)	73 (29–123)	.019	—	
HDL-Chol, mg/dL	42 (20–71)	47 (18–82)	.098	—	
FBS, mg/dL	94 (60–291)	96 (67–196)	.139	—	
Insulin, μU/mL	6.6 (0.7–23.7)	6.8 (2–23.7)	.039	—	
HOMA-IR	1.2 (0.3–11.7)	1.2 (0.4–7.2)	.697	—	
Fischer ratio	2.3 (1.5–3.3)	2.1 (1.5–2.8)	.005	8.91 (1.62–55.6)	.011
Other laboratory parameters					
AST level, IU/L	46 (18–258)	64 (21–283)	.017	—	
ALT level, IU/L	60 (16–376)	82 (18–345)	.052	—	
γ-GTP level, IU/L	36 (4–367)	75 (26–392)	<.001	—	
WBC, /mm^3	4800 (2100–11100)	4800 (2500–8200)	.551	—	
Hb level, g/dL	14 (9.3–16.6)	14.4 (11.2–17.2)	.099	—	
PLT, ×104/mm^3	15.7 (7–39.4)	15.2 (7.6–27.8)	.378	—	
Viral factors					
ISDR mutations ≤1 vs ≥2	80 vs 44	34 vs 9	.070	4.12 (1.25–15.9)	.019
HCV-RNA, KIU/mL	2300 (126–5000)	1930 (140–5000)	.725	—	
Treatment factors					
Total dose administered					
Peg-IFN, μg	3840 (960–7200)	3840 (1920–2880)	.916	—	
RBV, g	202 (134–336)	202 (36–336)	.531	—	
Achieved administration rate					
Peg-IFN, %					
≥80%	84	28	.975	—	
<80%	42	14			
RBV (%)					
≥80%	76	24	.745	—	
<80%	50	18			
Achievement of EVR	101/125 (81%)	0/43 (0%)	<.001	—	

BMI, body mass index; CI, confidence interval; FBS, fasting blood sugar; γ-GTP, gamma-glutamyl transpeptidase; Hb, hemoglobin; HDL-chol, high density lipoprotein cholesterol; LDL-chol, low density lipoprotein cholesterol; PLT, platelets; T-chol, total cholesterol; TG, triglycerides; WBC, leukocytes.

[a]IL-28B SNP at rs8099917.

instructions. The primer sequence for real-time detection PCR is given in the Supplementary Materials and Methods section. HCV RNA was detected as described previously[12] and expression was standardized to that of glyceraldehyde-3-phosphate dehydrogenase.

Reporter Assay

Construction of the interferon stimulated response element (ISRE)-luc reporter plasmid and Socs3-luc or Socs3 (FoxO binding element mutant [FBEmut])-luc reporter plasmids is described in the Supplementary Materials and Methods section.

Huh-7 cells were transfected with the ISRE-luc reporter plasmid 24 hours before IFN-alfa treatment. Cells were treated with IFN-alfa (0 or 100 U/mL) and BCAA (2 mmol/L) in low-amino-acid media. After 24 hours, luciferase activities were measured using the Dual Luciferase assay system (Promega, Madison, WI). For Socs3 promoter activities, Huh-7 cells were transfected with Socs3-luc or Socs3 (FBEmut)-luc reporter plasmids together with the Foxo3a expression plasmid, and luciferase activities were measured after 24 hours. Values were normalized to the luciferase activity of the co-transfected pGL4.75 Renilla luciferase-expressing plasmid (Promega).

Knockdown Experiments

Huh-7 cells were transfected with Ctrl (Stealth RNAi Negative Control Low GC Duplex #2; Invitrogen) or

targets (regulatory associated protein of mTOR [Raptor] and Foxo3a) (Supplementary Materials and Methods) small interfering RNA (siRNA) using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. After 48 hours, cells were cultured in normal or low-amino-acid media for a further 24 hours. The knockdown effect was confirmed by Western blotting.

Chromatin Immunoprecipitation Assay

Detailed experimental procedures are described in the Supplementary Materials and Methods section.

HCV Replication Analysis

pH77S3 is an improved version of pH77S, a plasmid containing the full-length sequence of the genotype 1a H77 strain of HCV with 5 cell culture–adaptive mutations that promote its replication in Huh-7 hepatoma cells.[13] pH77S.3/Gaussia luciferase (GLuc)2A is a related construct in which the GLuc sequence, fused to the 2A autocatalytic protease of foot-and-mouth virus RNA, was inserted in-frame between p7 and NS2[14] (Supplementary Materials and Methods). A signal sequence in GLuc directs its secretion into cell culture media, allowing real-time, dynamic measurements of GLuc expression to be performed without the need for cell lysis.

A 10-μg aliquot of synthetic RNA transcribed from pH77S.3/GLuc2A was used for electroporation. Cells were pulsed at 260 V and 950 μF using the Gene Pulser II apparatus (Bio-Rad Laboratories, Hercules, CA) and plated in fresh normal medium for 12 hours to recover. Cell medium was changed to ×1 DMEM without serum for 8 hours, then changed to low-amino-acid medium containing 0–8 mmol/L BCAA for a further 24 hours. Cells and culture medium were collected and used for GLuc assays, real-time detection PCR, and Western blotting. The number of viable cells was determined by a (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl] -2H-tetrazolium, inner salt) assay (Promega).

Continuously JFH-1–infecting Huh-7 cells were obtained by the infection of Huh-7 cells with JFH-1 cell culture-derived HCV at a multiplicity of infection of 0.01. Cells were maintained in normal medium by passaging every 3–4 days for approximately 6 months. About 20%–30% of the cells consistently were positive for HCV core protein (Supplementary Figure 4). Culture medium of JFH-1–infecting Huh-7 cells was changed to the low-amino-acid medium containing 0–8 mmol/L BCAA for 24 hours. Cells then were collected and used for assays.

Statistical Analysis

Results are expressed as mean ± standard deviation. Significance was tested by 1-way analysis of variance with the Bonferroni method, and differences were considered statistically significant at a P value of less than .05.

Results

Fischer's Ratio as a Predictive Factor for Treatment Response

The clinical characteristics of patients who received Peg-IFN and RBV combination therapy are shown in Table 1 and Supplementary Table 1, and explanations of these characteristics have been described previously.[4] All patients were infected with HCV genotype 1b and had a high viral load (>100 IU/mL). We compared patients with SVR + TR against those with NR, as assessed by the overall plausibility of treatment response groups using Fisher's C statistic as previously described.[4] We included data on the IL-28B polymorphism and plasma amino acid composition (aminogram).

Univariate regression analysis showed that no single amino acid was associated significantly with treatment response; however, using Fischer's ratio, the BCAA (Ile+Leu+Val)/aromatic amino acids (Phe+Tyr) ratio was associated significantly with treatment response (P = .005) (Table 1). Of the 121 patients with IL-28B major type, SVR, TR, and NR were observed in 53%, 37%, and 10%, respectively, and among 33 patients with IL-28B minor type, SVR, TR, and NR were observed in 15%, 17%, and 68%, respectively (P < .001) (data not shown). Fischer's ratio of SVR, TR, and NR was 2.35 ± 0.38, 2.30 ± 0.29, and 2.10 ± 0.31, respectively (P < .015) (data not shown).

We selected IL-28B polymorphism, hepatic ISG expression, fibrosis stage, HCV RNA, interferon sensitivity determining region mutation, and Fischer's ratio as factors for multivariate analysis. Multivariate analysis revealed that the minor type of IL-28B polymorphism (TG or GG at rs8099917) (odds ratio, 19.7; P < .001), advanced fibrosis stage of the liver (F3–4) (odds ratio, 6.35; P = .001), high hepatic ISGs (≧3.5) (odds ratio, 5.26; P = .005), low Fischer's ratio (continuous range, 1.5–3.3) (unit odds, 8.91; P = .011), and presence of ISDR mutation (≦1) (odds ratio, 4.12; P = .019) independently contributed to NR (Table 1).

The distribution of the Fischer's ratio according to fibrosis stage is shown in Supplementary Figure 1. The ratio decreased significantly in advanced fibrosis stage (F3–4) compared with early fibrosis stage (F1). No significant association between major or minor type of IL-28B polymorphism and different fibrosis stages of the liver was observed (Supplementary Figure 1*A*). In early fibrosis (F1–2) (Supplementary Figure 1*B*), 90% (80 of 89) of SVR+TR cases had the major type of IL-28B polymorphism, and 94% (16 of 17) of NR cases had the minor type. However, in the advanced fibrosis stage of the liver (F3–4) (Supplementary Figure 1*C*), 85% (23 of 27) of SVR+TR cases had the major type of IL-28B polymorphism and 50% (10 of 20) of NR cases had the minor type. Thus, in advanced fibrosis stages, factors other than the IL-28B polymorphism appear to contribute to NR. Interestingly, the Fischer's ratio was significantly lower in NR patients than SVR+TR pa-

tients in the advanced fibrosis stage of the liver. Therefore, Fischer's ratio could be an important predictor for NR that is independent of IL-28B polymorphism and histologic stage of the liver.

Fischer's Ratio and mTORC1 Signaling in CH-C Livers

Hepatic gene expression in 91 of 168 patients (Supplementary Table 1) was obtained using Affymetrix genechip analysis as described previously.[4] To examine the relationship between the plasma Fischer's ratio and mTORC1 signaling in the liver we evaluated the expression of key regulatory genes related to mTORC1 signaling. We found that expression of branched chain amino-acid transaminase 1 (BCAT1), an important catalytic enzyme of BCAA, was significantly negatively correlated with Fischer's ratio (Figure 1*A*). This indicates that the plasma Fischer's ratio is regulated in the liver as well as in peripheral muscle. Interestingly, the expression of c-myc, a positive regulator of BCAT1 (Figure 1*C*),[15] was correlated negatively with the Fischer's ratio (Figure 1*B*). The expression of PDCD4, a negative transcriptional target of ribosomal *p70 S6 protein kinase (S6K)*, downstream of mTORC1, was correlated significantly with BCAT1 (Figure 1*D* and *E*). Thus, in CH-C livers, BCAT1 is induced with progressive liver disease and mTORC1 signaling is repressed, a process that might involve c-myc. Fischer's ratio of the plasma therefore can be seen to reflect mTORC1 signaling in the liver.

Impaired IFN Signaling in Huh-7 Cells Grown in Low-Amino-Acid Medium

Recent reports have shown the functional relevance of mTOR on IFN signaling and antiviral responses.[9,10] To evaluate IFN-alfa signaling and the mTOR pathway, we used Huh-7 cells grown in different amino acid conditions (×1 DMEM, ×1/5 DMEM, ×1/30 DMEM, and ×1/100 DMEM). The phosphorylated forms of mTOR (p-mTOR) and S6K (pS6K), an important downstream regulator of mTORC1 signaling, were decreased substantially in ×1/30 DMEM and ×1/100 DMEM (Figure 2*A*). Interestingly, the expression of the phosphorylated form of signal transducer and activator of transcription 1 (pSTAT1), an essential transducer of type 1 IFN signaling, also was decreased in these conditions (Figure 2*A*). Similarly, the expression of p-mTOR and pSTAT1 was repressed significantly in CH-C livers with a low Fischer's ratio compared with those with a high Fischer's ratio (Supplementary Figure 2, Supplementary Table 2).

To examine whether decreased pSTAT1 expression might be owing to repressed mTORC1 signaling, we knocked down the expression of Raptor, a specific subunit of mTORC1. We achieved more than 50% knockdown of Raptor by specific siRNA (Figure 2*B*). Under these conditions, the expression of p-mTOR and pSK6 were repressed, which is consistent with previous reports.[16] The expression of pSTAT1 also was repressed after Raptor knockdown (Figure 2*B*).

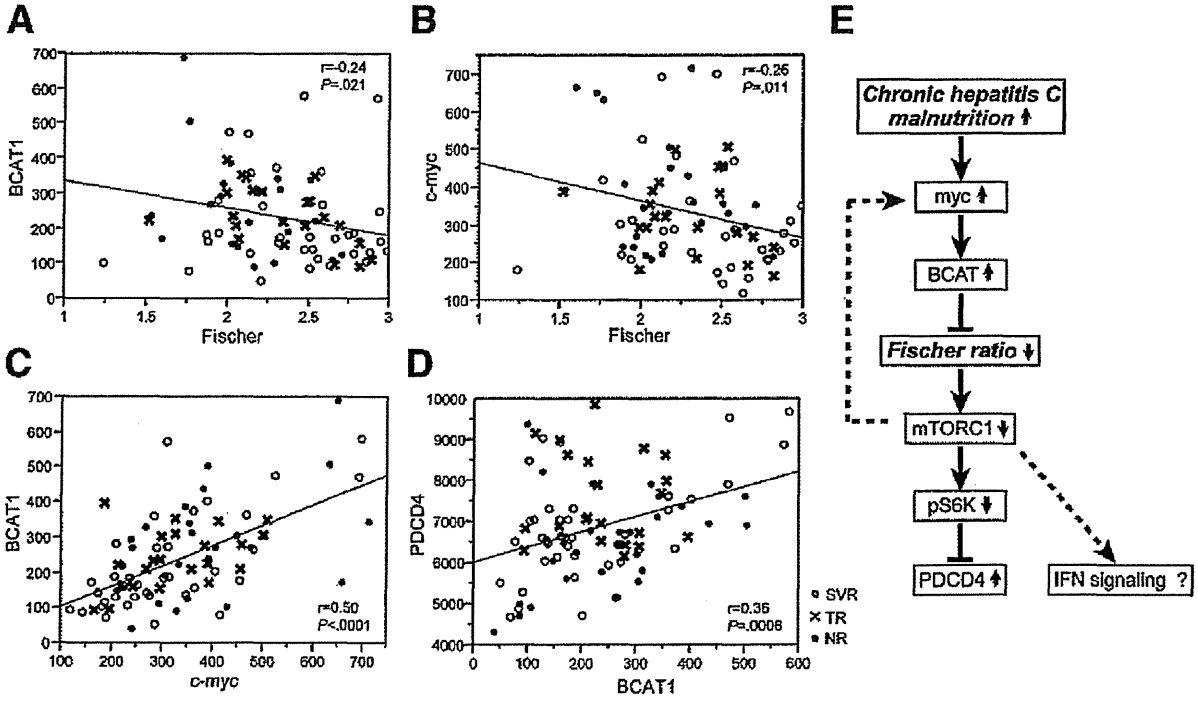

Figure 1. Regression analysis of mTORC1-related gene expression in liver. Gene expression values were determined by probe intensities. (*A*) BCAT1 and Fischer's ratio. (*B*) c-myc and Fischer's ratio. (*C*) BCAT1 and c-myc. (*D*) PDCD4 and BCAT1. (*E*) Putative signaling of mTORC1-related genes in CH-C.

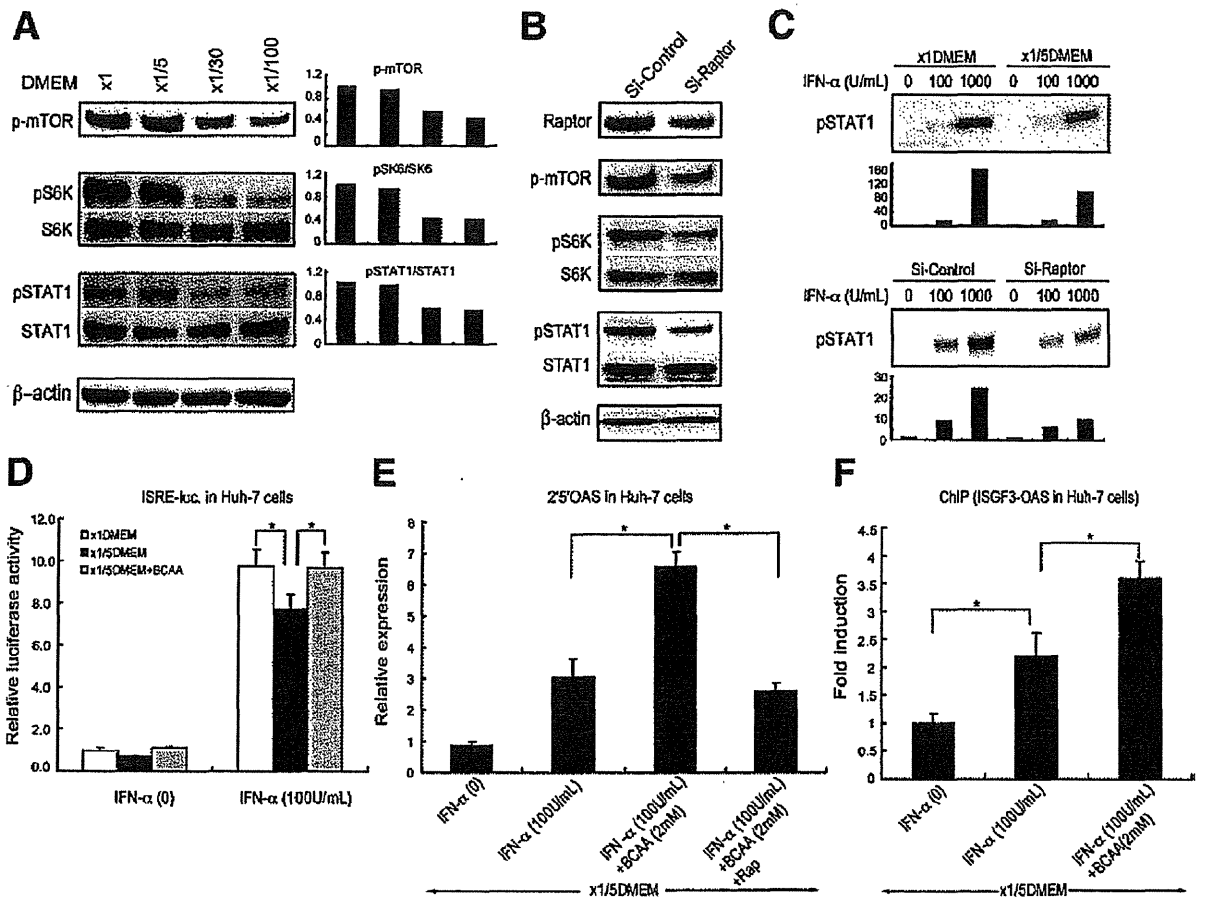

Figure 2. mTORC1 and IFN signaling in Huh-7 cells in low-amino-acid medium. (*A*) p-mTOR, pS6K, and pSTAT1 expression in different amino acid media. (*B*) p-mTOR, pS6K, and pSTAT1 expression under Raptor knock-down conditions. (*C*) IFN-alfa stimulation and pSTAT1 expression in low-amino-acid media or under Raptor knock-down conditions. (*D*) IFN-alfa stimulation and ISRE reporter activities in normal and low-amino-acid media. (*E*) IFN-alfa stimulation and 2'5'OAS expression supplemented with BCAA or rapamycin in low-amino-acid medium. (*F*) Chromatin immunoprecipitation of 2'5'OAS promoter region by ISGF3γ.

The induction of pSTAT1 by IFN-alfa (1000 U/mL) stimulation was impaired in ×1/5 DMEM or in Raptor knocked-down condition, compared with the control (Figure 2*C*). Consistent with these results, IFN-alfa–induced ISRE-dependent transcriptional activity, as measured using an ISRE-luciferase reporter assay, was impaired significantly in ×1/5 DMEM compared with ×1 DMEM (Figure 2*D*). However, this activity could be rescued by the addition of 2 mmol/L BCAA (Figure 2*D*). These results were confirmed by determining the expression of the endogenous IFN-alfa responsive gene, 2'5'OAS, using quantitative reverse-transcription PCR. Figure 2*E* shows that BCAA treatment augmented 2'5'OAS expression in low levels of amino acids, and that this could be reversed by the addition of rapamycin, an inhibitor of mTORC1 (Figure 2*E*). Furthermore, chromatin immunoprecipitation (ChIP) experiments revealed that transcriptional augmentation by BCAA was mediated by the binding of the IFN-alfa–inducible transcription factor, ISGF3γ, to the promoter region of 2'5'OAS (Figure 2*F*). These results indicate that amino acids in culture media play an essential role in IFN-alfa signaling through mTORC1 signaling, and that the addition of BCAA can overcome impaired IFN-alfa signaling in Huh-7 cells.

Induction of Socs3 in Low-Amino-Acid Medium in Huh-7 Cells

Besides being involved in mTOR signaling, Foxo transcriptional factors mediate another important branch of nutrition-sensing signaling pathway.[17] Therefore, we evaluated forkhead box O3A (Foxo3a) expression in low-amino-acid conditions in Huh-7 cells. After 6 hours culture in ×1/5, ×1/30, and ×1/100 DMEM, expression of the phosphorylated form of Foxo3a (pFoxo3a) decreased, whereas that of total Foxo3a increased in ×1/5 and ×1/30 DMEM, and the ratio of pFoxo3a to Foxo3a (pFoxo3a/Foxo3a) substantially decreased (Figure 3*A* and *B*). It has been reported that dephosphorylated Foxo3a is translocated to the nucleus before activation of its target genes.[18] In the present study, immunofluorescent staining

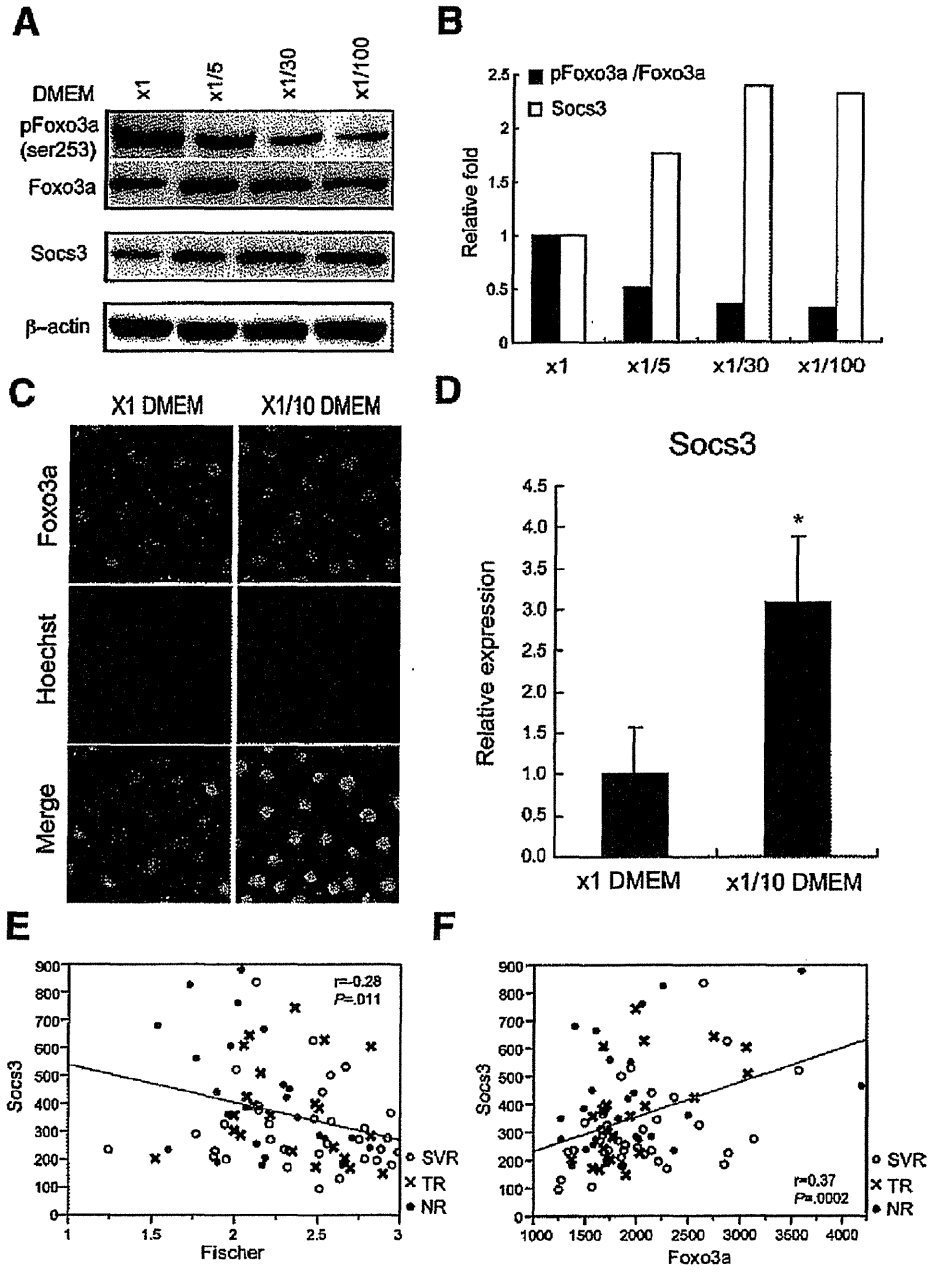

Figure 3. Foxo3a and Socs3 signaling in Huh-7 cells in low-amino-acid medium. (*A*) Foxo3a and Socs3 expression in different amino acid media. (*B*) Relative change of pFoxo3a/Foxo3a and Socs3 expression in different amino acid media. (*C*) Immunofluorescence staining of Foxo3a in Huh-7 cells in normal and low-amino-acid media. (*D*) Relative change of Socs3 messenger RNA in Huh-7 cells in normal and low-amino-acid media. (*E*) Regression analysis of Socs3 in liver and Fischer's ratio. (*F*) Regression analysis of Socs3 and Foxo3a in liver.

with an anti-Foxo3a antibody showed that Foxo3a diffused in both the cytoplasm and nucleus in normal amino acid medium, but localized in the nucleus in low-amino-acid medium (×1/10 DMEM) (Figure 3*C*).

Interestingly, in low-amino-acid medium, transcription and protein expression of Socs3 increased significantly (Figure 3*A*, *B*, and *D*). The induction of Socs3 in a state of malnutrition also was confirmed in clinical samples. In CH-C livers there was a significant negative correlation between the plasma Fischer's ratio and Socs3 expression, implying that Socs3 expression increases during the malnutrition state induced by CH-C. There was also a significant correlation between Foxo3a and the transcriptional level of Socs3 in CH-C livers (Figure 3*E* and *F*), suggesting an in vitro and in vivo biological role for Foxo3a in the activation of Socs3 expression. These findings also were confirmed by Western blotting of CH-C livers (Supplementary Figure 2, Supplementary Table 2).

Socs3 Is a Transcriptional Target of Foxo3a

The significant correlation between Socs3 and Foxo3a in CH-C livers prompted us to analyze the Socs3 promoter sequence and, in doing so, we identified a putative Foxo binding element (FBE) (Figure 4*A*). To investigate the functional relevance of Foxo3a in the transcriptional regulation of Socs3, we constructed reporter plasmids containing a luciferase coding region fused to the Socs3 promoter region (Socs3-luc). Socs3-luc promoter activity was increased substantially by the overexpression of Foxo3a (Figure 4*B*). The mutations introduced in the putative FBE (FBEmut) in the Socs3 promoter significantly reduced Foxo3a-induced Socs3 promoter activation (Figure 4*B*).

Foxo3a then was knocked down by siRNA and Socs3 induction was evaluated. After suppression of Foxo3a (Supplementary Figure 3), Socs3 promoter activity was repressed significantly in low-amino-acid medium (×1/10 DMEM) (Figure 4*C*). Thus, Foxo3a appears to be indispensable for activating the Socs3 promoter under low-amino-acid conditions. Correlating with these results, ChIP assays using an anti-Foxo3a antibody showed a significant increase in the association between Foxo3a and the FBE of the Socs3 promoter in low-amino-acid conditions (×1/10 DMEM) (Figure 4*D*). Taken together, these results suggest that, besides mTORC1 signaling, the Foxo3a-mediated Socs3 signaling pathway might contribute to impaired IFN signaling in a state of malnutrition in CH-C. BCAA potentially restores this signaling (Figure 4*E*).

Effect of BCAA on HCV Replication in Huh-7 or Huh-7.5 Cells

Based on the earlier-described results, we used 2 HCV in vitro replication systems to examine whether BCAA affects HCV replication in Huh-7 or Huh-7.5 cells. The first system used a recombinant infectious genotype 1a clone, H77S.3/GLuc2A (Supplementary Materials and Methods, Supplementary Figure 4), including reporter genes, whereas the second used continuously JFH-1–infecting Huh-7 cells (Supplementary Materials and Methods).

The synthetic RNA transcribed from pH77S.3/GLuc2A was introduced into Huh-7.5 cells and replication of H77S.3/GLuc2A was evaluated in normal or low-amino-acid medium supplemented with BCAA. H77S.3/GLuc2A increased significantly by 2.6-fold in Huh-7.5 cells grown in low-amino-acid medium (×1/5 DMEM) compared with normal amino acid medium (×1 DMEM). Interestingly, BCAA repressed H77S.3/GLuc2A replication in a dose-dependent manner (Figure 5*A*). In agreement with these results, the expression of Mx-1 was increased significantly by the addition of BCAA (Figure 5*B*). Similar findings were observed in JFH-1–infecting Huh-7 cells (Materials and Methods, Supplementary Figure 4). Although no obvious increase in HCV replication was observed in low-amino-acid medium (×1/5 DMEM) compared with normal amino acid medium (×1 DMEM), JFH-1 replication was repressed significantly by the addition of BCAA in a dose-dependent manner (Figure 5*D*). The expression of Mx-1 was increased substantially by the addition of BCAA (Figure 5*E*), suggesting that BCAA significantly repressed HCV replication in cells with either naive or persistent HCV infection. Importantly, there were no significant differences in cell viability between the conditions (Figure 5*C* and *F*).

To validate these findings, signaling pathways in HCV replicating cells were examined (Figure 6*A* and *B*). BCAA increased pS6K in a dose-dependent manner, implying its involvement in the activation of mTORC1 signaling. Related to this, expression of pSTAT1 was shown to be increased and the ratio of pSTAT1 to total STAT1 (pSTAT1/STAT1) increased 2.5- to 3-fold after the addition of BCAA. Thus, BCAA activated mTORC1 and the JAK-STAT signaling pathway in HCV-infected cells. In addition, the expression ratio of pFoxo3a to total Foxo3a (pFoxo3a/Foxo3a) increased 3- to 4-fold, indicating an increase in the cytoplasmic form of Foxo3a that is exposed to proteasome degradation. Concordant with these findings, we observed a decrease in the expression of Socs3. In addition, expression of the HCV core protein decreased as shown in Figure 6*A* and *B*. Thus, these results clearly show that BCAA repressed HCV replication through activation of IFN signaling and repression of Socs3-mediated IFN inhibitory signaling, as proposed in Figure 4*E*.

Discussion

Thompson et al[5] showed that the IL-28B polymorphism, HCV RNA, nationality (Caucasian/Hispanic vs African American), hepatic fibrosis stage, and fasting blood sugar level are all significant variables for achieving SVR in patients infected with genotype 1 HCV. However, the significance of variable factors for treatment response in conjunction with the IL-28B polymorphism has not been evaluated fully. In the present study, in addition to previously examined variables,[4] we included the plasma Fischer's ratio as a nutritional parameter. Multivariate analysis showed that the minor type of IL-28B polymorphism, advanced fibrosis stage, high hepatic ISGs, low Fischer's ratio, and ISDR mutation (≤1) independently contributed to NR (Table 1). Interestingly, among patients of similar fibrosis stage (F3–4), the Fischer's ratio was significantly lower in NR than SVR+TR cases. Therefore, the plasma value of Fischer's ratio was associated with the treatment response that was independent of the IL-28B polymorphism and histologic stage of the liver, although patients with advanced hepatic fibrosis are likely to be nutritionally affected.

As a nutrient sensor signaling pathway, the protein kinase mTOR plays an essential role in maintaining homeostasis and regulates protein synthesis in response to nutrient conditions. mTOR is the catalytic subunit of 2 distinct complexes, mTORC1 and mTORC2. In addition

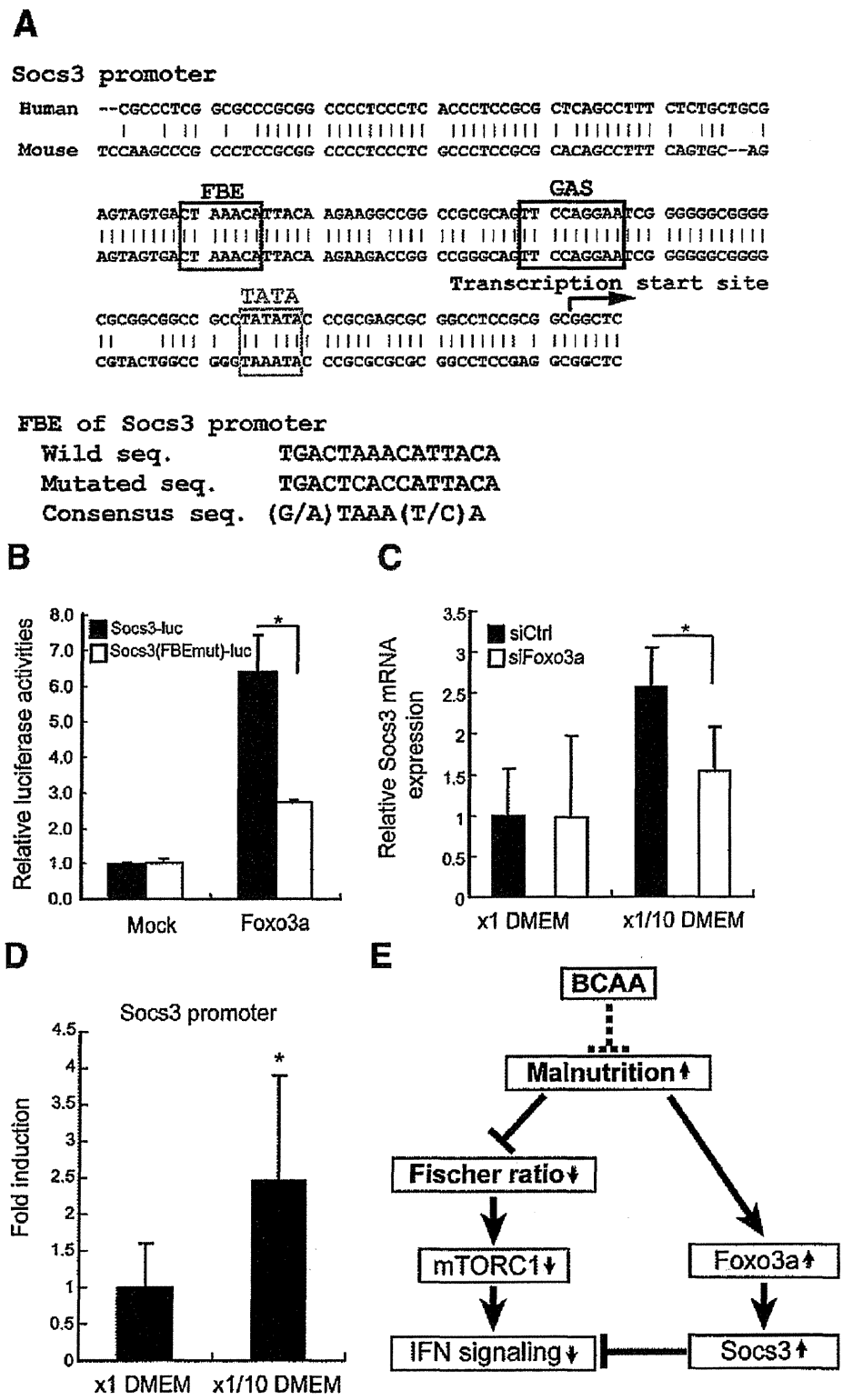

Figure 4. Socs3 promoter assay. (*A*) Primary structure of putative Foxo binding element in Socs3 promoter region. (*B*) Socs3-luc and Socs3 (FBEmut)-luc activities after overexpression of Foxo3a in Huh-7 cells. (*C*) Relative Socs3 messenger RNA (mRNA) expression after knockdown of Foxo3a in normal and low-amino-acid media. (*D*) Chromatin immunoprecipitation of Socs3 promoter region by Foxo3a in normal and low-amino-acid media. (*E*) Model of impaired IFN signaling by repressed mTORC1 signaling and increased Socs3 signaling under CH-C state of malnutrition.

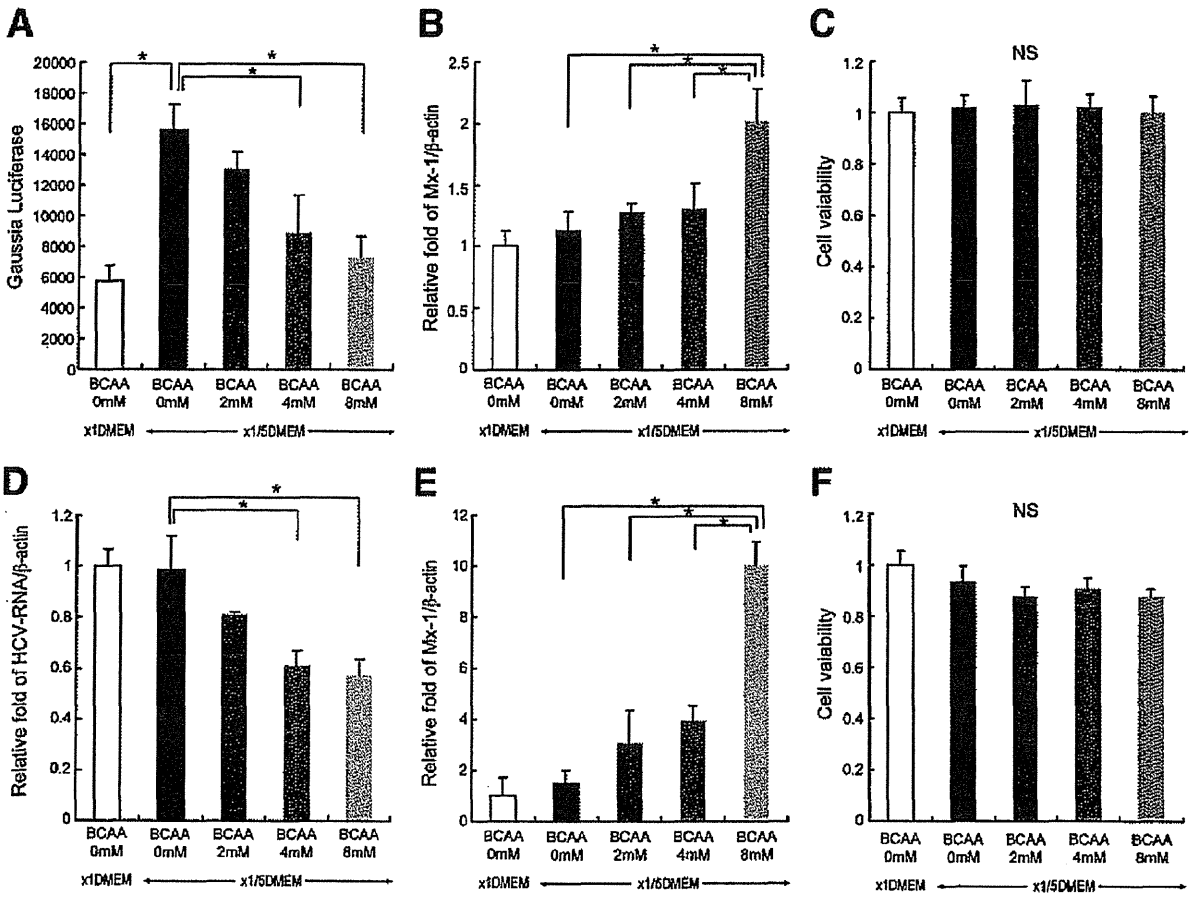

Figure 5. Effect of BCAA on HCV replication in cells in low-amino-acid medium. (*A*) Effect of BCAA on H77S.3/GLuc2A replication in Huh-7.5 cells. (*B*) Mx-1 expression in H77S.3/GLuc2A-transfected Huh-7.5 cells supplemented with BCAA. (*C*) Viability of Huh-7.5 cells. (*D*) Effect of BCAA on JFH-1 replication 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 1C) 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 2C). It therefore could be speculated that IFN treat-