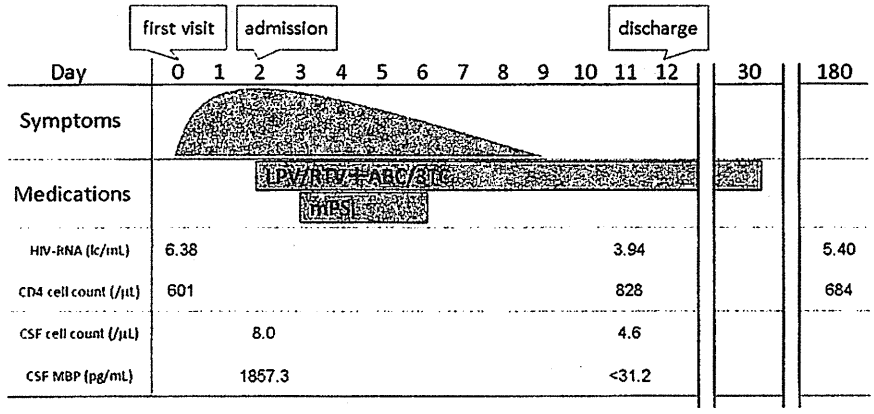

Figure 1. Clinical course. LPV: Lopinavir, RTV: Ritonavir, ABC: Abacabir, 3TC: Lamivudine, mPSL: methylprednisolone, CSF: cerebrospinal fluid, MBP: myelin basic protein

(4.6/μL) in the CSF decreased to the normal ranges at treatment day 9. Methylprednisolone was given for 3 days and cART was continued for one month, which resulted in no relapse of ATM-related symptoms in the subsequent 6 months (Fig. 1), and also no resistant mutation was seen in Protease and Reverse Transcriptase lesions of HIV (data not shown).

Discussion

ATM is a segmental spinal cord injury caused by acute inflammation characterized by acute or subacute motor, sensory, and autonomic (genitourinary and digestive systems) spinal cord dysfunction (4, 5). Although preceding infection is noted in 20-50% of cases (4, 6-9) ATM associated with PHI was only reported before the era of cART (3). There is currently no standard treatment for ATM. Although corticosteroids are the first-line treatment due to the probable mechanisms such as molecular mimicry and the development of autoantibodies, approximately 30 to 50% of patients develop severe sequelae (4, 10, 11).

In the present patient, ATM was diagnosed by the rapid development of symptoms, neurological findings, including clearly-defined bilateral sensory deficits below Th7 level, autonomic dysfunction, and MBP elevation, suggestive of spinal cord inflammation (4, 5). Furthermore, PHI was diagnosed just before the onset of ATM, based on the absence of bands in Western blot analysis and a high titer of HIV-RNA level. It was concluded that PHI was the trigger of ATM.

Although direct cytopathic effects of the virus and immune-mediated toxicity are suggested, pathogenesis of PHI is not fully understood (12-14). There are case reports of rapid improvement of PHI-related symptoms after cART initiation, even though the HIV-RNA level was not completely suppressed at the time of clinical resolution (15, 16). Regarding these phenomena, it is assumed that inhibition of viral replication by cART induces the resolution of symptoms. In the present case, complete recovery was achieved by the combination of steroids and cART (4, 11, 12).

In conclusion, concurrent use of cART and corticosteroids was effective against PHI accompanied by ATM. The immediate improvement in ATM allowed the subsequent discontinuation of treatment.

The authors state that they have no Conflict of Interest (COI).

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INTERNAL MEDICINE

□ CASE REPORT □

Antiretroviral Therapy for Treatment-naïve Chronic HIV-1 Infection with an Axonal Variant of Guillain-Barré Syndrome Positive for Anti-ganglioside Antibody: A Case Report

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Abstract

Guillain-Barré syndrome sometimes manifests as immune reconstitution inflammatory syndrome. We report a treatment-naïve male with chronic HIV-1 infection who presented with an axonal variant of Guillain-Barré syndrome. Antiretroviral therapy commenced one month later and no rapid improvement or deterioration of tetraparesis was noted. This is the first report that describes the detection and serial measurements of anti-ganglioside antibody in a patient with HIV infection. This case suggests a limited role for T-cell immunity in the production of anti-ganglioside antibody and the pathogenesis of axonal variants, since the antiretroviral therapy-induced improvement in T-cell immunity neither re-elevated anti-ganglioside antibody titer nor worsened tetraparesis.

Key words: human immunodeficiency virus, Guillain-Barré syndrome, anti-ganglioside antibody, acute motor axonal neuropathy, immune reconstitution inflammatory syndrome

(Intern Med 50: 2427-2429, 2011)
(DOI: 10.2169/internalmedicine.50.5883)

Introduction

Guillain-Barré syndrome (GBS) is a well-known but rare complication of primary human immunodeficiency virus (HIV) infection (1). GBS can also manifest as immune reconstitution inflammatory syndrome, a condition sometimes seen in patients with HIV infection after the introduction of antiretroviral therapy, which is characterized by recovery of T-cell immunity with an overwhelming response to pre-existing antigen (usually opportunistic infections) leading to worsening of symptoms (1, 2). Thus, CD4+ T cell-mediated cellular immunity appears to play a role in the pathogenesis of GBS. However, little is known about the effect of CD4+ T-cell immunity activated by antiretroviral therapy on the pathogenesis and clinical course of pre-existing GBS. To our knowledge, there are only two case reports that described such patients (3, 4). Here, we report a case of a treatment-naïve patient with chronic HIV infection who presented with acute motor axonal neuropathy (AMAN), an axonal variant of GBS, after an episode of Herpes zoster.

Case Report

A 33-year-old Japanese male with treatment-naïve HIV-1 infection visited the emergency room, complaining of progressive tetraparesis. He had been diagnosed with HIV infection five years earlier, and the latest CD4 count was 334/μL with a viral load of 7.9×10^3 copies/mL. He reported developing the first episode of Herpes zoster on the chest 12 days earlier, and was treated with famciclovir. There was no history of diarrhea. On admission, the head CT scan and MRI of the head and spinal cord showed no abnormalities. Cerebrospinal fluid (CSF) examination showed a cell count

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Received for publication May 24, 2011; Accepted for publication July 19, 2011
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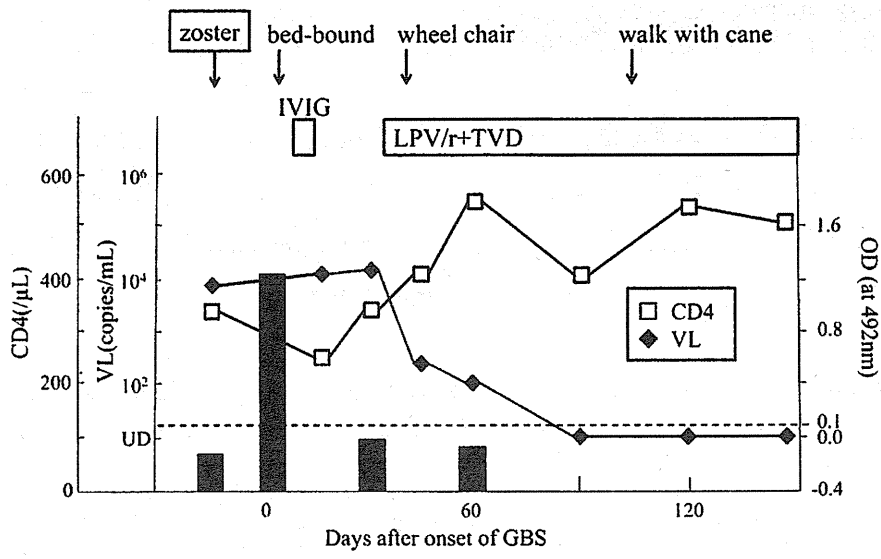

Figure 1. Clinical course after the onset of GBS. *Solid bars*: titer of serum anti-GM1 IgG antibody measured with ELISA (cut-off value =0.1 OD, dashed line) (5). *Open squares*: CD4 cell count, *solid diamonds*: viral load (VL). Anti-ganglioside antibody became positive at the onset of GBS but it was negative thereafter. GBS: Guillain-Barré syndrome, IVIG: intravenous immunoglobulin, LPV/r: lopinavir/ritonavir, TVD: tenofovir/emtricitabine, OD: optical density

of 6/μL and protein 17 mg/dL, and polymerase chain reaction was negative for varicella zoster virus and herpes simplex virus. Tetraparesis worsened after admission and the patient became bed-bound on day 3. However, the trunk muscles were intact and no respiratory distress was evident. On day 4, a 5-day course of high-dose intravenous immunoglobulin (IVIG) was started with a tentative diagnosis of GBS. Electrophysiological studies on day 5 showed conduction blocks of motor nerves and markedly reduced F-wave frequencies: 0% in the median nerve (normal >40%), 37.5% in the ulnar nerve (>40%), and 0% in the tibial nerve (> 99%). We collected blood samples on day 2 to measure IgG and IgM antibodies to gangliosides GM1, GD1a, GD1b, GT 1b, GQ1b, asialo-GM1, GM2, GM3, GD2, and GD3 with ELISA as described elsewhere (5). Anti-GM1 IgG antibody, a diagnostic marker for AMAN, was positive, whereas other IgG antibodies were negative (6). Neither serum anti-*Campylobacter jejuni* IgG antibody nor stool culture for campylobacter was positive, suggesting that the preceding zoster was the trigger. On day 15, a marked reduction in the compound muscle action potentials of motor nerves was noted: 1.3 mV in the median nerve, 1.2 mV in the ulnar nerve, and 0.09 mV in the tibial nerve, whereas sensory nerve action potentials were intact: 26.5 μV in the ulnar nerve. CSF re-sampling on day 30 showed albuminocytological dissociation (protein: 73 mg/dL, cell count: 7/μL). The clinical symptoms, electrophysiological examination, CSF analysis, and positivity for anti-GM1 IgG antibody confirmed the diagnosis of AMAN (7).

Arrest of symptom progression was noted after day 1 of IVIG; however, it was not clear whether this represented the natural course of the disease or was IVIG-related. Antiretroviral therapy with lopinavir/ritonavir and tenofovir/emtricitabine was started on day 33, one month after the onset of GBS (CD4: 349/μL, viral load: 1.7×10^3 copies/mL). The treatment suppressed the viral load within 8 weeks to an undetectable level and increased CD4 count to 414/μL. However, tetraparesis remained stable with no rapid improvement or deterioration after initiation of antiretroviral therapy. Recovery of compound muscle action potentials of motor nerves was also slow: 1.27 mV on day 28, 2.7 mV on day 56, and 2.6 mV on day 98 in the left median nerve. Serum samples obtained 14 days before the onset of GBS, on day 1 of antiretroviral therapy (day 33), and 4 weeks later (day 61) were negative for anti-ganglioside antibodies (AGA) as shown in Fig. 1. The recovery of tetraparesis was slow; the patient needed 15 months of rehabilitation to be able to walk without a cane.

Discussion

We described a treatment-naïve patient with chronic HIV infection who presented with AMAN due to preceding Herpes zoster. Herpes zoster reactivation is known to sporadically trigger the occurrence of GBS (8). Antiretroviral therapy was introduced one month after the onset of GBS, which resulted in a substantial rise in the CD4 cell count. However, elevated T-cell immunity did not improve or worsen the symptoms of GBS; it was 15 months after onset that the patient could walk independently. HIV-related GBS often occurs as a complication of primary HIV infection or immune reconstitution inflammatory syndrome (1, 2). The uniqueness of this case is that the clinical course of pre-existing GBS, which was triggered by Herpes zoster, not by

HIV *per se*, after the commencement of antiretroviral therapy was described, and that CD4+ T-cell immunity activated by antiretroviral therapy modulated neither the clinical course of pre-existing GBS nor AGA titer.

To our knowledge, this is the first report that describes the detection and serial measurements of AGA in a GBS patient with HIV infection. AGA was negative in a previously reported case of AMAN (4). In the present patient, the AGA titer was highest at the onset of GBS but rapidly decreased afterwards (Fig. 1). This trend is similar to that of patients free of HIV infection (9). In this patient, antiretroviral therapy did not seem to affect either clinical recovery from GBS or AGA titer.

GBS is classified into two forms based on clinical, electrophysiological, pathological, and immunological criteria: axonal variants including AMAN, and acute inflammatory demyelinating polyneuropathy (AIDP) (7). The pathogenesis of axonal variants is thought to be different from that of AIDP. AGA is an antibody against lipopolysaccharide in the cell wall of infectious pathogens and it is known to cross-react with oligosaccharide epitopes of gangliosides in the peripheral nervous tissue. AGA plays a major role in the pathogenesis of axonal variants (7). In contrast, T-cell-mediated cellular immunity was found to play a major role in AIDP, with knowledge earned through experimental autoimmune neuritis, an autoimmune disease with clinical and pathological features similar to AIDP (10). One report described a patient with advanced AIDS and a very low CD4 count (24/μL) who presented with Fisher/Guillain-Barré overlap syndrome and was positive for AGA, suggesting that axonal variants might occur independent of T-cell immunity (11).

Antibody production against lipopolysaccharides is generally T-cell independent (11). Such antibodies produced by B-cells are the IgM isotypes, and the intervention of T-cells has been considered to be essential in switching class to IgG1 and IgG3 subclasses, to which most AGA belongs (12). However, it was recently discovered that subsets of B cells could produce class-switched IgG T-cells independently, with the help of B-cell activating factor and a proliferation-inducing ligand secreted by dendritic cells (13). Although the role of T-cells in the production of AGA in axonal variants of GBS has yet to be determined, it appears that recovery of CD4+ T-cell immunity did not alter the clinical course of pre-existing GBS or AGA titer, at least in this patient.

In conclusion, we reported a treatment-naïve patient with chronic HIV infection who presented with AMAN due to Herpes zoster. Antiretroviral therapy was introduced one month after the onset of GBS and resulted in substantial improvement of CD4+ T-cell immunity, but it had no effect on the prognosis of tetraparesis or trend of AGA titer. It is important to distinguish axonal variants from AIDP especially in patients with HIV infection, because the risk of immune reconstitution inflammatory syndrome after the introduction of antiretroviral therapy might be lower in the axonal variants. The measurement of AGA titer during the acute phase of GBS should help establishing the diagnosis.

The authors state that they have no Conflict of Interest (COI).

Acknowledgement

The authors thank all the clinical staff at AIDS Clinical Center.

All authors contributed to the concept, design, and writing of this submission.

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5 石川県の肝癌撲滅計画

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肝癌撲滅には背景にある肝炎ウイルスに対する治療導入が重要である。石川県では肝炎ウイルス検診初年度より協議会を設立し，陽性者をフォローアップしてきた。インターフェロン療法導入率向上を目指してさまざまな施策を講じ，導入率は30%を越えるようになった。2010年度よりかかりつけ医と専門医の連携を強化した「石川県肝炎診療連携」を新たに開始して専門医受診勧奨，抗ウイルス療法導入を図ることにより肝癌撲滅を目指している。

はじめに

2009年度人口動態統計では肝癌による死亡者数は男性で第4位，女性では第6位であり，年間3万人を越えている。肝癌の多くはウイルス性慢性肝疾患を背景に発生しており，肝癌撲滅には肝炎ウイルス感染者を早期に発見し，早期に治療することが重要である。国は2002年度より5年間で肝炎ウイルス検診を行い，肝炎ウイルス感染者の発見に努めたが，検診受診率は決して高くなく，また医療機関を受診しても適切な観察，治療導入すなわち抗ウイルス療法が行われてきたとは言い難い。本稿では肝炎ウイルス検診開始当初より石川県で取り組んできた肝炎ウイルス症例への対策について述べる。

肝炎ウイルス検診の方針 1

2002年肝炎ウイルス検診会誌当初より，石川県では肝炎協議会を設置し，県健康福祉部・医師会・保健所・検査センター・学術経験者が一体となって協力した検診体制を確立した。地域により専門医療機関の過不足があるため，精密検査は特に指定医療機関とはせず，かかりつけ医でも可とした。このため検診精度の向上と経過観察の重要性を考え，以下7つの項目を検診事業の柱とした。

1. 検診陽性者への行政の関与することの通知と同意
2. 精密検査の全県での統一
3. 住民，担当医用の診断手引きの作成
4. 精密検査での画像検査の義務付け
5. 全症例を対象とした事例検討会
6. 前年度陽性者に対する事後調査
7. 保健師などを対象とした研修会の開催

このなかで石川県として独自性の高いと考えているものは検診陽性者を行政が継続フォローするために必要な1，6および担当医の肝炎への理解を深めた5である。毎年検診陽性者の医療機関受診・治療状況を把握することと，担当のかかりつけ医が正しく診断，治療導入することへの意識が高まるようこれら事業を継続した。

肝炎ウイルス検診の状況 2

石川県では5年間の肝炎ウイルス検診受診率は36.6～41.5%と全国平均[1]と比べると10%ほど受診率がよかったが半数には満たない。検診陽性者の精密検査受診状況は男性67.6%，女性75.0%，年齢では若年（65歳未満）66.1%，高齢（65歳以上）74.7%であった。性年齢でわけると若年男性53.4%，若年女性71.9%，高齢男性74.0%，高齢女性74.0%と若年男性で精密検査の受診率が低いことが明らかであり，仕事等で忙しく受診機会をつくりにくい状況がうかがえる。図1に性・年齢・医療圏別での精検受診状況を示す。検診自体の受診率は能登地方および南加賀で低い傾向にあった。しかし能登地方はウイルスキャリアと判明すると医療機関をきちんと受診する傾向にある。一方，南加賀ではウイルスキャリアと判明しても医療機関への受診率が悪い。能登地方ではキャリアの発掘が重要であり，南加賀ではキャリアの発掘と受診勧奨の両面が必要なことがう

PROFILE

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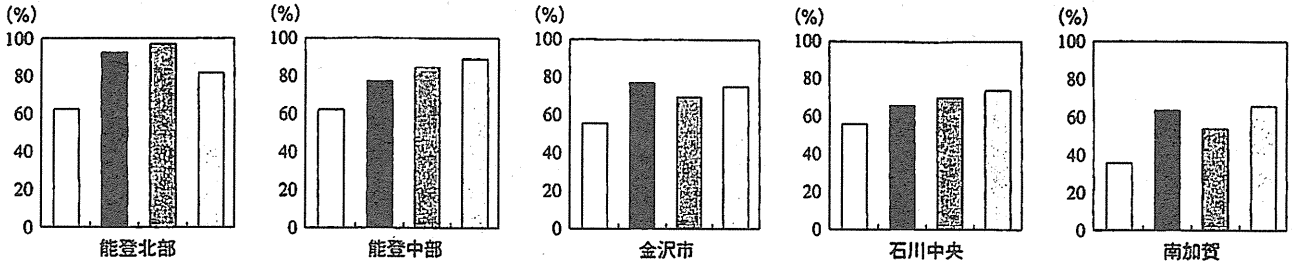

図1◎検診陽性者の精密検査受診状況
□：男性・65歳未満 ■：男性・65歳以上 ▨：女性・65歳未満 □：女性・65歳以上

表1◎石川県精検未受診者のその後の状況

	検診初年度 精検未受診	翌年以降 医療機関受診	IFN療法/受診者
能登北部	18（14.8%）	12（66.7%）	3（25.0%）
能登中部	32（17.5%）	17（53.1%）	2（11.8%）
石川中央	71（31.8%）	45（63.4%）	7（15.6%）
南加賀	88（40.6%）	52（59.1%）	10（19.2%）
金沢市	147（28.1%）	39（26.5%）	2（ 5.1%）
合計	356（28.1%）	165（46.3%）	24（14.5%）

かがえる。また医療機関受診の時間がとりにくい若年男性の受診率が悪いのは地域で共通しており，受診動機を促す啓蒙活動が必要である。

フォローアップ事業の有用性 3

前述したように石川県では保健師が面談，電話，手紙などの方法で検診陽性者の状況把握に努めている。継続して医療機関で経過観察されているのはC型肝炎では48.7～63.7%であった。一方，各市町で少なくともフォロー期間（2～7年）中に1度は医療機関を受診した症例はB型肝炎ウイルス陽性者で49～100%，C型肝炎ウイルス陽性者で80～100%であった。表1に初年度精密検査未受診者のその後の状況を示す。受診勧奨を行った結果未受診者のうち能登北部66.7%，能登中部53.1%，金沢市26.5%，石川中央63.4%，南加賀59.1%がその後に医療機関を受診し，さらに受診者のうち能登北部25.0%，能登中部11.8%，金沢市5.1%，石川中央15.6%，南加賀19.2%がインターフェロン（IFN）療法を行っていた。継続した状況把握，受診勧奨が適切な医療へと結びつくことが明らかとなった。

IFN治療状況 4

肝癌撲滅という目標に対してC型肝炎であればIFN療法によりウイルスが排除されることが一番である。年齢，合併症などにより全ての症例でIFN療法を行うのは困難であるが，検診症例のIFN療法の施行率が低いことが問題となっている。厚生労働省研究班の報告では当初3年間では13.8～18.2%であった[1]。石川県でも2002年131例中5例（3.8%），2003年164例中14例（8.5%）とIFN療法施行率は低かった。特に65歳以上の高齢者ではIFN施行率は2.6%と，65歳未満の9.6%に対して有意に低かった[2]。IFN導入率が高齢者を含めて低い理由を検討するために，石川県全下で内科標榜医療機関にアンケート調査を行った。設問「一度はIFN療法を患者に説明するか（複数回答可）」に肝臓専門医の約8割は条件を問わずIFN療法について説明するが，非専門医師は約5割しか条件を問わずにIFN療法を説明していなかった。また「IFN療法を行わない理由」としては高齢であることをあげる医師が多数を占めたが，「何歳までがIFN療法の適応と考えるか」という設問では専門医は70～75歳までを適応と考えているが，非専門医はおおむね70歳以下と考えており，IFN適応年齢を非専門医は低く考えがちであることも明らかとなった[2]。このような実態を踏まえ，一例ごとの事例検討会，IFN療法をテーマにした講習会などを繰り返し行い，2004年102例中24例

表2㉒全国および石川県の検診C型肝炎陽性者のIFN施行率

		初年度IFN療法施行率	
		精検受診者中	慢性肝炎中
全国[1]	2002年	13.8%	
	2003年	13.3%	
	2004年	18.2%	
	2005年		
	2006年		
石川県	2002年	3.0%	3.8%
	2003年	5.7%	8.5%
	2004年	14.7%	23.5%
	2005年	24.5%	35.3%
	2006年	23.7%	31.0%

表3㉓「肝炎診療連携」で把握された75歳以下検診C型肝炎陽性症例のIFN治療状況

	キャリア（n：13）＋慢性肝炎（n：75） n＝88
IFN過去にあり	28（著効 6例）
現在投与中	7
投与開始	7
IFN施行数（率）	42/88（48%）
合併症不可（IP，うつなど）	4
IFN可能症例施行数（率）	42/84（50%）
IFN検討中	8

（23.5%），2005年68例中24例（35.3%），2006年71例中22例（31.0%）と後半2年間はIFN療法施行率が30%を超えていた（表2）。

石川県肝炎診療連携 5

年々IFN施行率は上昇してきたが，さらに向上させるには専門医が関わることが重要である。石川県では精密検査を専門医が行った症例では144例中53名（36.8%）がすぐにIFN導入され，翌年以降にさらに26例でIFN療法が施行，計79例（54.9%）でIFN療法が導入されていた。一方，かかりつけ医で診られた41症例では計8例（19.5%）のIFN導入にとどまり，IFN療法施行率をあげるには専門医がその診断，治療方針決定に関わることが重要であった。2007年にでた厚生労働省の肝炎検査後診療体制のガイドラインでも「状態に変化がなくとも年一回の専門医療機関受診が望ましい」とされており，かかりつけ医から患者を年1回の専門医に受診勧奨する「石川県肝炎診療連携」を立案した。個人情報保護の問題をクリアし，行政の保持する検診データを拠点病院と専門医療機関で構成する肝炎診療連携協議会に移行するために，行政・各市町と協議の上，患者より「石川県肝炎診療連携」への参加，データ移行に関して再同意をとり，専門医療機関を受診，順次データ移管することとなった。非同意，または返答のなかった症例は引き続き行政でフォローアップをすることとした。

2,570人の肝炎ウイルス検診陽性者に同意書・調査票が送付され494人が同意，非同意が90人，専門医療機関受診し調査票が回収されたのは328人であった。HBs抗原陽性148人，HCV抗体陽性174人であった。HBs抗原陽性では無症候性キャリアと診断されたのが79例で，そのうち5例でALT31IU/L以上の異常値であったが，4例ではHBV-DNA低値の情報が付加されており，診断が妥当であることが確認された。また核酸アナログ使用率も14%とHBs抗原陽性で治療を必要とする従来の割合と合致しているデータと考えられた。HCV抗体陽性者のうち慢性肝炎またはキャリアと診断された症例の治療方針をみると専門医がIFN療法が望ましいとしたのは全体の33%であった。一方経過観察が選択された症例では，ALT値が低いか，超高齢者が多く含まれていた。今回の専門医受診を契機にIFN療法導入が7例あり，過去のIFN歴も踏まえて現在までにIFN療法が行われたのは75歳以下の検診症例で48%であった（表3）。

おわりに

肝癌撲滅には背景となるウイルス性肝疾患への適切な経過観察，治療の導入が重要である。県下の肝炎ウイルス検診症例を専門医受診勧奨とデータ管理により早期に適切な治療導入に図りたい。

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GASTROENTEROLOGY 2011;141:128–140

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

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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*).

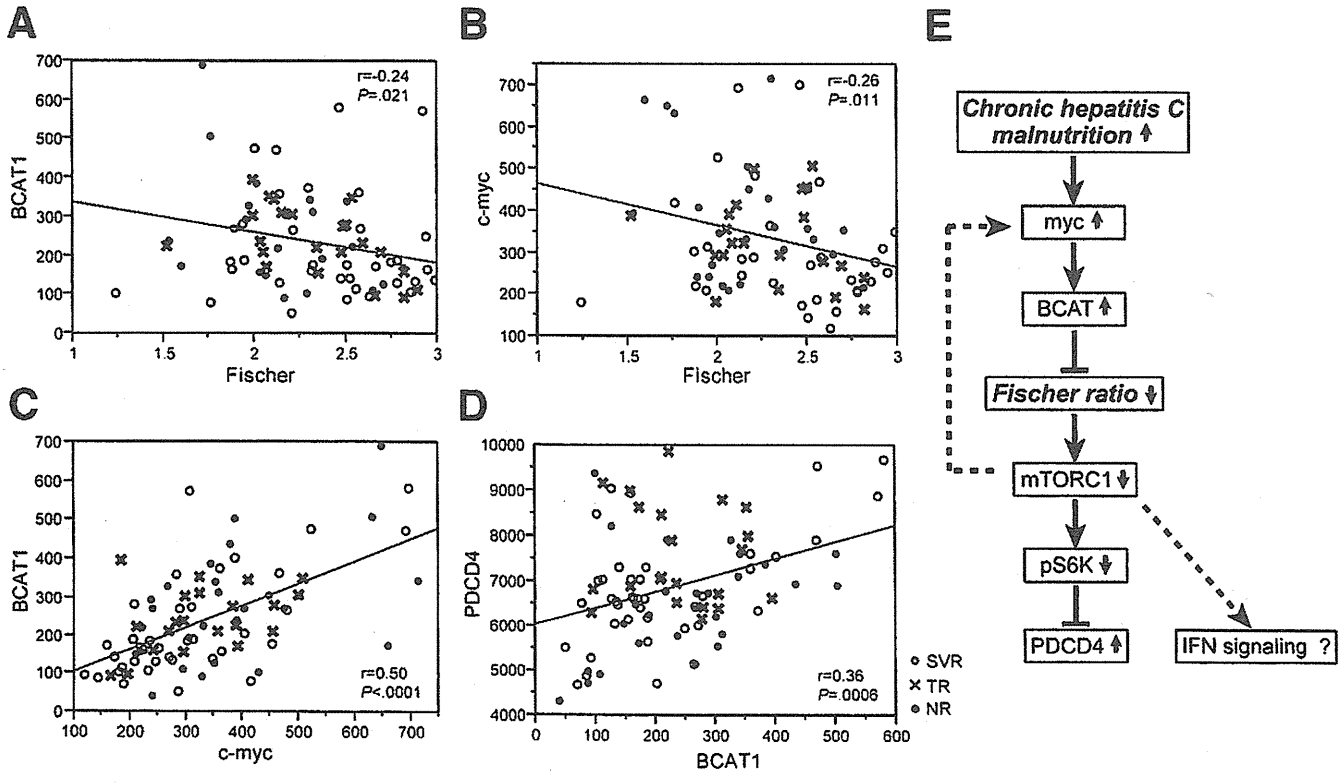

Figure 1. Regression analysis of mTORC1-related gene expression in liver. Gene expression values were determined by probe intensities. (*A*) BCAT1 and Fischer's ratio. (*B*) c-myc and Fischer's ratio. (*C*) BCAT1 and c-myc. (*D*) PDCD4 and BCAT1. (*E*) Putative signaling of mTORC1-related genes in CH-C.

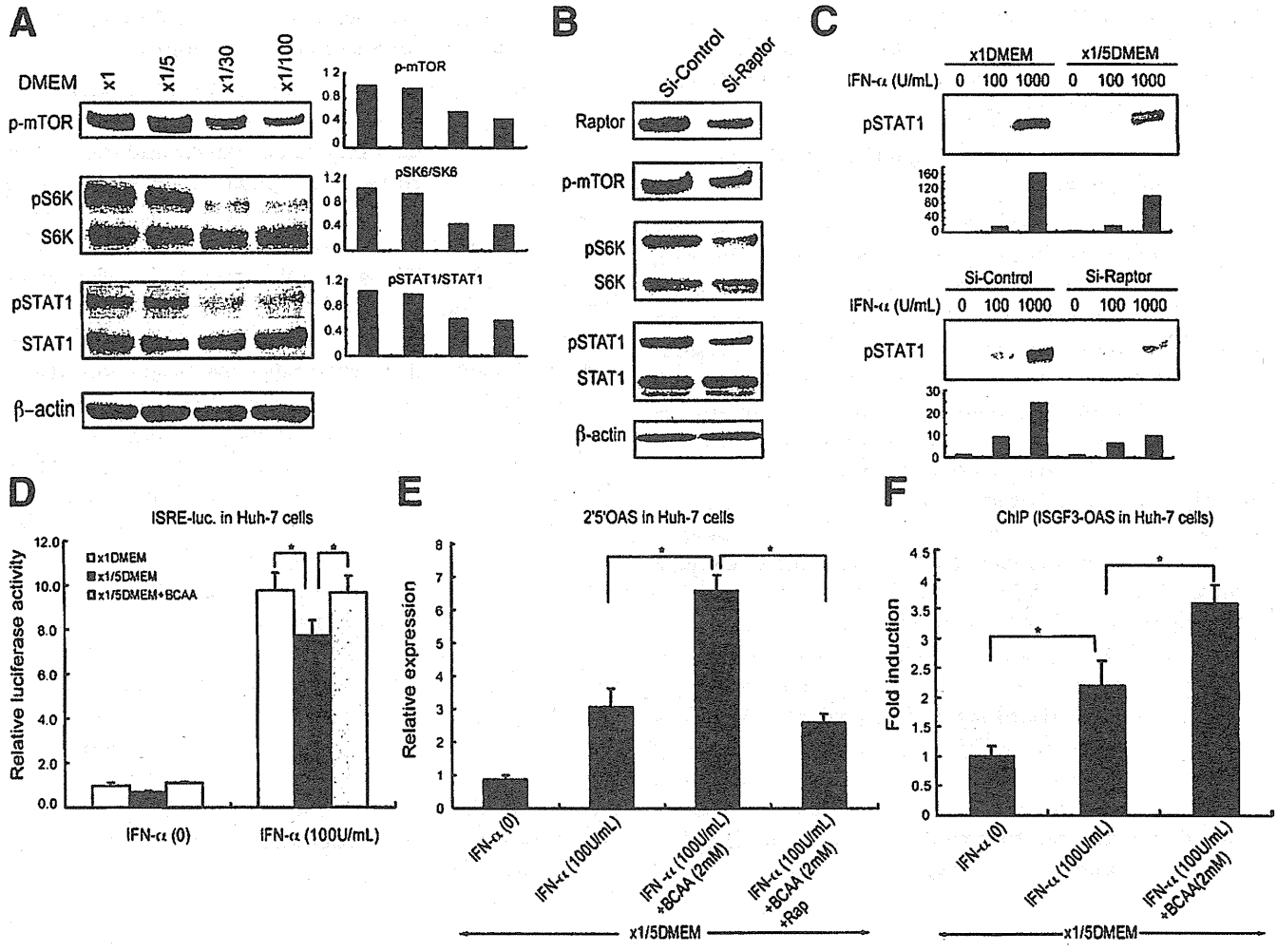

Figure 2. mTORC1 and IFN signaling in Huh-7 cells in low-amino-acid medium. (*A*) p-mTOR, pS6K, and pSTAT1 expression in different amino acid media. (*B*) p-mTOR, pS6K, and pSTAT1 expression under Raptor knock-down conditions. (*C*) IFN-alfa stimulation and pSTAT1 expression in low-amino-acid media or 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

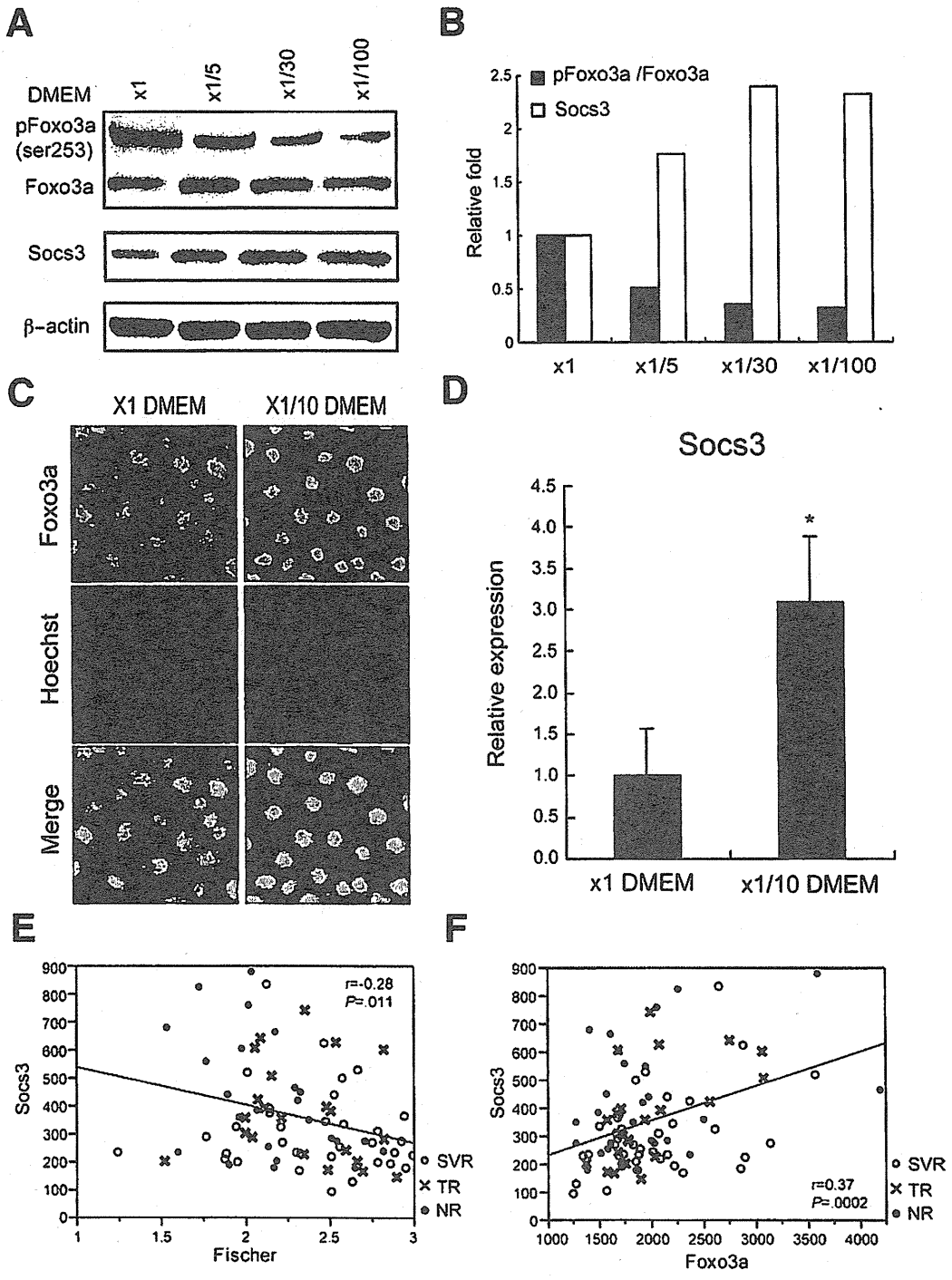

Figure 3. Foxo3a and Socs3 signaling in Huh-7 cells in low-amino-acid medium. (*A*) Foxo3a and Socs3 expression in different amino acid media. (*B*) Relative change of pFoxo3a/Foxo3a and Socs3 expression in different amino acid media. (*C*) Immunofluorescence staining of Foxo3a in Huh-7 cells in normal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

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

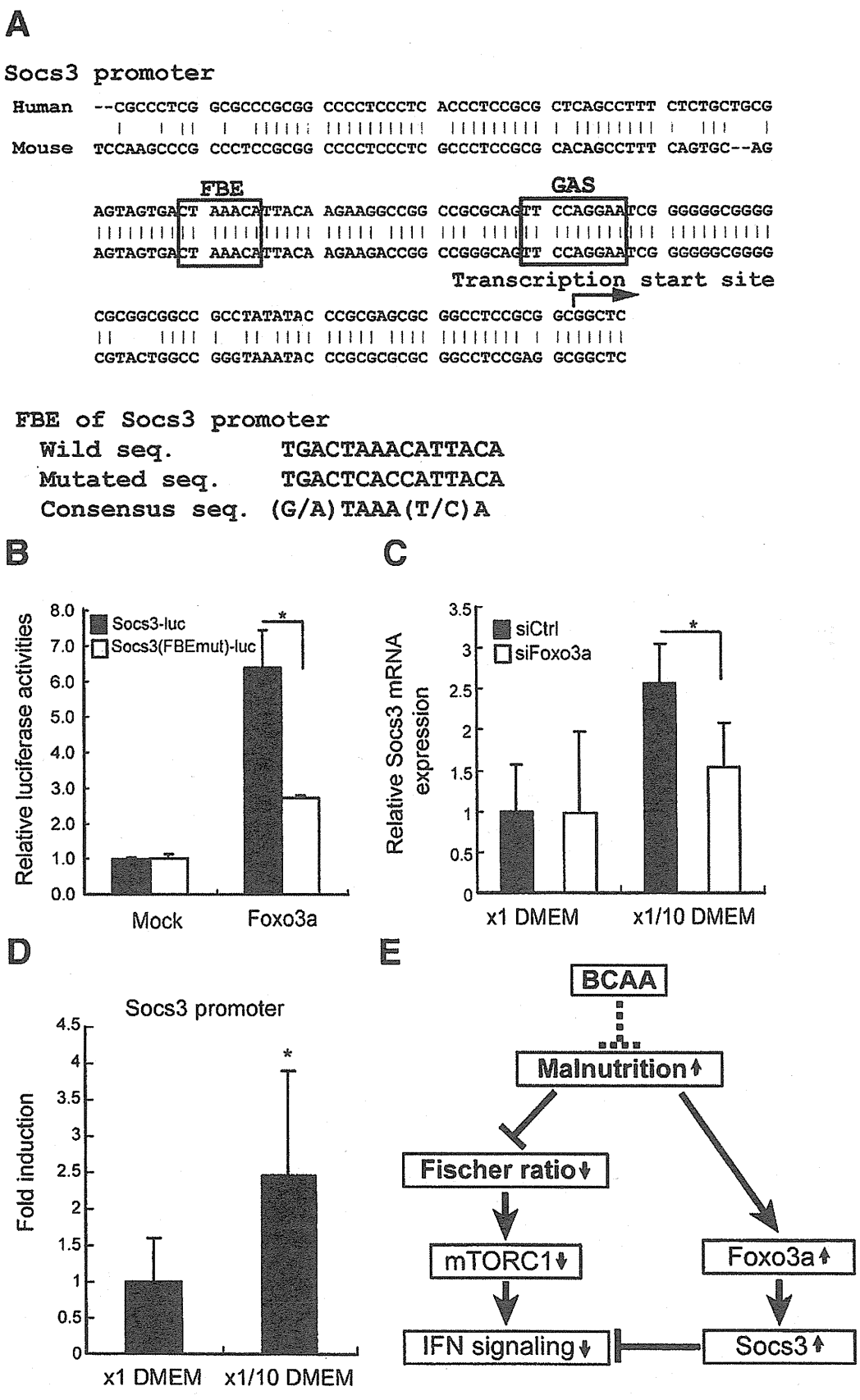

Figure 4. Socs3 promoter assay. (*A*) Primary structure of putative Foxo binding element in Socs3 promoter region. (*B*) Socs3-luc and Socs3 (FBEmut)-luc activities after overexpression of Foxo3a in Huh-7 cells. (*C*) Relative Socs3 messenger RNA (mRNA) expression after knockdown of Foxo3a in normal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

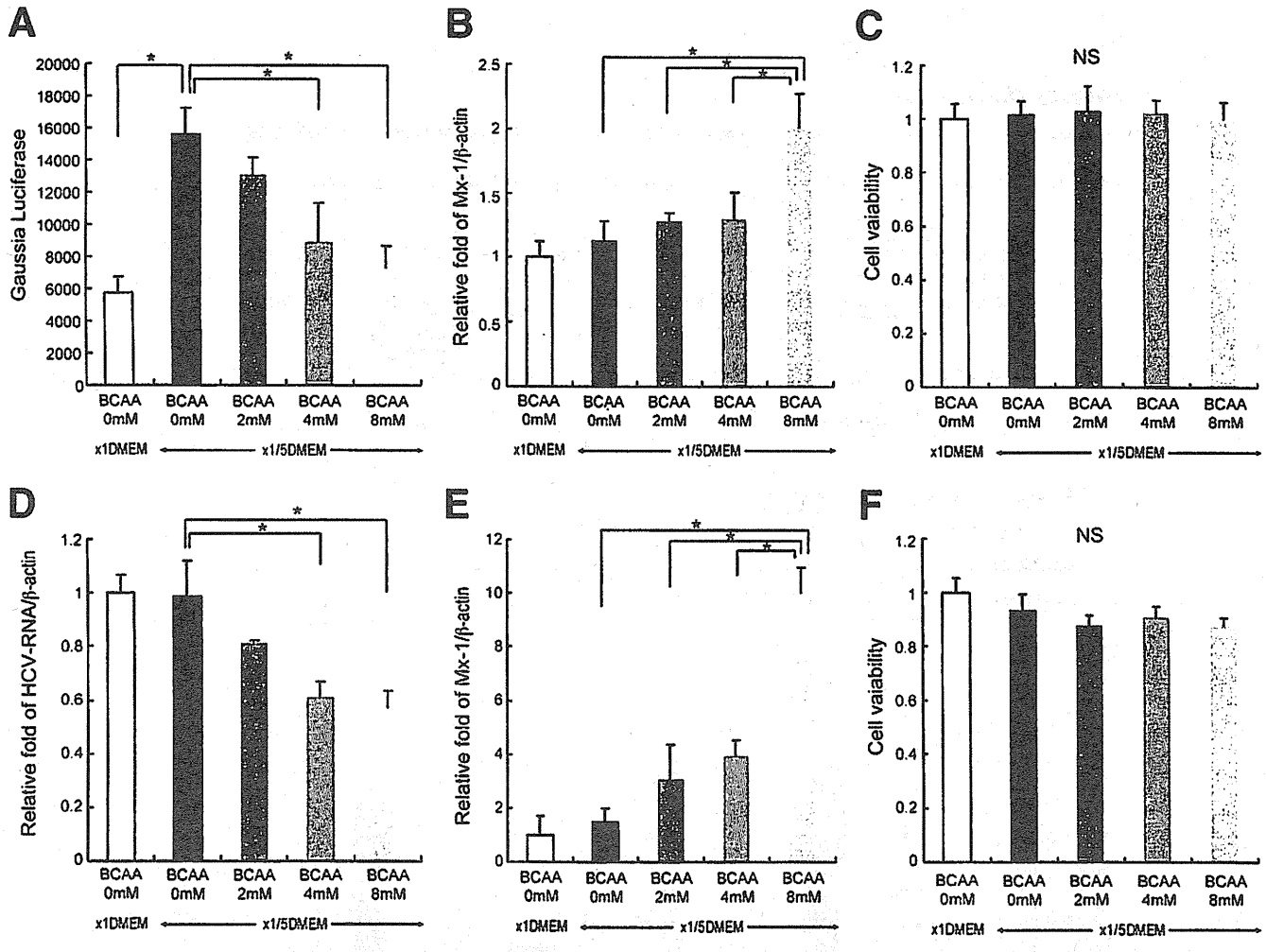

Figure 5. Effect of BCAA on HCV replication in cells in low-amino-acid medium. (*A*) Effect of BCAA on H77S.3/GLuc2A replication in Huh-7.5 cells. (*B*) Mx-1 expression in H77S.3/GLuc2A-transfected Huh-7.5 cells supplemented with BCAA. (*C*) Viability of Huh-7.5 cells. (*D*) Effect of BCAA on JFH-1 replication 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 1*C*) as reported previously.[15] Several studies observed up-regulated c-myc expression in advanced stages of CH-C[19] but, on the other hand, c-myc recently was shown to be a target of mTORC1 in hepatic cells.[17] The existence of a feedback mechanism between c-myc and mTORC1 signaling to maintain liver homeostasis (Figure 1*E*) is plausible, although the precise mechanisms need to be confirmed.

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

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

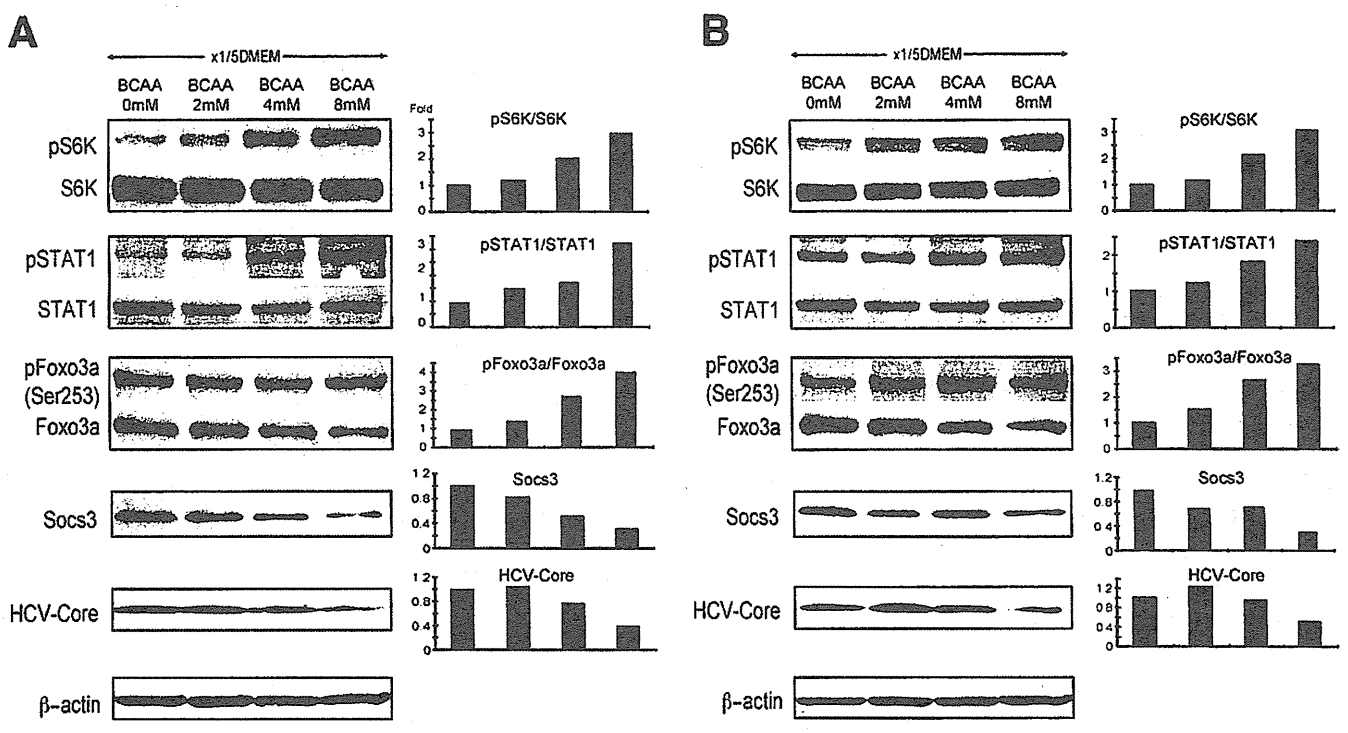

Figure 6. Expression of S6K, STAT1, Foxo3a, Socs3, and HCV core in H77S.3/GLuc2A-transfected Huh-7.5 cells or continuously JFH-1–infecting Huh-7 cells supplemented with BCAA.

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

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

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

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

Supplementary Material

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

Appendix A

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