

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 \pm SD of specific spots. (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 tissue⁷; 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

- Deuffic S, Poynard T, Buffat L, Valleron AJ. Trends in primary liver cancer. *Lancet* 1998;351:214-215.
- Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. *Int J Cancer* 2001;94:153-156.
- Lencioni R. Loco-regional treatment of hepatocellular carcinoma. *HEPATOLOGY* 2010;52:762-773.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- Tanaka H, Tsunoda T, Nukaya I, Sette A, Matsuda K, Umamo 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.
- 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.
- Umamo 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.
- 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.
- 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.
- 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.
- 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.
- Ribas A, Butterfield LH, Glaspy JA, Economou JS. Current developments in cancer vaccines and cellular immunotherapy. *J Clin Oncol* 2003;21:2415-2432.
- 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.
- Itoh K, Yamada A. Personalized peptide vaccines: a new therapeutic modality for cancer. *Cancer Sci* 2006;97:970-976.
- Butterfield LH, Ribas A, Meng WS, Disette 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.
- Butterfield LH, Ribas A, Disette 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.
- Butterfield LH. Recent advances in immunotherapy for hepatocellular cancer. *Swiss Med Wkly* 2007;137:83-90.
- Dougan M, Dranoff G. Immune therapy for cancer. *Annu Rev Immunol* 2009;27:83-117.
- 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.
- 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. Zerbin 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. Zerbin 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.

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.

© 2011 by the AGA Institute

0016-5085/\$36.00

doi:10.1053/j.gastro.2011.03.051

primary hepatocytes⁶ and cirrhotic rat liver⁷ 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- α -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- α 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 NaHCO₃, 1 g glucose, and 0.5 mL 1M (mol/L) sodium pyruvate in 500 mL Milli-Q water, then sterilizing with a 0.22- μ m filter (Millipore, Billerica, MA). Low amino acid media ($\times 1/5$, $\times 1/10$, $\times 1/30$, and $\times 1/100$ DMEM) were prepared by diluting $\times 1$ 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 μ g/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- α 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 <i>P</i> value	Multivariate odds (95% CI)	Multivariate <i>P</i> 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, ×10 ⁴ /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.

^aIL-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 previously¹² 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 NS2¹⁴ (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- μ g aliquot of synthetic RNA transcribed from pH77S.3/GLuc2A was used for electroporation. Cells were pulsed at 260 V and 950 μ F 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 \times 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 \pm 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 1A). In early fibrosis (F1–2) (Supplementary Figure 1B), 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 1C), 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 1A). 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 1C),¹⁵ was correlated negatively with the Fischer's ratio (Figure 1B). The expression of PDCD4, a negative transcriptional target of ribosomal p70 S6 protein kinase (S6K), downstream of mTORC1, was correlated significantly with BCAT1 (Figure 1D 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- α signaling and the mTOR pathway, we used Huh-7 cells grown in different amino acid conditions ($\times 1$ DMEM, $\times 1/5$ DMEM, $\times 1/30$ DMEM, and $\times 1/100$ DMEM). The phosphorylated forms of mTOR (p-mTOR) and S6K (pS6K), an important downstream regulator of mTORC1 signaling, were decreased substantially in $\times 1/30$ DMEM and $\times 1/100$ DMEM (Figure 2A). 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 2A). 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 2B). Under these conditions, the expression of p-mTOR and pS6K were repressed, which is consistent with previous reports.¹⁶ The expression of pSTAT1 also was repressed after Raptor knockdown (Figure 2B).

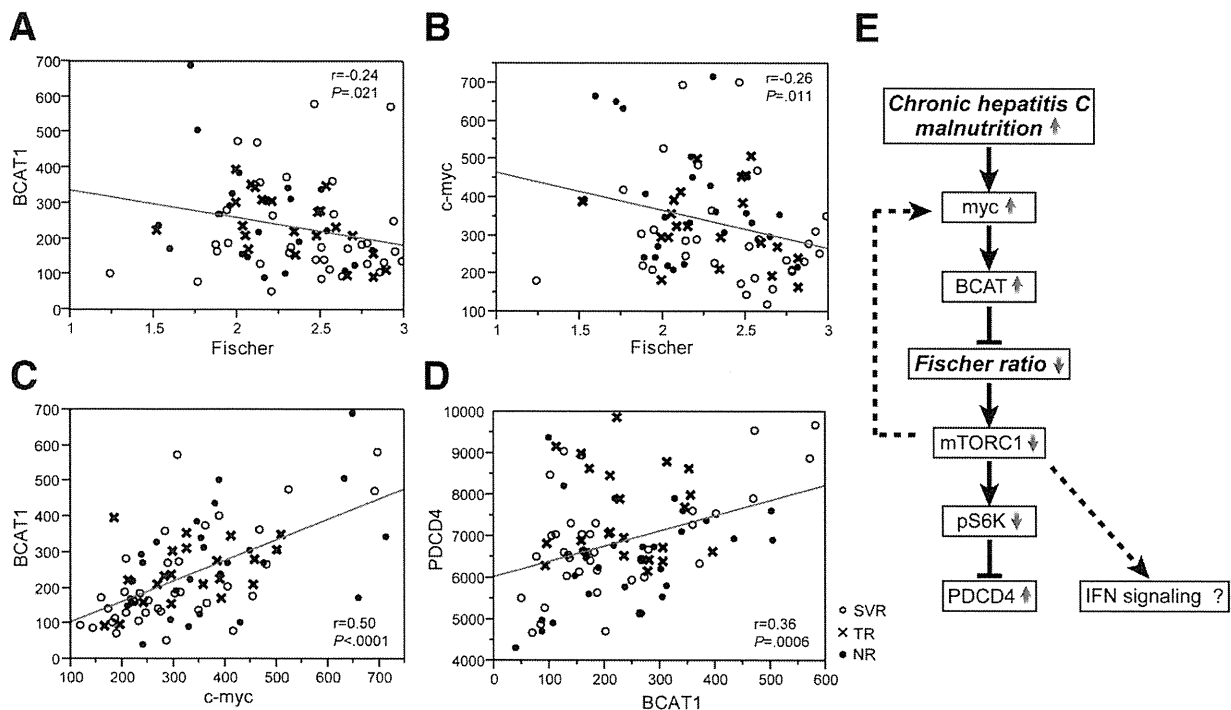


Figure 1. Regression analysis of mTORC1-related gene expression in liver. Gene expression values were determined by probe intensities. (A) BCAT1 and Fischer's ratio. (B) *c-myc* and Fischer's ratio. (C) BCAT1 and *c-myc*. (D) PDCD4 and BCAT1. (E) Putative signaling of mTORC1-related genes in CH-C.

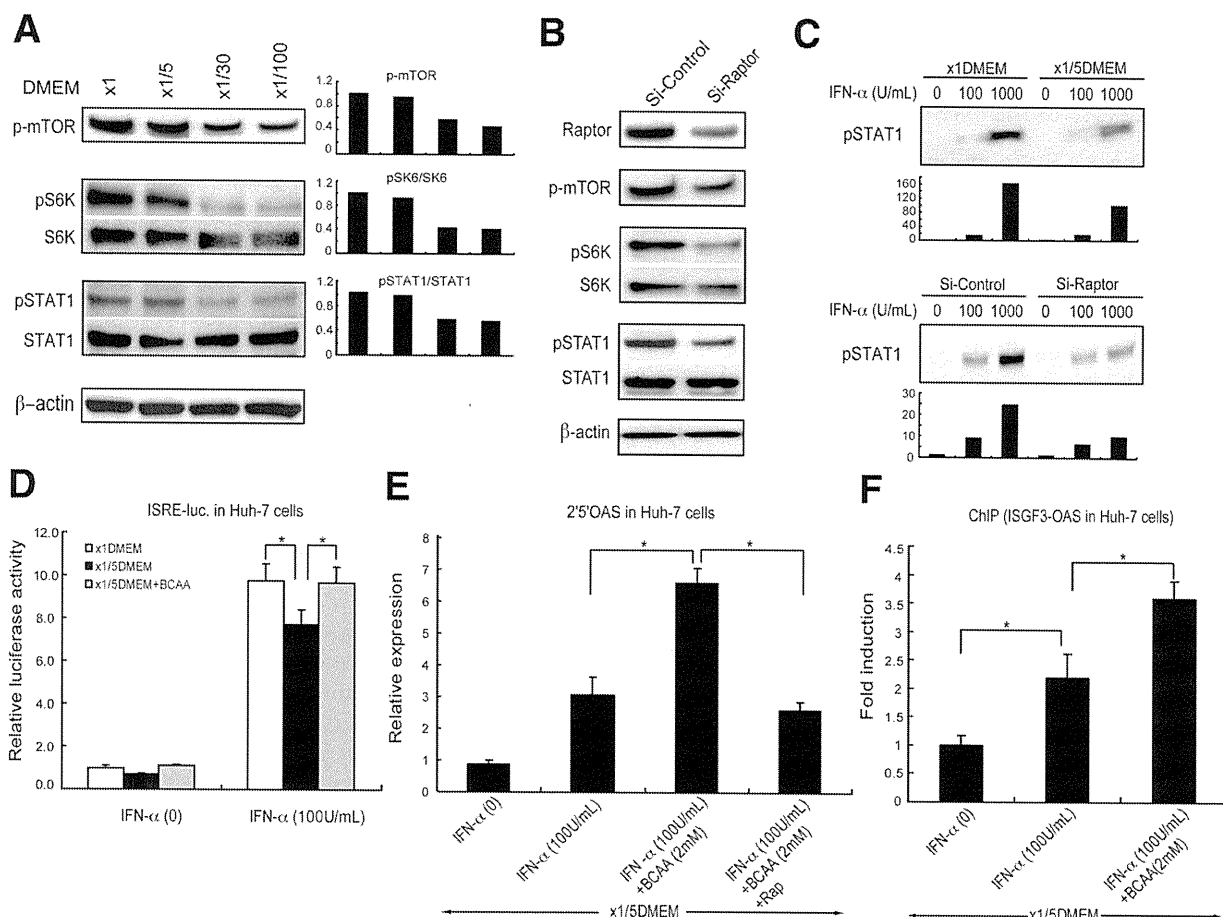


Figure 2. mTORC1 and IFN signaling in Huh-7 cells in low-amino-acid media. (A) p-mTOR, pS6K, and pSTAT1 expression in different amino acid media. (B) p-mTOR, pS6K, and pSTAT1 expression under Raptor knock-down conditions. (C) IFN- α stimulation and pSTAT1 expression in low-amino-acid media or under Raptor knock-down conditions. (D) IFN- α stimulation and ISRE reporter activities in normal and low-amino-acid media. (E) IFN- α 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 ISGF3 γ .

The induction of pSTAT1 by IFN- α (1000 U/mL) stimulation was impaired in $\times 1/5$ DMEM or in Raptor knocked-down condition, compared with the control (Figure 2C). Consistent with these results, IFN- α -induced ISRE-dependent transcriptional activity, as measured using an ISRE-luciferase reporter assay, was impaired significantly in $\times 1/5$ DMEM compared with $\times 1$ DMEM (Figure 2D). However, this activity could be rescued by the addition of 2 mmol/L BCAA (Figure 2D). These results were confirmed by determining the expression of the endogenous IFN- α responsive gene, 2'5'OAS, using quantitative reverse-transcription PCR. Figure 2E 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 2E). Furthermore, chromatin immunoprecipitation (ChIP) experiments revealed that transcriptional augmentation by BCAA was mediated by the binding of the IFN- α -inducible transcription factor, ISGF3 γ , to the promoter region of 2'5'OAS (Figure 2F). These results indicate that

amino acids in culture media play an essential role in IFN- α signaling through mTORC1 signaling, and that the addition of BCAA can overcome impaired IFN- α 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 $\times 1/5$, $\times 1/30$, and $\times 1/100$ DMEM, expression of the phosphorylated form of Foxo3a (pFoxo3a) decreased, whereas that of total Foxo3a increased in $\times 1/5$ and $\times 1/30$ DMEM, and the ratio of pFoxo3a to Foxo3a (pFoxo3a/Foxo3a) substantially decreased (Figure 3A 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

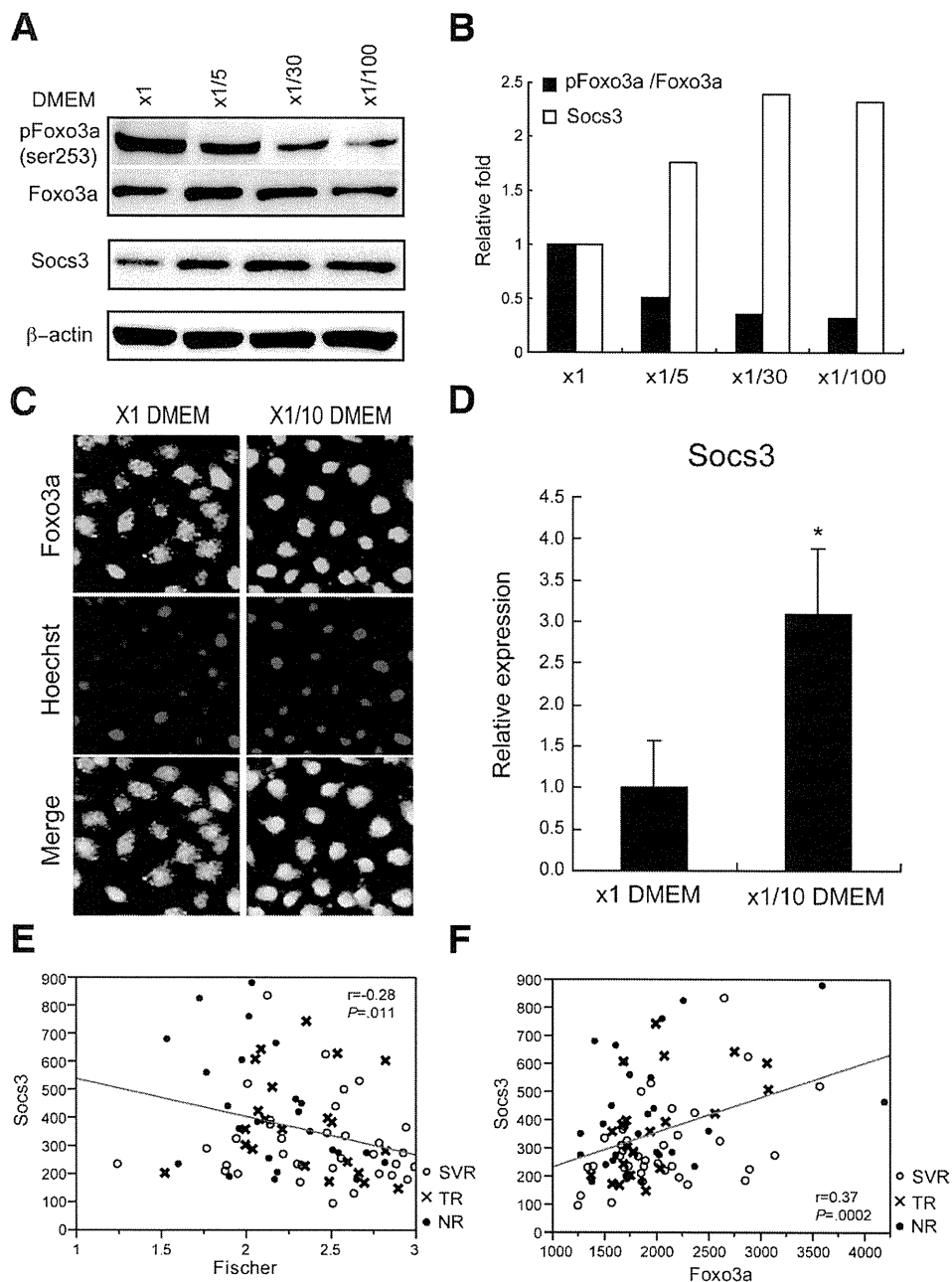


Figure 3. Foxo3a and Socs3 signaling in Huh-7 cells in low-amino-acid medium. (A) Foxo3a and Socs3 expression in different amino acid media. (B) Relative change of pFoxo3a/Foxo3a and Socs3 expression in different amino acid media. (C) Immunofluorescence staining of Foxo3a in Huh-7 cells in normal and 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 ($\times 1/10$ DMEM) (Figure 3C).

Interestingly, in low-amino-acid medium, transcription and protein expression of Socs3 increased significantly (Figure 3A, 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 3E 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 4A). 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 4B). The mutations introduced in the putative FBE (FBE_{mut}) in the *Socs3* promoter significantly reduced *Foxo3a*-induced *Socs3* promoter activation (Figure 4B).

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 ($\times 1/10$ DMEM) (Figure 4C). 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 ($\times 1/10$ DMEM) (Figure 4D). 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 4E).

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 ($\times 1/5$ DMEM) compared with normal amino acid medium ($\times 1$ DMEM). Interestingly, BCAA repressed H77S.3/GLuc2A replication in a dose-dependent manner (Figure 5A). In agreement with these results, the expression of Mx-1 was increased significantly by the addition of BCAA (Figure 5B). 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 ($\times 1/5$ DMEM) com-

pared with normal amino acid medium ($\times 1$ DMEM), JFH-1 replication was repressed significantly by the addition of BCAA in a dose-dependent manner (Figure 5D). The expression of Mx-1 was increased substantially by the addition of BCAA (Figure 5E), 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 5C and F).

To validate these findings, signaling pathways in HCV replicating cells were examined (Figure 6A 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 6A 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 4E.

Discussion

Thompson et al⁵ 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

A

Socs3 promoter

```

Human  --CGCCCTCG GGGCCCGGG CCCTCCCTC ACCCTCCGG CTCAGCCTTT CTCTGCTGG
      |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
Mouse  TCCAAGCCGG CCCTCCGGG CCCTCCCTC GCCCTCCGG CACAGCCTTT CAGTGC--AG

      FBE                               GAS
AGTAGTGA CT AAACATTACA AGAAGGCCGG CCGCGCAGTT CCAGGAATCG GGGGGCGGGG
      |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
AGTAGTGA CT AAACATTACA AGAAGGCCGG CCGCGCAGTT CCAGGAATCG GGGGGCGGGG

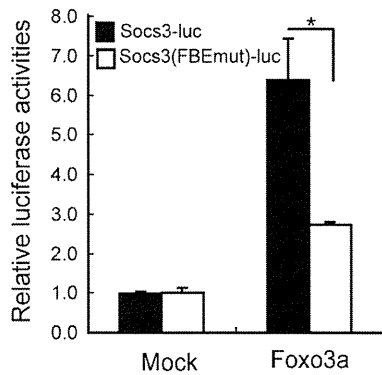
      TATA                               Transcription start site
CGCGCGGGCC GCCTATATAC CCGCGAGCGC GGCCTCCGG GCGGCTC
      |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
CGTACTGGCC GGTAAATAC CCGCGCGCGC GGCCTCCGAG GCGGCTC
    
```

FBE of Socs3 promoter

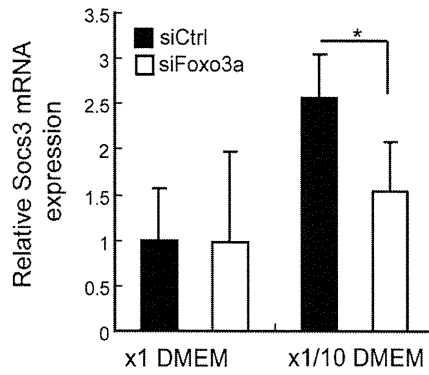
```

Wild seq.      TGACTAAACATTACA
Mutated seq.   TGACTCACCATTACA
Consensus seq. (G/A)TAAA(T/C)A
    
```

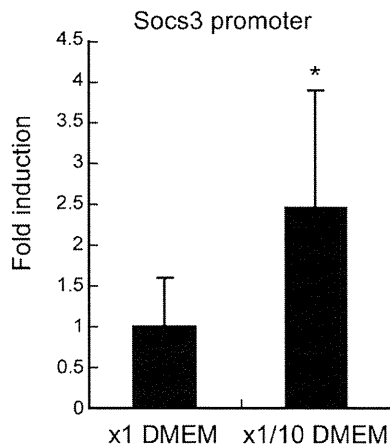
B



C



D



E

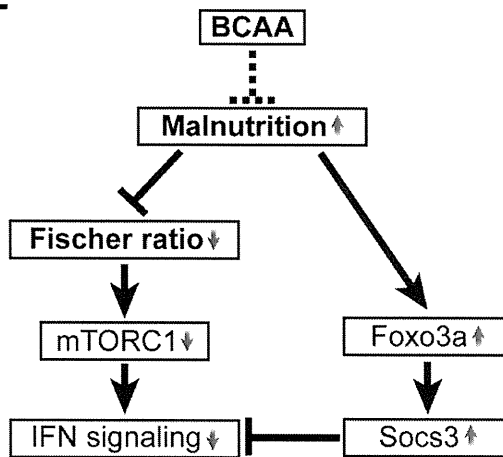


Figure 4. Socs3 promoter assay. (A) Primary structure of putative Foxo binding element in Socs3 promoter region. (B) Socs3-luc and Socs3 (FBEmut)-luc activities after overexpression of Foxo3a in Huh-7 cells. (C) Relative Socs3 messenger RNA (mRNA) expression after knockdown of Foxo3a in normal and low-amino-acid 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.

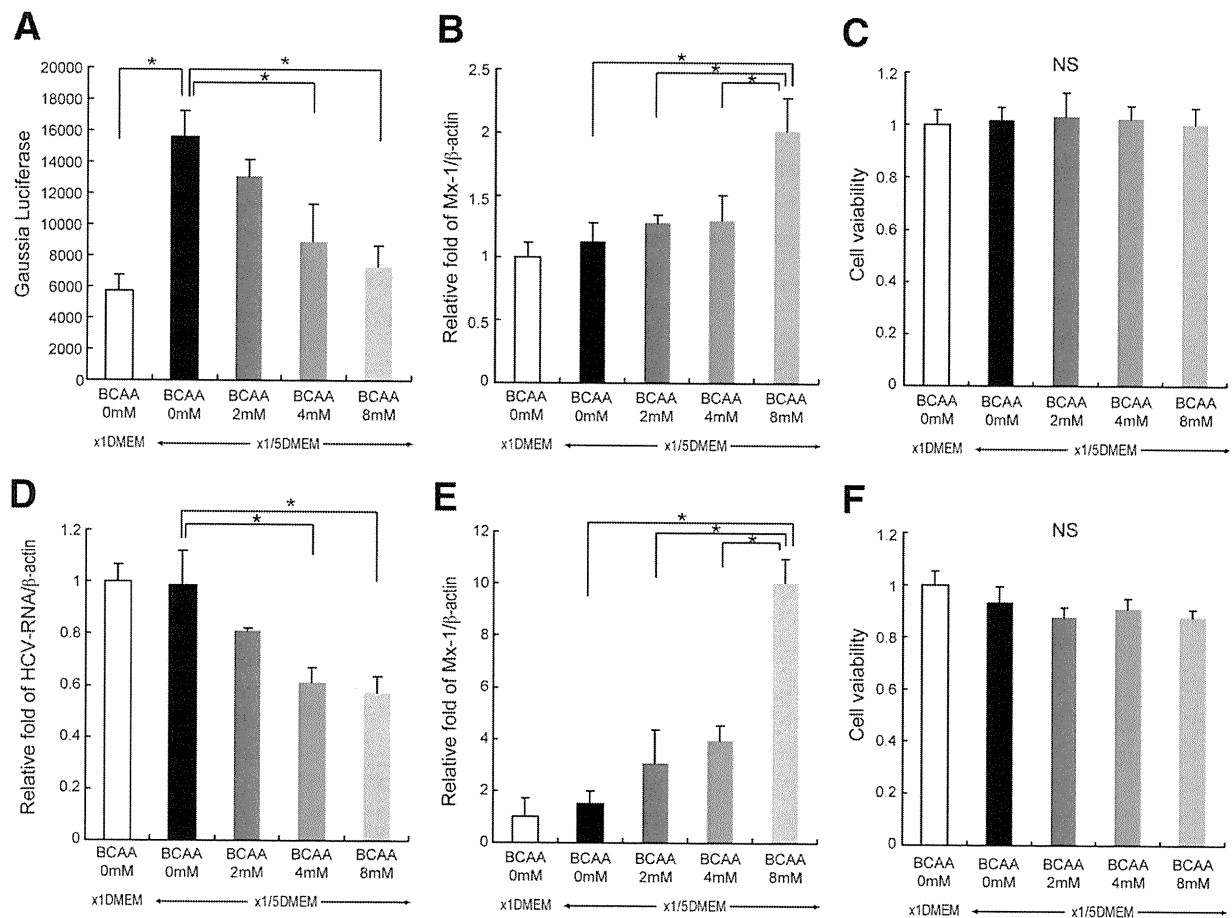


Figure 5. Effect of BCAA on HCV replication in cells in low-amino-acid medium. (A) Effect of BCAA on H77S.3/GLuc2A replication in Huh-7.5 cells. (B) Mx-1 expression in H77S.3/GLuc2A-transfected Huh-7.5 cells supplemented with BCAA. (C) Viability of Huh-7.5 cells. (D) Effect of BCAA on JFH-1 replication 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 1A). Moreover, BCAT1 expression was correlated positively with PDCD4 expression, which in turn is regulated negatively by pS6K at the transcriptional level (Figure 1D).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-C¹⁹ 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 1E) is plausible, although the precise mechanisms need to be confirmed.

Impaired mTORC1 signaling is suggested to affect the IFN- α -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 ($\times 1/5$, $\times 1/30$, and $\times 1/100$ 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 2A).

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

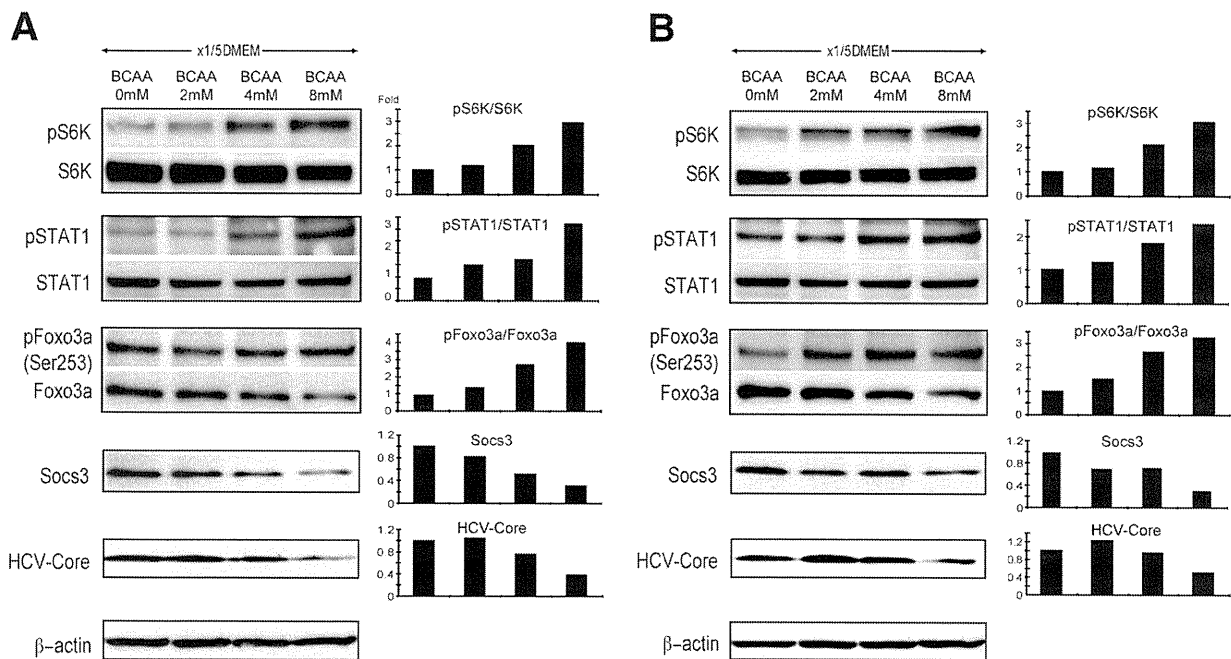


Figure 6. Expression of S6K, STAT1, Foxo3a, Socs3, and HCV core in H77S.3/GLuc2A-transfected Huh-7.5 cells or continuously JFH-1-infected Huh-7 cells supplemented with BCAA.

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 ISGF3 γ to its targets (Figure 2D–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 3C). The expression of Foxo3a was significantly positively correlated with that of Socs3 in CH-C liver (Figure 3F). These findings prompted us to identify a putative FBE in the Socs3 promoter region (Figure 4A). 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 4D). 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 4E).

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 5A). H77S.3/GLuc2A RNA produces infectious virus¹⁴ 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 Toru 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 Tatsuhiko Sugimoto and Koji Adachi (Tonami General Hospital, Tonami, 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 Yukihiko 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 Hirotohi 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).

References

1. Fried MW, Shiffman ML, Reddy KR, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002;347:975–982.
2. Tanaka Y, Nishida N, Sugiyama M, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009;41:1105–1109.
3. Ge D, Fellay J, Thompson AJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009;461:399–401.
4. Honda M, Sakai A, Yamashita T, et al. Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. *Gastroenterology* 2010;139:499–509.
5. Thompson AJ, Muir AJ, Sulkowski MS, et al. Interleukin-28B polymorphism improves viral kinetics and is the strongest pre-treatment predictor of sustained virologic response in genotype 1 hepatitis C virus. *Gastroenterology* 2010;139:120–129 e18.
6. Nishitani S, Ijichi C, Takehana K, et al. Pharmacological activities of branched-chain amino acids: specificity of tissue and signal transduction. *Biochem Biophys Res Commun* 2004;313:387–389.
7. Matsumura T, Morinaga Y, Fujitani S, et al. Oral administration of branched-chain amino acids activates the mTOR signal in cirrhotic rat liver. *Hepato Res* 2005;33:27–32.
8. Kim DH, Sarbassov DD, Ali SM, et al. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* 2002;110:163–175.
9. Colina R, Costa-Mattioli M, Dowling RJ, et al. Translational control of the innate immune response through IRF-7. *Nature* 2008;452:323–328.
10. Kaur S, Lal L, Sassano A, et al. Regulatory effects of mammalian target of rapamycin-activated pathways in type I and II interferon signaling. *J Biol Chem* 2007;282:1757–1768.
11. Shimbo K, Kubo S, Harada Y, et al. Automated precolumn derivatization system for analyzing physiological amino acids by liquid chromatography/mass spectrometry. *Biomed Chromatogr* 2009;24:683–691.
12. Shirasaki T, Honda M, Mizuno H, et al. La protein required for internal ribosome entry site-directed translation is a potential therapeutic target for hepatitis C virus replication. *J Infect Dis* 2010;202:75–85.
13. Yi M, Villanueva RA, Thomas DL, et al. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. *Proc Natl Acad Sci U S A* 2006;103:2310–2315.
14. Shimakami T, Welsch C, Yamane D, et al. Protease inhibitor-resistant hepatitis C virus mutants with reduced fitness from impaired production of infectious virus. *Gastroenterology* 2011;140:667–675.
15. Eden A, Simchen G, Benvenisty N. Two yeast homologs of ECA39, a target for c-Myc regulation, code for cytosolic and mitochondrial branched-chain amino acid aminotransferases. *J Biol Chem* 1996;271:20242–2045.
16. Dowling RJ, Topisirovic I, Alain T, et al. mTORC1-mediated cell proliferation, but not cell growth, controlled by the 4E-BPs. *Science* 2010;328:1172–1176.
17. Teleman AA, Hietakangas V, Sayadian AC, et al. Nutritional control of protein biosynthetic capacity by insulin via Myc in *Drosophila*. *Cell Metab* 2008;7:21–32.
18. Zhang X, Gan L, Pan H, et al. Phosphorylation of serine 256 suppresses transactivation by FKHR (FOXO1) by multiple mechanisms. Direct and indirect effects on nuclear/cytoplasmic shuttling and DNA binding. *J Biol Chem* 2002;277:45276–45284.
19. Farinati F, Cardin R, Bortolami M, et al. Oxidative damage, pro-inflammatory cytokines, TGF-alpha and c-myc in chronic HCV-related hepatitis and cirrhosis. *World J Gastroenterol* 2006;12:2065–2069.

Received October 19, 2010. Accepted March 18, 2011.

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.

The authors thank Mina Nishiyama and Yuki Hatayama for excellent technical assistance.

Conflicts of interest

The authors disclose no conflicts.

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 pH77S² 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 Gln₄₁ 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).

References

1. Shimbo K, Kubo S, Harada Y, et al. Automated precolumn derivatization system for analyzing physiological amino acids by liquid chromatography/mass spectrometry. *Biomed Chromatogr* 2009; 24:683–691.
2. Yi M, Villanueva RA, Thomas DL, et al. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. *Proc Natl Acad Sci U S A* 2006;103:2310–2315.
3. Shetty S, Kim S, Shimakami T, et al. Hepatitis C virus genomic RNA dimerization is mediated via a kissing complex intermediate. *RNA* 2010;16:913–925.
4. Shimakami T, Welsch C, Yamane D, et al. Protease inhibitor-resistant hepatitis C virus mutants with reduced fitness from impaired production of infectious virus. *Gastroenterology* 2011; 140:667–675.

Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in *IL28B* and viral factors

Masayuki Kurosaki¹, Yasuhito Tanaka², Nao Nishida³, Naoya Sakamoto⁴, Nobuyuki Enomoto⁵, Masao Honda⁶, Masaya Sugiyama², Kentaro Matsuura², Fuminaka Sugauchi², Yasuhiro Asahina¹, Mina Nakagawa⁴, Mamoru Watanabe⁴, Minoru Sakamoto⁵, Shinya Maekawa⁵, Akito Sakai⁶, Shuichi Kaneko⁶, Kiyooki Ito⁷, Naohiko Masaki⁷, Katsushi Tokunaga³, Namiki Izumi^{1,*}, Masashi Mizokami^{2,7}

¹Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan; ²Department of Virology, Liver Unit, Nagoya City University, Graduate School of Medical Sciences, Nagoya, Japan; ³Department of Human Genetics, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; ⁴Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan; ⁵First Department of Internal Medicine, University of Yamanashi, Yamanashi, Japan; ⁶Department of Gastroenterology, Kanazawa University, Graduate School of Medicine, Kanazawa, Japan; ⁷Research Center for Hepatitis and Immunology, International Medical Center of Japan, Konodai Hospital, Ichikawa, Japan

Background & Aims: Pegylated interferon and ribavirin (PEG-IFN/RBV) therapy for chronic hepatitis C virus (HCV) genotype 1 infection is effective in 50% of patients. Recent studies revealed an association between the *IL28B* genotype and treatment response. We aimed to develop a model for the pre-treatment prediction of response using host and viral factors.

Methods: Data were collected from 496 patients with HCV genotype 1 treated with PEG-IFN/RBV at five hospitals and universities in Japan. *IL28B* genotype and mutations in the core and IFN sensitivity determining region (ISDR) of HCV were analyzed to predict response to therapy. The decision model was generated by data mining analysis.

Results: The *IL28B* polymorphism correlated with early virological response and predicted null virological response (NVR) (odds ratio = 20.83, $p < 0.0001$) and sustained virological response (SVR) (odds ratio = 7.41, $p < 0.0001$) independent of other covariates. Mutations in the ISDR predicted relapse and SVR independent of *IL28B*. The decision model revealed that patients with the minor *IL28B* allele and low platelet counts had the highest NVR (84%) and lowest SVR (7%), whereas those with the major *IL28B* allele and mutations in the ISDR or high platelet counts had the lowest NVR (0–17%) and highest SVR (61–90%). The model had high reproducibility and predicted SVR with 78% specificity and 70% sensitivity.

Conclusions: The *IL28B* polymorphism and mutations in the ISDR of HCV were significant pre-treatment predictors of response to PEG-IFN/RBV. The decision model, including these host and viral factors may support selection of optimum treatment strategy for individual patients.

© 2010 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.

Introduction

Hepatitis C virus (HCV) infection is the leading cause of cirrhosis and hepatocellular carcinoma worldwide [1]. The successful eradication of HCV, defined as a sustained virological response (SVR), is associated with a reduced risk of developing hepatocellular carcinoma. Currently, pegylated interferon (PEG-IFN) plus ribavirin (RBV) is the most effective standard of care for chronic hepatitis C but the rate of SVR is around 50% in patients with HCV genotype 1 [2,3], the most common genotype in Japan, Europe, the United States, and many other countries. Moreover, 20–30% of patients with HCV genotype 1 have a null virological response (NVR) to PEG-IFN/RBV therapy [4]. The most reliable method for predicting the response is to monitor the early decline of serum HCV-RNA levels during treatment [5] but there is no established method for prediction before treatment. Because PEG-IFN/RBV therapy is costly and often accompanied by adverse effects such as flu-like symptoms, depression and hematological abnormalities, pre-treatment predictions of those patients who are unlikely to benefit from this regimen enables ineffective treatment to be avoided.

Recently, it has been reported through a genome-wide association study (GWAS) of patients with genotype 1 HCV that single nucleotide polymorphisms (SNPs) located near the *IL28B* gene are strongly associated with a response to PEG-IFN/RBV therapy in

Keywords: *IL28B*; ISDR; Peg-interferon; Ribavirin; Data mining; Decision tree.
Received 14 March 2010; received in revised form 22 June 2010; accepted 7 July 2010;
available online 19 September 2010

* Corresponding author. Address: Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, 1-26-1 Kyonan-cho, Musashino-shi, Tokyo 180-8610, Japan. Tel.: +81 422 32 3111; fax: +81 422 32 9551.
E-mail address: nizumi@musashino.jrc.or.jp (N. Izumi).

