例のうちgenotype 1bは3例のみと少なく，その他の症例では多彩なgenotypeを認めたが，このことはHIV，HCV重複感染におけるgenotypeが多様であるという側面と，genotype 1の症例での治療導入率が低いという2点が関与していたと考えられる．

今回の治療効果としてはgenotype 1または4の症例ではSVRを認めたのは1例のみで，結果としては芳しいものではなかった．一方，genotype 1または4以外の症例では治療効果は良好で，多くの症例でSVRを認めた．この結果は，HCV単独感染症例で予測されるものと同様な結果であった[16)~18)]．

SVRが得られなかった症例を検討すると，症例1のgenotype 1b症例では，薬剤のadherenceも良好であったが，48週投与にてHCV-RNA量はほとんど低下を認めず，難治症例と考えられた．また，症例3のgenotype 1b症例では，治療開始当初はHCV-RNAの早期陰性化を認めたが，その後は治療中にもかかわらず，HCV-RNAの再活性化を認めbreakthrough hepatitisを呈し32週で治療中断となった．なお，本症例におけるHCV-RNAの再陽性化の理由は不明であった．症例8のgenotype 3b症例では，治療当初から好中球減少が著しく，PEG-IFNのadherenceが低く12週の時点でHCV-RNA量の変化をほとんど認めなかったため治療中断となった．症例9のgenotype 4a症例においては，16週でHCV-RNAの陰性化を認め，48週以上の投与を目標に治療を行っていたがインフルエンザ様症状による全身倦怠感が高度で，24週の時点で治療を中断した．そして，中断後に短期間で再燃している．一般にgenotype 4については，比較的難治であることが報告されており，genotype 1に準じた長期間の治療が必要とされている[19)]．

同療法の治療期間については，2011年に発表されたわが国の抗HIV治療ガイドラインにおいて，HCV-HIV International Panelによる2007年の勧告に準じたresponse guided therapyが推奨されている[10)20)]．具体的には，HCV-RNAが4週目に陰性のrapid virological response（RVR）を認めれば，genotype 1または4で48週間，1または4以外で24週間の投与が推奨されている．一方，4週目に陽性で12週目に2log以上の低下を認め24週までに陰性化した場合は，genotype 1または4で72週間，1または4以外で48週間の投与が推奨されている．なお，上記の条件に当てはまらない場合は，その時点での治療の中止が妥当とされている．今回の症例のうち副作用や治療反応性不良で中止した3症例（症例3，8，9）を除いた7症例中6症例でSVRを認めているが，それら6症例についてガイドラインで示された内容と比較検討すると，genotype 1または4でSVRを達成した1症例（症例2）では，RVRを認めた後，ガイドラインと合致した48週投与にてSVRを達成している．一方，genotype 1または4以外でSVRを達成した5症例では，1例（症例7）でRVRを経て24週投与，3例（症例4，6，10）で8週目のウイルス陰性化を経て48週投与と，ガイドラインに合致した治療でいずれもSVRを認めた．一方で，1例（症例5）では8週目のウイルス陰性化を経て，ガイドラインより短い24週投与にてSVRを認めている．ここで注目すべきなのは，genotype 1または4以外の2例（症例4，6）でPEG-IFN，RBVのadherenceは決して良好ではなかったが，単独感染の投与期間より長い48週間の投与を行ったところSVRを認めていることである．両症例ともHCV-RNAの陰性化時期は5週目以降で，結果的には現在のガイドラインに沿った形での治療を行っていた．ガイドラインに示されているresponse guided therapyでは，単独感染よりやや長めの投与期間が設定されているが，adherenceが比較的不良な症例に対しても対応可能な内容と考えられ，現在当院でも，この内容に沿った治療が行われている．

今回治療を受けたgenotype 1bの症例のうち，PEG-IFNα2a，RBV併用療法の保険適応が認められた2007年以降に治療を開始された症例が1例（症例2）のみあったが，全例PEG-IFNα2bを用いた治療が行われた．一般にPEG-IFN，RBV併用療法において，PEG-IFNα2aを用いる方がPEG-IFNα2bでの治療よりもわずかにSVR率が

高く有害事象は同程度との報告が多い[21][22]．一方，HIV，HCV重複感染症例では，両薬剤のSVR率に差がなく，PEG-IFNα2aの方がより血球減少が高度であるとの報告もある[23]．前出のガイドラインにおいても，PEG-IFN，RBV併用療法においてPEG-IFNαの種類にまでは言及しておらず，症例ごとに各薬剤の特徴を考慮して使用すべきと考えられる[10]．

また，本療法の注意事項として，PEG-IFNおよびRBVと抗HIV剤との相互作用についての報告も散見されている．一般に抗HIV剤はPEG-IFNとの相互作用はないとされているが，一方でいくつかの抗HIV剤はRBVとの相互作用が報告されており注意が必要である[24]．特に核酸系逆転写酵素阻害剤であるAZTは血液毒性が比較的高度であるが，RBVとの併用で重篤な貧血をおこすことから原則併用禁忌である[3][25]．今回，AZTを使用した状態でPEG-IFN，RBV併用療法が開始されたことにより，急速に貧血を呈し一時PEG-IFN，RBV併用療法が中止となった症例（症例6）が存在した．その後，AZTをTDFに変更することで速やかに貧血は改善しPEG-IFN，RBVの48週間投与を行うことができた[26]．当院ではHIVの治療を感染症科が，HCVの治療を消化器科が担当している．消化器科医師はHAARTに慣れておらず，AZTとRBV併用の危険性に対する認識がなかった．今後，HIVとHCVに対する治療者が異なる場合は，消化器科の医師もHAARTに対する十分な理解が必要で，HIVに対する治療者との綿密な情報交換が必須と思われた．また，核酸系逆転写酵素阻害剤であるジダノシン（ddi）とサニルブジン（d4T）は，ミトコンドリア障害による乳酸アシドーシスや膵炎を生ずる危険があり，RBVとの併用で細胞内濃度が上昇することでその頻度が増大すると報告されており，特にddiは併用禁忌である[27]．今回，1例（症例10）において，患者の希望で前医からの処方を変更できずにd4Tを使用したままPEG-IFN，RBV併用療法を行った．幸い重篤な合併症は生じなかったが，PEG-IFN，RBV併用療法開始前にddiやd4Tを使用している症例は他の薬剤に変更してから治療を開始するべきと思われる．ただし，他の核酸系逆転写酵素阻害剤においても乳酸アシドーシスや膵炎のリスクは存在しており，血中乳酸値を測定するなどの注意を払って治療に当たるべきである．一方，核酸系逆転写酵素阻害剤であるアバカビル（ABC）はRBVと代謝経路が一部同じであるため，競合的リン酸化阻害によるRBVの効果減弱の可能性が指摘されているが，実際の治療効果への影響は不明である[28]．また，プロテアーゼ阻害剤であるアタナザビル（ATV）を使用中にPEG-IFN，RBV併用療法を行うと，高度のビリルビン血症をきたすことがあるとの報告もあり注意が必要である[29]．

相互作用ではないが，非核酸系逆転写酵素阻害剤であるEFVは抑鬱などの精神神経症状をきたしやすいため，PEG-IFN投与に当たっては他剤への変更が望ましいとされている[30]．今回の症例でも3症例でEFVが使用されていたが，PEG-IFN，RBV併用療法の開始に当たって2症例で薬剤の変更が行われていた．

今後の展望としては，HCVに対するプロテアーゼ阻害剤であるテラプレビルとの併用によるC型慢性肝炎の治癒率向上が期待されている[31]．HIV，HCV genotype 1重複感染例に対するテラプレビル，PEG-IFN，RBV併用療法のphase II trialの途中経過の報告では，重篤な有害事象はなく，4週，12週の時点でHCV-RNA陰性化率はいずれも約70%と報告されており，最終の結果が期待されている[32]．ただし，抗HIV剤とテラプレビルとの薬剤相互作用も報告されており注意が必要である．テラプレビルは，リトナビルをブースターとした各種抗HIVプロテアーゼ阻害剤やEFVの使用で血中濃度が低下するとの報告がある[33]．また，テラプレビルはTDFの血中濃度の上昇をもたらすため定期的な腎機能の観察が必要とも報告されている[33][34]．しかし，今のところHIV，HCV重複感染症例に対するテラプレビルの投与についての報告は限られており，抗HIV剤との相互作用の全貌が明らかになっているとはいい難く，現在も検討途中の段階である[35]．

結 論

HIVとHCVの重複感染症例の治療は，HIV感染症治療の中でも重要な位置づけを有しており，消化器科医の果たす役割は大きい．今回の検討でHIV，HCVの重複感染症例に対するPEG-IFN，RBV併用療法は，適切な抗HIV療法のもとで行えば安全で有効な治療と考えられた．特に，genotype 1または4以外の症例では，単独感染より長期間の治療を行うことで良好な治療成績を得ることができた．今後は，genotype 1高ウイルス症例を主とした難治例に対するテラプレビル，PEG-IFN，RBV併用療法による治療成績の向上が期待されている．

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本論文内容に関連する著者の利益相反
：後藤秀実（MSD株式会社）

文 献

1) Koike K, Tsukada K, Yotsuyanagi H, et al : Prevalence of coinfection with human immunodeficiency virus and hepatitis C virus in Japan. Hepatol Res 37 ; 2-5 : 2007
2) Tatsunami S, Mimaya J, Shirahata A, et al : Current status of Japanese HIV-infected patients with coagulation disorders : coinfection with both HIV and HCV. Int J Hematol 88 ; 304-310 : 2008
3) Fuster D, Huertas JA, Gómez G, et al : Baseline factors associated with haematological toxicity that leads to a dosage reduction of pegylated interferon-alpha2a and ribavirin in HIV- and HCV-coinfected patients on HCV antiviral therapy. Antivir Ther 10 ; 841-847 : 2005
4) Núñez M, Ocampo A, Aguirrebengoa K, et al : Incidence of anaemia and impact on sustained virological response in HIV/HCV-coinfected patients treated with pegylated interferon plus ribavirin. J Viral Hepat 15 ; 363-369 : 2008
5) Felsenstein J : Evolutionary trees from DNA sequences : a maximum likelihood approach. J Mol Evol 17 ; 368-376 : 1981
6) Li C, Fu Y, Lu L, et al : Complete genomic sequences for hepatitis C virus subtypes 6e and 6g isolated from Chinese patients with injection drug use and HIV-1 co-infection. J Med Virol 78 ; 1061-1069 : 2006
7) Avidan NU, Goldstein D, Rozenberg L, et al : Hepatitis C viral kinetics during treatment with peg IFN-alpha-2b in HIV/HCV coinfected patients as a function of baseline CD4+ T-cell counts. J Acquir Immune Defic Syndr 52 ; 452-458 : 2009
8) European Association for the Study of the Liver : EASL Clinical Practice Guidelines : management of hepatitis C virus infection. J Hepatol 55 ; 245-264 : 2011
9) Cargnel A, Angeli E, Mainini A, et al : Open, randomized, multicentre italian trial on PEG-IFN plus ribavirin versus PEG-IFN monotherapy for chronic hepatitis C in HIV-coinfected patients on HAART. Antivir Ther 10 ; 309-317 : 2005
10) 平成22年度厚生労働科学研究費補助金エイズ対策研究事業，HIV感染症及びその合併症の課題を克服する研究班：抗HIV治療ガイドライン，104-105 : 2011
11) Torriani FJ, Rodriguez-Torres M, Rockstroh JK, et al : Peginterferon Alfa-2a plus ribavirin for chronic hepatitis C virus infection in HIV-infected patients. N Engl J Med 351 ; 438-450 : 2004
12) Taylor JM, Fahey JL, Detels R, et al : CD4 percentage, CD4 number and CD4 : CD8 ratio in HIV infection : which to choose and how to use. J Acquir Immune Defic Syndr 2 ; 114-124 : 1989
13) Zein NN : Clinical significance of hepatitis C virus genotypes. Clin Microbiol Rev 13 ; 223-235 : 2000
14) Yamada G, Tanaka E, Miura T, et al : Epidemiology of genotypes of hepatitis C virus in Japnanese patients with type C chronic liver diseases : a multi-institution analysis. J Gastroenterol Hepatol 10 ; 538-545 : 1995
15) 小池和彦，高松純樹，菅原寧彦，他：我が国におけるHIV感染症に合併するHCV感染症の実態．厚生労働省科学研究費助成金 エイズ対策研究事業 平成15年度～17年度 総合研究報告書，23-34 : 2005
16) Fried MW, Shiffman ML, Reddy KR, et al : Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347 ; 975-982 : 2002
17) Zeuzum S : Heterogeneous virologic response rates to interferon-based therapy in patients with chronic hepatitis C : who responds less well? Ann Intern Med 140 ; 370-381 : 2004
18) Zhao S, Cheng D, Liu E, et al : Peginterferon vs.

interferon in the treatment of different HCV genotype infections in HIV patients. Eur J Clin Microbiol Infect Dis 27 ; 1183–1192 : 2008
19) Martín-Carbonero L, Puoti M, García-Samaniego J, et al : Response to pegylated interferon plus ribavirin in HIV-infected patients with chronic hepatitis C due to genotype 4. J Viral Hepat 15 ; 710–715 : 2008
20) Soriano V, Puoti M, Sulkowski M, et al : Care of patients coinfected with HIV and hepatitis C virus : 2007 updated recommendations from the HCV-HIV International Panel. AIDS 21 ; 1073–1089 : 2007
21) Rumi MG, Aghemo A, Prati GM, et al : Randomized study of peginterferon-alpha2a plus ribavirin vs peginterferon-alpha2b plus ribavirin in chronic hepatitis C. Gastroenterology 138 ; 108–115 : 2010
22) Awad T, Thorlund K, Hauser G, et al : Peginterferon alpha-2a is associated with higher sustained virological response than peginterferon alfa-2b in chronic hepatitis C : systematic review of randomized trials. Hepatology 51 ; 1176–1184 : 2010
23) Laguno M, Cifuentes C, Murillas J, et al : Randomized trial comparing pegylated interferon alpha-2b versus pegylated interferon alpha-2a, both plus ribavirin, to treat chronic hepatitis C in human immunodeficiency virus patients. Hepatology 49 ; 22–31 : 2009
24) Seden K, Back D, Khoo S : New directly acting antivirals for hepatitis C : potential for interaction with antiretrovirals. J Antimicrob Chemother 65 ; 1079–1085 : 2010
25) Mira JA, López-Cortés LF, Merino D, et al : Predictors of severe haematological toxicity secondary to pegylated interferon plus ribavirin treatment in HIV-HCV-coinfected patients. Antivir Ther 12 ; 1225–1235 : 2007
26) 高橋昌明, 藤崎誠一郎, 伊部史朗, 他 : HIV, HCV 重複感染に対するリバビリン併用ペグインターフェロン療法により CD4, CD8 陽性リンパ球数が減少した1症例. 新薬と臨床 56 ; 332–335 : 2007
27) Bani-Sadr F, Carrat F, Pol S, et al : Risk factors for symptomatic mitochondrial toxicity in HIV/hepatitis C virus-coinfected patients during interferon plus ribavirin-based therapy. J Acquir Immune Defic Syndr 40 ; 47–52 : 2005
28) Mira JA, López-Cortés LF, Barreiro P, et al : Efficacy of pegylated interferon plus ribavirin treatment in HIV/hepatitis C virus co-infected patients receiving abacavir plus lamivudine or tenofovir plus either lamivudine or emtricitabine as nucleoside analogue backbone. J Antimicrob Chemother 62 ; 1365–1373 : 2008
29) Rodríguez-Nóvoa S, Morello J, González M, et al : Increase in serum bilirubin in HIV/hepatitis-C virus-coinfected patients on atazanavir therapy following initiation of pegylated-interferon and ribavirin. AIDS 22 ; 2535–2537 : 2008
30) Laguno M, Blanch J, Murillas J, et al : Depressive symptoms after initiation of interferon therapy in human immunodeficiency virus-infected patients with chronic hepatitis C. Antivir Ther 9 ; 905–909 : 2004
31) Soriano V, Sherman KE, Rockstroh J, et al : Challenges and opportunities for hepatitis C drug development in HIV-hepatitis C virus-co-infected patients. AIDS 25 ; 2197–2208 : 2011
32) Sulkowski M, Dietrich D, Sherman K, et al : Interim analysis of a phase 2a double-blind study of TVR in combination with pegIFN-α2a and RBV in HIV/HCV co-infected patients. 18th CROI, 146LB : 2011
33) Van Heeswijk R, Vandevoorde A, Boogaerts G, et al : Pharmacokinetic Interactions between ARV Agents and the Investigational HCV Protease Inhibitor TVR in Healthy Volunteers. 18th CROI, 119 : 2011
34) Van Heeswijk R, Gysen V, Boogaerts G, et al : The pharmacokinetic interaction between tenofovir disoproxil fumarate and the investigational HCV protease inhibitor telaprevir. 48th ICAAC, 13–17 : 2008
35) Thomas DL, Bartlett JG, Peters MG, et al : Provisional guidance on the use of hepatitis C virus protease inhibitors for treatment of hepatitis C in HIV-infected persons. Clin Infect Dis 54 ; 979–983 : 2011

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Clinical evaluation of peginterferon α plus ribavirin for patients co-infected with HIV and HCV at Nagoya Medical Center

Tomoyuki TSUZUKI, Hiroaki IWASE, Masaaki SHIMADA[1], Noboru HIRASHIMA[2], Yusuke HIBINO, Nobumitsu RYUGE, Masashi SAITO, Dai TAMAKI, Asako KAMIYA, Misaki YOKOI[1], Yoshiyuki YOKOMAKU[3], Seiichiro FUJISAKI[4], Wataru SUGIURA[5] and Hidemi GOTO[6]

[1] *Department of Gastroenterology, National Hospital Organization, Nagoya Medical Center*
[2] *Department of Gastroenterology, National Hospital Organization, East Nagoya Hospital*
[3] *Department of Infectious Diseases, National Hospital Organization, Nagoya Medical Center*
[4] *Influenza Virus Research Center, National Institute of Infectious Disease*
[5] *Clinical Research Center, National Hospital Organization, Nagoya Medical Center*
[6] *Department of Gastroenterology, Nagoya University Graduate School of Medicine*

At Nagoya Medical Center, 10 patients co-infected with HIV and HCV received peginterferon α (PEG-IFNα) plus ribavirin therapy. Three of the cases were HCV genotype 1b, 2 cases were HCV 3b, and 1 case each were 2b, 2c, 3a, 4a and 6n. Nine patients received anti HIV therapy from the beginning. In 5 of these patients, anti HIV therapy was modified when PEG-IFNα plus ribavirin treatment was started. Of the above, 7 patients completed the protocol. No patients had severe adverse effects. Sustained virological response was achieved in 1 of 4 (25%) of the patients with genotypes 1 or 4, and in 5 of 6 (83%) of the patients with other genotypes. PEG-IFNα plus ribavirin therapy is considered a safe and efficacious treatment for patients co-infected with HIV and HCV.

第150回

クリティカルパスを活用した肝臓病チーム医療の実践

国立病院機構熊本医療センター　消化器内科部長　**杉 和洋**
（日本医療マネジメント学会会員）

はじめに

診療が高度かつ煩雑になるにつれ、医師による医療行為以外に、生活、服薬あるいは栄養指導・管理、さらには医療費助成手続きや病院経営管理など多職種による役割分担と相互の連携が重要になってきている。クリティカルパスは関係する職種スタッフの共同作業により作成された診療・看護・リハビリテーションなどの時系列の計画書であり、医療の標準化とともにチーム医療の向上にも効果がある。

クリティカルパスの作成・見直しに当たっては、医師、看護師、薬剤師、栄養士、検査技師のみならず医療ソーシャルワーカーや医事課職員等、関連職種の共同作業が求められる。

今日では入院から外来への切れ目ないさまざまな地域連携クリティカルパスが運用されており、クリティカルパスは当初の診療計画書から、医療者と患者、院内および院外の医療者間の情報共有のツールとしての役割を果たしている。

当院でのクリティカルパスを用いた肝臓病チーム医療の実践と、それによりみえてきたアウトカムを紹介する。

国立病院機構熊本医療センターにおける肝臓病クリティカルパスの変遷

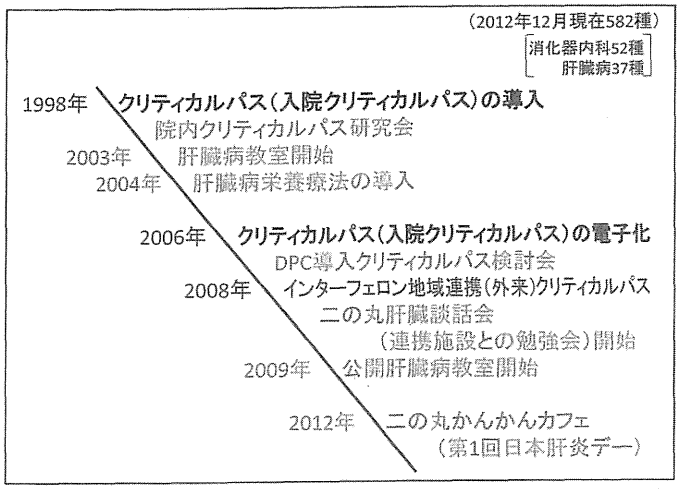
当院では一九九八年に全国に先駆けてクリティカルパスを導入した〈図1〉。これは現在の日本医療マネジメント学会設立の契機となり、これに伴い院内では定例のクリティカルパス研究会を開始した。クリティカルパスは新規作成とともに定期的に内容の見直しがなされ、実情に合わないものは削除される。これらの作業は、医師、看護師はもとより、薬剤師、栄養士、検査技師、あるいは医療ソーシャルワーカーや医事課職員等、関連職種が共同で行う。

二〇一二年一二月で院内全体の作成数は五八〇以上で、消化器内科五二種、肝臓病関係では経皮的肝生検、経皮的ラジオ波焼灼療法、肝動脈塞栓術、C型慢性肝炎に対するインターフェロン療法など合計三七種にのぼっている。

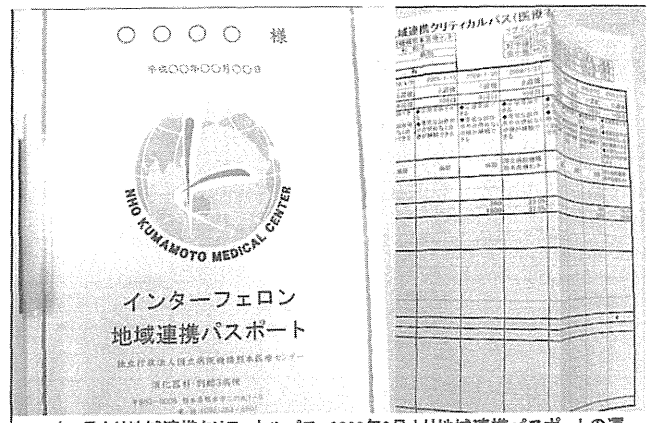
二〇〇六年には電子カルテの導入に伴いクリティカルパスも電子化された。診療報酬は出来高算定よりDPCへ移行し、在院日数管理が病院経営へ大きくかかわることになった。毎週早朝に開催されるクリティカルパス検討会では病院幹部、各職場長、当該診療科長および病棟看護師長が一堂に会し、当院の診療実績とともに全国のDPC導入病院での診療実績がベンチマークとして公開され、経営面からみたクリティカルパスの在院日数、検査・治療内容あるいは指導内容が吟味され、必要に応じて改訂が促される。

クリティカルパスの病院運営における役割

クリティカルパスは、発足当初の医療の標準化と質の向上はもとより、現在では病院全体の運営・経営向上のためのツールとしての役割を併せ持つことになった。実例として、C型慢性肝炎に対するインターフェロン治療を紹介する。作成当初は入院期間を一六日に設定していた。すなわち入院一日目より週一回のペグインターフェロン投与を開始し、一六日目三回目の投与後に退院する設定だった。二〇〇八年のDPCでは期間Ⅰが七日、期間Ⅱが一三日に設定されていたが、二〇一〇年の改定で期間Ⅰが六日、期間Ⅱが一一日に短縮された。それに応えるべく二〇一〇年二月に入院期間を九日すなわち二回目の注射後の退院に短縮するクリティカルパスに改訂した。改訂に至った理由としてDPCのみならず、



＜図１＞国立病院機構熊本医療センターにおける肝臓病チーム医療の変遷



2008年4月より地域連携クリティカルパス、2009年2月より地域連携パスポートの運用開始。医療者用・患者用クリティカルパスをファイルして患者に渡し医療機関受診時に持参する。2011年より患者記録、栄養指導および服薬指導をファイルしている。

＜図２＞インターフェロン地域連携パスポート

仕事や家事のため短い入院を希望する患者からの要望や、当院が救急病院として病床確保が必要だったことがある。

一方、短縮することで患者に不利益が生じてはならない。すでに運用されてから約二年がすぎていた地域連携クリティカルパスの使用実績があり、短期間の入院でも緊密な連携のもと、外来でも安全な医療が提供可能と考えられ改訂に踏み切った。執筆時点まで改訂されたクリティカルパスを運用して問題は生じていない。平均在院日数短縮により病院全体の病床確保に寄与している。

二〇〇八年四月よりインターフェロン地域連携クリティカルパスの運用を開始した。これはC型慢性肝炎診療上の多くの問題点の解決と連携に対する要望に応えるものだった。治療導入時に患者ごとに電子カルテ内で作成し、入院中の事項を記入し、退院時に印刷し連携医療機関に送る。そこでデータを記入し、当院受診時に持参してもらう。当院では前医でのデータと当日のデータとを併せて電子カルテ内で上書きし、保存する。同時に印刷し連携医療機関に送る。これを繰り返すことで医療情報の共有化を図った。

＜図３＞

二〇〇九年二月より患者用クリティカルパスと医療者用クリティカルパスを併せ、ファイルに綴じて「インターフェロン地域連携パスポート」とし、退院時に患者に渡すことにした〈図２〉。患者はそれを持参して医療機関を行き来し最新の情報を得ることになり、医療連携機関と患者の三位一体の情報共有ができるようになった。詳細は本誌二〇〇九年五月号に記載されている。

これまで入院診療においてのみチーム医療がなされていたが、地域連携クリティカルパスの運用により外来看護師、外来医療クラーク、さらには院外医療機関および院外調剤薬局スタッフとの多職種連携がなされることになった。また、これを契機に肝臓病チームは院外医療機関（かかりつけ医）、患者あるいは地域住民に対する啓発活動に目を向けることになった。

かかりつけ医、患者・住民啓発活動

インターフェロン地域連携クリティカルパスを運用するなかで、多数の施設より顔のみえる勉強会の要望があり、二〇〇八年九月に年四回の事例検討会と一回の特別講演会を行う「二の丸肝臓談話会」を発足した。本稿の執筆時点で一七回の定例会と四回の特別講演会が開催されている。回を重ねるごとに医師のみならず看護師、薬剤師、栄養士および医療ソーシャルワーカー等コメディカルが積極的に参加し、それぞれの視点に立った発表を行い、院外からも多職種の参加者が増えている。これは地域ぐるみのチーム医療の構築に寄与するであろう。

当院では二〇〇三年より肝臓病教室が開催され、翌二〇〇四年に肝臓病栄養療法が導入され、これにより肝臓病患者に対して医師、看護師、薬剤師、検査技師および栄養士による肝臓病チーム医療の基礎が築かれた。これまでお互いがそれぞれの職種の肝臓病における専門的な知識を

ペグインターフェロン・リバビリン併用療法地域連携クリティカルパス（医療者用）

患者様名		ID番号		導入機関名	国立病院機構熊本医療センター
生年月日		PEG-IFN名	ペグイントロン・ペガシス	医師名	
セロタイプ	1型・2型	リバビリン名	レベトール・コペガス	連携機関名	
ウイルス量	高・低	初回/再治療	初回・再治療	医師名	
肝生検結果	A(0・1・2・3) F(0・1・2・3・4)	予定期間	24週・48週・72週	公費助成	無・有

ペグインターフェロン

WBC	1500>	半量	1000>	中止
好中球	750>	半量	500>	中止
血小板	*8万>	半量	5万>	中止

リバビリン

Hb	10未満	減量
	2以上低下	減量
	8.5未満	中止

*ペグイントロンのみ

日時		yyyy/mm/dd	/ /	/ /	/ /	/ /	/ /	/ /	/ /	/ /	/ /	/ /	/ /	/ /
経過		治療開始時	1週後	2週後	3週後	4週後	5週後	6週後	7週後	8週後	9週後	10週後	11週後	12週後
		投与1回目	2回目	3回目	4回目	5回目	6回目	7回目	8回目	9回目	10回目	11回目	12回目	13回目
達成目標			◆病気に対して理解している ◆治療の理解ができ、同意している ◆重篤な副作用や合併症なく治療が開始できる	◆重篤な副作用や合併症なく治療が継続できる	◆重篤な副作用や合併症なく治療が継続できる ◆外来での治療に移行できる ◆内服薬の自己管理ができる	◆定期受診ができる ◆重篤な副作用や合併症なく治療が継続できる	◆定期受診できる ◆重篤な副作用や合併症なく治療が継続できる ◆超早期ウィルス消失(RVR)が得られる	◆定期受診できる ◆重篤な副作用や合併症なく治療が継続できる	◆定期受診できる ◆重篤な副作用や合併症なく治療が継続できる	◆定期受診できる ◆重篤な副作用や合併症なく治療が継続できる	◆定期受診できる ◆重篤な副作用や合併症なく治療が継続できる	◆定期受診できる ◆重篤な副作用や合併症なく治療が継続できる	◆定期受診できる ◆重篤な副作用や合併症なく治療が継続できる	◆定期受診できる ◆重篤な副作用や合併症なく治療が継続できる ◆早期ウィルス消失(EVR)が得られる
医療機関		国立病院機構熊本医療センター	国立病院機構熊本医療センター			国立病院機構熊本医療センター				国立病院機構熊本医療センター				国立病院機構熊本医療センター
治療	PEG-IFN μg													
	リバビリン mg													
	PEG-IFN積算量（予定%）				μg	%			μg	%			μg	%
	リバビリン積算量（予定%）				mg	%			mg	%			mg	%
検査	白血球数 /μl													
	好中球数 /μl													
	Hb g/dl													
	血小板数 x10⁴/μl													
	AST IU/L													
	ALT IU/L													
	Cr mg/dl													
	Glu mg/dl													
	TG mg/dl													
	HCV-RNA量 LogIU/ml													
	定性 +・−													
	AFP ng/ml													★
	腹部超音波													
	眼科					★								
観察項目	体重 Kg													
	食欲不振													
	不眠													
	貧血症状													
	咳													
	発疹													
	その他													
バリアンス			無・有	無・有	無・有	無・有	無・有	無・有	無・有	無・有	無・有	無・有	無・有	無・有	無・有

*有害事象　これらはあくまで代表例であり、患者様によっては症状や発症時期が異なる場合があります　*4週以降の眼科受診日は、眼科医の指示に従ってください

初期症状 ～1w	インフルエンザ様症状 食欲不振、発疹、かゆみ
中期症状 ～12w	倦怠感、口内炎、不眠、不安、躁鬱病 間質性肺炎(乾咳、呼吸困難、労作時息切れ、微熱など) 貧血、不整脈、糖尿病悪化
後期症状 3ヶ月以降	脱毛 甲状腺機能異常(動悸、汗かき、むくみ)

国立病院機構熊本医療センター 消化器内科 2008年12月改訂

ペグインターフェロン・リバビリン併用療法地域連携クリティカルパス（医療者用）

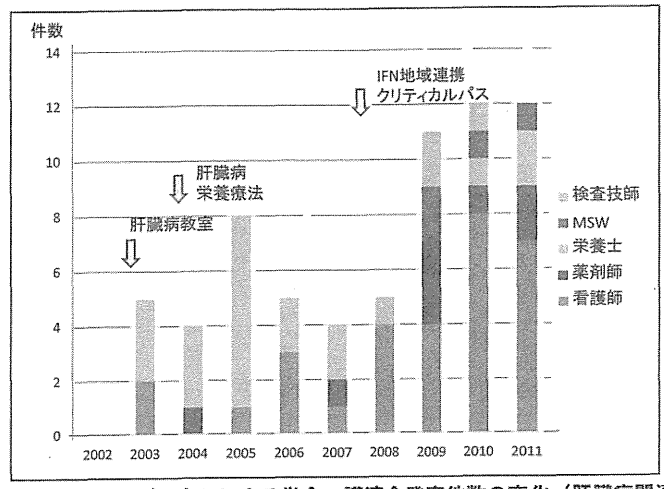
知る機会に乏しかったが、肝臓病教室で一堂に会して講義を聴講することによりお互いの知識を共有し、他職種の役割に目を向けることになり、なによりお互いの顔を知るきっかけになった。肝臓病教室は月に一回、入院あるいは外来患者を対象に開催されているが、二〇〇八年より年に一回患者のみならずその家族や知人あるいは一般市民を対象に公開肝臓病教室が開催されている〈図3〉。

これはいわゆる市民公開講座を模したもので、一般講演の後に医療・健康相談を行うものである。医師、看護師、薬剤師、栄養士に加え、検査技師および医療ソーシャルワーカーがそれぞれの専門性を活かして対応している。開催のきっかけは、二〇〇八年に国の肝炎総合対策事業として肝疾患拠点病院を中心とした肝疾患診療ネットワークの整備が進められ、当院が熊本県央ブロックの地域中核病院として、かかりつけ医との医療連携を緊密にするとともに地域住民の啓発活動を行う役割を担うことになったことによる。

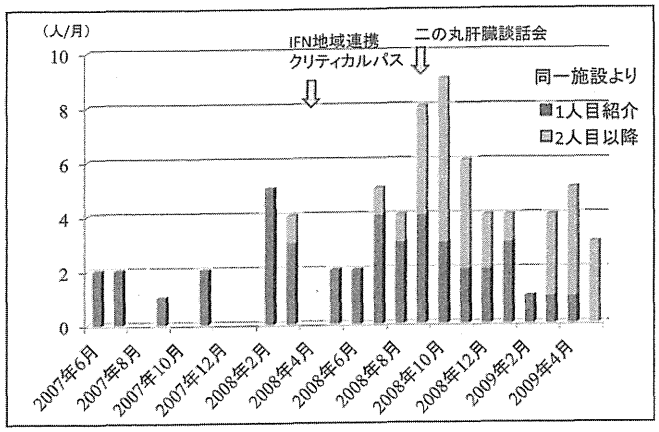
すでに地域連携クリティカルパスを運用し、かかりつけ医との顔のみえる勉強会を立ち上げていたので、肝臓病チームとしての運営は円滑に行われた。開催当初は医師主導で企画されたが、回を重ねるごとに関係職種での話し合いのもと内容の見直しが行われ、現在では栄養士が中心となって運営されている。

世界保健機関（WHO）は、二〇一〇年に世界的レベルでのウイルス性肝炎のまん延防止と患者・感染者に対する差別・偏見の解消や感染予防の推進を図ることを目的として、七月二八日を"World Hepatitis Day"（世界肝炎デー）と定め、肝炎に関する啓発活動等の実施を提唱した。二〇一一年が第一回世界肝炎デーとなったが、同年国は毎年七月二八日を日本肝炎デーと定め、二〇一二年七月二八日第一回日本肝炎デーを迎えた。当院ではそれに合わせて、肝臓病患者や肝臓病に関心のある一般住民を対象に交流と情報交換の場を提供するというコンセプトで「二の丸かんかんカフェ」を開催した〈図4〉。肝臓病のトピックスの講演の後に、一グループ六～八人に分かれ、それぞれの体験や疑問を紹介し、助言あるいは解決法を話し合い、最後に全体で質疑応答を行う内容である。国が肝炎対策推進事業として全国に開催を呼びかけている肝炎患者サロンに沿ったもので、肝臓病チームの運営により毎年開催したいと考えている。

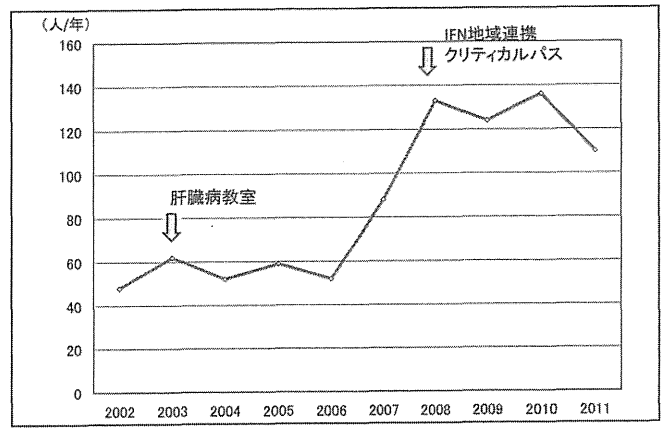
<図4>



<図5>コメディカルによる学会・講演会発表件数の変化（肝臓病関連）



<図6>C型慢性肝炎紹介患者の推移



<図7>C型慢性肝炎入院患者数

肝臓病チーム医療のアウトカム

はじめに述べた院内クリティカルパス研究会は一年に五回開催され、毎回四～五例の一般演題と一題の教育講演がある。消化器内科関連の発表は、二〇〇八年を契機にそれまでの年間約一件から約四件へと飛躍的に増加した。また、肝臓病に関する医師を除くコメディカルの学会や講演会発表数も二〇〇九年よりそれまでの年間五件から一一～一二件に増加した〈図5〉。これは肝臓病地域連携クリティカルパスの作成・運用、肝臓病に関するかかりつけ医や患者、住民に対する啓発活動への取り組みにより関係職種がそれぞれのモチベーションを高めるとともに専門性を深め、その成果を外に向かって発信する、すなわち肝臓病チーム医療の職員教育に関する成果である。C型慢性肝炎紹介患者数が増加し〈図6〉、入院患者数も増加する〈図7〉ことで診療実績が向上したことも啓発活動の成果と考えられる。

終わりに

クリティカルパスを用いた肝臓病チーム医療を実践してみえてきたことは、

1. 地域連携クリティカルパスを作成・運用する過程で、各職種の役割が明確になり、情報共有がなされている。

2. 診療における病院実績の向上のみならず、患者および一般住民への啓発活動を活性化させ、かかりつけ医との連携を深める機会ができた。

3. 医師以外の医療職種におけるモチベーション向上の成果として、学会・講演会への発表数が増加し、教育的効果がみられた。

4. クリティカルパスは単なる診療計画としての役割以上に、活用することで医療チームとしての一体化、さらには各職種のモチベーションの向上につながり、チーム医療の向上に貢献していると考えられる。

以上、当院におけるクリティカルパスを活用した肝臓病チーム医療の実践と現時点でのアウトカムについて紹介した。目指すところは病院機能の向上はもとより、地域の肝臓病診療レベルの向上および患者をはじめ地域住民の生活の質の向上である。肝臓病はウイルス肝炎、肝硬変、さらには肝がんを包括しており、がん対策を含めた総合的なチーム医療の実践が望まれる。そして、ここに示されたごとく、クリティカルパスはそのツールとなり得る。

Hepatology Research

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Original Article

Prevalence of restless legs syndrome in Japanese patients with chronic liver disease

Toshihisa Matsuzaki,[1] Tatsuki Ichikawa,[1] Hideaki Kondo,[2] Naota Taura,[1] Hisamitsu Miyaaki,[1] Hajime Isomoto,[1] Fuminao Takeshima[1] and Kazuhiko Nakao[1]

[1]*Department of Gastroenterology and Hepatology, Nagasaki University Graduate School of Biomedical Sciences, and* [2]*Center for Sleep Medicine, Saiseikai Nagasaki Hospital, Nagasaki, Japan*

Aim: Sleep disturbance is a major complication in patients with chronic liver disease, but causes are unclear. The aim of this study was to clarify the prevalence of restless legs syndrome (RLS) in Japanese chronic liver disease patients and investigate the influence on sleep and quality of life.

Methods: The study included 149 consecutive outpatients with chronic liver disease at Nagasaki University Hospital between September 2008 and March 2010. The presence of RLS was evaluated by a written survey using the questionnaire for the epidemiological surveillance of the international RLS research group in 2003. In addition, 89 cases, including all RLS patients, were evaluated for sleep quality and health-related quality of life. Sleep quality was evaluated by using the Japanese version of the Pittsburgh Sleep Quality Index (PSQI), and health-related quality of life was evaluated by the Japanese SF-36 Health Survey.

Result: Twenty-five of the 149 patients (16.8%) fulfilled the diagnostic criteria for RLS. The median global PSQI score of the RLS group was significantly higher than the non-RLS group (9 vs 5, $P < 0.01$). The number of poor sleepers (global PSQI score, >5) in the RLS group was significantly higher than in the non-RLS group ($P < 0.05$). In SF-36, the mental component summary score of the RLS group was 43.8 ± 10.8, which was significantly lower than the non-RSL group (49.8 ± 10.5; $P < 0.05$).

Conclusion: This is the first report that clarifies the prevalence of RLS in Japanese chronic liver disease patients. RLS worsens quality of sleep and life in chronic liver disease patients.

Key words: chronic liver disease, quality of life, restless legs syndrome, sleep disturbance

INTRODUCTION

RESTLESS LEGS SYNDROME (RLS) is a disease characterized by an urge to move the extremities, and paresthesias such as a burning, "creepy-crawly" sensation.[1] The symptoms worsen during periods of rest or inactivity such as lying or sitting. The prevalence of RLS in North America and Europe among the general adult population is reported to be 4.1–11.5%.[2–6] In contrast, the prevalence of RLS in Asian nations, including Japan, is reported to be 0.6–1.8%, which is lower than that in Europe and the USA.[7–9]

The symptoms of RLS worsen in the evening or night, especially during sleep. Therefore, patients with RLS often complain of sleep disturbance.[1] By polysomnography (PSG), the impaired sleep quality in RLS patients was revealed objectively.[10,11] Periodic leg movements (PLM) are observed during sleep in more than 80% of patients with RLS.[1] It is known that PLM makes the sympathetic nervous system and arousal system active

Correspondence: Dr Toshihisa Matsuzaki, Department of Gastroenterology and Hepatology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. Email: tmatsuzaki6@gmail.com

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on electroencephalograms.[12–15] In addition, it is reported that daytime drowsiness and anxiety are observed at a high frequency among RLS patients, and it impairs their quality of life.[5,7]

Restless legs syndrome has been reported to be a frequent complication of physical disorders. RLS is especially prevalent in patients with end-stage renal disease, with North America and European patients having an estimated prevalence of 21.5–68%.[16,17] However, the reported prevalence in Asian nations, including Japan, is much lower at 6.6–12.2%.[18,19] In collagen disease,[20] diabetes[21] and inflammatory bowel disease,[22] the prevalence of RLS is also more frequent than that in the general population.

It was reported for the first time in 2008 that the prevalence of RLS symptoms in patients with chronic liver disease (CLD) was 62%.[23] Moreover, the complaint of insomnia, such as the difficulties in falling asleep and nocturnal awakening, is reported to be high in cirrhosis,[24] and this might be related to RLS. However, these studies were of patients in Europe and the USA. Considering the differences in the populations of patients with CLD and RLS, these reports cannot be applied to our country.[1,7,25] The aim of the present study was to clarify the prevalence and the clinical features of RLS in CLD patients of Japan. In addition, we wanted to clarify the influence on the patient's quality of life and sleep due to RLS.

METHODS

OUR STUDY INCLUDED 149 consecutive outpatients with CLD at the Nagasaki University Hospital recruited from September 2008 to March 2010. The protocol for this study was approved by the Nagasaki University Ethics Committee (approval no. 09112732). All participating subjects provided their informed consent. The study was conducted according to the 2008 version of the Declaration of Helsinki. The characteristics of the patients with CLD are shown in Table 1. The mean age was 62.0 ± 11.5 years, and 70 (47%) patients were male and 79 (53%) were female. The etiology of the liver disease was determined by use of clinical, laboratory, radiological and histological variables. The underlying liver disease of 46 (30.9%) patients was chronic hepatitis C, liver cirrhosis (LC) in 25 (16.8%) and chronic hepatitis B in 20 (13.4%).

Blood biochemical tests were performed in all patients, and liver function was evaluated. The severity of the liver dysfunction was assessed using Pugh's modification of Child's scoring system.[26] The patients with severe anemia (hemoglobin, <7.0 g/dL; reference interval, 11.3–15.2)[27] and those with renal dysfunction (creatinine, >2.0 mg/dL; reference interval, 0.40–1.10) were excluded from the study. Serum ferritin levels (ng/mL; reference interval, 6–138) were measured by latex agglutination test for the evaluation of iron metabolism. Blood ammonia was measured by a colorimetric method (μg/dL; reference interval, 0–75), and the molar ratio of total branched-chain amino acids to tyrosine (BTR) was measured by an enzyme assay (μmol/L; reference interval, 4.41–10.05).

Table 1 Underlying liver disease ($n = 149$)

CH	93 (62.4%)
HCV	46 (30.9%)
HBV	20 (13.4%)
NASH	13 (8.7%)
AIH	11 (7.4%)
Primary biliary cirrhosis	3 (2.0%)
LC	46 (30.9%)
HCV	25 (16.8%)
HBV	10 (6.7%)
Alcohol	4 (2.7%)
Primary sclerosing cholangitis	2 (1.3%)
NBNC	5 (3.4%)
Others	10 (2.7%)

AIH, autoimmune hepatitis; CH, chronic hepatitis; HBV, hepatitis B virus; HCV, hepatitis C virus; LC, liver cirrhosis; NASH, non-alcoholic steatohepatitis; NBNC, non-B non-C. Others includes two Wilson's disease, two congestive hepatopathy, two alcoholic liver disease, two Caroli's disease, one glycogen storage disease type III and one portal vein anomaly.

All subjects were evaluated for the presence of RLS by a written survey using the questionnaire for the epidemiological surveillance of the international restless legs syndrome research group in 2003.[1] In this study, patients who gave positive answers to all four criteria and had symptoms of RLS that occurred at least twice per week were diagnosed as RLS.

In addition, 89 cases, including all RLS patients, agreed with further examinations of sleep quality and health-related quality of life. Sleep quality was evaluated by using the Japanese version of the Pittsburgh Sleep Quality Index (PSQI).[28,29] Questionnaire responses are used to generate seven components, each of which is scored 0–3, where 3 represents the extreme negative and over 1 is abnormal. The component scores are added to provide the global PSQI score (range, 0–21); scores of over 5 identify "poor" sleepers. Health-related quality of life was evaluated by the Japanese SF-36v2 Health Survey (Medical Outcomes Trust, Health Assessment

Lab, QualityMetric and Shunichi Fukuhara).[30,31] One item is designed to assess the perceived change in health status, and the remaining 35 items are used to generate eight subscales (0–100 scale): physical functioning (PF); role limitations due to poor physical health (RP); bodily pain (BP); general health perception (GH); vitality (VT); social functioning (SF); role limitations due to poor emotional health (RE); and role limitations due to mental health (MH). The subscale scores were standardized by using the general Japanese population to generate a corresponding z-score. Aggregate physical and mental component summary scores of the SF-36 (PCS and MCS, respectively) were obtained by multiplying each z-score by its respective physical and mental factor score coefficient and summing these eight products. Finally, each aggregate component score was transformed to a norm-based score with a Japanese population mean of 50 and standard deviation (SD) of 10.

The analysis was performed using JMP ver. 8.0 statistical discovery software from SAS. Differences between normally distributed variables were examined by Student's *t*-test. Differences between non-normally distributed variables were examined by Mann–Whitney *U*-test. Categorized indices were compared with Pearson's χ^2-test and Fisher's exact test. Data are expressed as frequency (percentage), mean ± SD and median (interquartile range). All *P*-values are two tailed and less than 0.05 was considered to be statistically significant.

RESULTS

TWENTY-FIVE OF THE 149 patients (16.8%) fulfilled the diagnostic criteria for RLS (Table 2). This result showed a much higher frequency than the prevalence of RLS in the general population of Japanese adults.[7,9] As shown in Table 2, there were no differences between patients with and without RLS in terms of age, sex and the presence of cirrhosis, overt hepatic encephalopathy (HE), Child–Pugh classification, hemoglobin, creatinine, ferritin, ammonia and BTR. There were also no differences in the morbidity rate of diabetes, collagen disease and Parkinson's disease between the two groups. The frequency of hepatitis C virus (HCV) infection and the interferon treatment (assumed to be a cause of RLS)[32] were also not significantly different between the two groups. The prevalence rates of RLS in chronic hepatitis and LC were 17.2% (16/93) and 17.4% (8/46), respectively.

Table 3 shows the results of the PSQI. In the global score, the mean of all patients was 6.31. A total of 48% (43/89) of cases had a sleep disturbance (poor sleeper; global PSQI score, >5). RLS was present in 48% of the poor sleepers (18 cases of RLS, 43 cases of poor sleeper). In the component of subjective sleep quality and sleep latency, the number of patients with abnormal subscore (≥2) was significantly higher in the RLS group. The median global PSQI score of the RLS group was significantly higher than the non-RLS group (median [interquartile range]: RLS group, 9 (5.75–11.25); non-RLS group, 5 [3.0–8.0]; $P < 0.01$]. Additionally, the number of poor sleepers (global PSQI score) of the RLS group was significantly higher than the non-RLS group ($P < 0.05$).

Next, the SF-36 was used to examine whether RLS influenced the patient quality of life. In the RLS group, there was a significantly lower physical functioning score, higher body pain, and lower vitality and mental health. There was no significant differences in the physi-

Table 2 Demographics and clinical characteristics of the study population

	RLS ($n = 25$)	Non-RLS ($n = 124$)
Age	59.6 (12.5)	62.5 (11.3)
Male : female	15:10	55:69
Cirrhosis	8 (32.0%)	38 (30.6%)
HCV-related	13 (52.0%)	58 (46.8%)
IFN therapy	6 (24.0%)	26 (21.0%)
NASH	2 (8.0%)	11 (8.9%)
AIH	1 (4.0%)	10 (8.1%)
Overt HE	2 (8.0%)	7 (5.6%)
Diabetes	7 (28.0%)	27 (21.8%)
Collagen disease	0 (0.0%)	2 (1.6%)
Parkinson's disease	1 (4.0%)	2 (1.6%)
BMI (kg/m^2)	22.8 (3.6)	23.6 (4.2)
Child–Pugh classification (A/B/C)	2/3/20	3/17/104
Hemoglobin (g/dL)	12.1 (2.5)	12.8 (2.0)
Creatinine (mg/dL)	0.76 (0.18)	0.80 (0.27)
Ferritin (ng/mL)†	141 (64–267)	121 (40–230)
NH_3 (μg/dL)†	36 (34–65)	39 (21–84.5)
BTR	4.38 (2.02)	4.83 (1.71)

No data were significantly different between RLS and non-RLS groups. Student's *t*-test for normally distributed variables, expressed as mean (standard deviation).
†Mann–Whitney *U*-test for non-normally distributed variables, expressed as median (25–75 percentile). Pearson's χ^2-test or Fisher's exact test for categorical variables.
AIH, autoimmune hepatitis; BMI, body mass index; BTR, the molar ratio of total branched-chain amino acids to tyrosine; HCV, hepatitis C virus; HE, hepatic encephalopathy; IFN, interferon; NASH, non-alcoholic steatohepatitis; RLS, restless legs syndrome.

Table 3 Results of the questionnaire for sleep quality (PSQI)

	RLS ($n = 25$)	Non-RLS ($n = 64$)	*P*-value
Subjective sleep quality (≥2)	15 (60.0%)	17 (26.5%)	<0.01
Sleep latency (≥2)	16 (64.0%)	16 (25.0%)	<0.01
Sleep duration (≥2)	8 (32.0%)	11 (17.2%)	NS
Habitual sleep efficiency (≥2)	5 (20.0%)	8 (32.0%)	NS
Sleep disturbance (≥2)	6 (24.0%)	9 (14.1%)	NS
Use of sleep medication (≥2)	10 (40.0%)	15 (23.4%)	NS
Daytime dysfunction (≥2)	7 (28.0%)	6 (9.4%)	NS
Global PSQI Score (≥6)	18 (72.0%)	25 (39.1%)	<0.01

NS, not significant; PSQI, Pittsburgh Sleep Quality Index; RLS, restless legs syndrome.

cal component summary, but the mental component summary of the RLS group was 43.8 ± 10.8, which was significantly lower than the 49.8 ± 10.5 of the non-RLS group ($P = 0.026$) (Fig. 1).

DISCUSSION

IN THIS STUDY, we determined the prevalence of RLS in Japanese patients with CLD, and reported that the prevalence of RLS was 16.8%, which was more frequent than that of the general Japanese population.[7,9] This is the first report that clarifies the prevalence of RLS in Japanese CLD patients. In addition, it clarifies that RLS worsens quality of sleep and life in CLD patients.

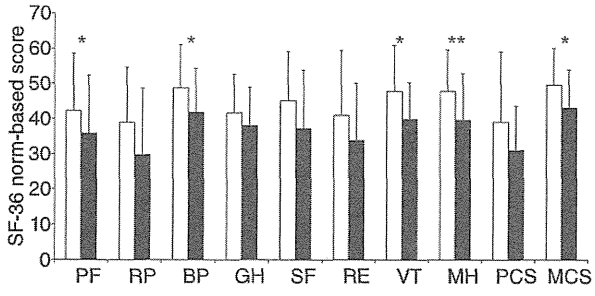

Figure 1 SF-36 scores of the patients with and without restless leg syndrome (RLS). Scores are calculated by norm-based algorithms. The white box and black box represent the mean value of the non-RLS groups and RLS, respectively. The bars represent the standard deviation. $*P < 0.05$ and $**P < 0.01$ indicate the significant differences between the RLS groups and non-RLS. BP, bodily pain; GH, general health perception; MCS, mental component summary scores; MH, role limitations due to mental health; PCS, physical component summary scores; PF, physical functioning; RE, role limitations due to poor emotional health; RP, role limitations due to poor physical health; SF, social functioning; VT, vitality.

It was previously reported in the USA that 88 of 141 CLD patients (62%) were judged by a questionnaire to have RLS.[23] However, it has been reported that racial differences exist in not only idiopathic RLS, but also secondary RLS.[16,33,34] In this study, the RLS complication rate of 16.8% was considerably lower compared with the previous Western population report. However, this lower level is still higher than the general level in Japan, and given the racial differences between Japan and the USA, does not contradict the findings of the previous study.[7–9]

Cordoba *et al.* reported that sleep disturbance was seen in 47.7% of cirrhotic patients.[24] Sleep disturbance is a major complication in CLD patients. Although the cause of sleep disturbance in CLD patients is still unclear, RLS should be considered as one of the causes of sleep disturbance in these patients. In this study, RLS was diagnosed in 48% of the patients in the poor sleeper group. However, it is known that there are many cases of obstructive sleep apnea syndrome in CLD patients.[35] Moreover, hepatic encephalopathy causes sleep disturbance and daytime drowsiness,[24] thus RLS alone may not explain the poor sleep experienced by CLD patients. Nevertheless, in our study, the rate of RLS in Japanese CLD patients is still several-fold more than the general population, and we observed some patients with RLS with highly decreased sleep efficiency because of over 100 PLM/h, extended sleep latency and arousal during sleep (data not shown). Therefore, it will be necessary to examine by PSG whether RLS contributes to the insomnia of CLD patients.

Restless legs syndrome has been reported to be associated with impaired dopaminergic neurotransmission,[36] and the utility of dopamine agonists has been established.[37,38] It was also reported that the existence of impaired dopaminergic neurotransmission in CLD patients might cause Parkinsonism.[39,40] These studies

suggested that the cause of RLS in CLD is associated with impaired dopaminergic neurotransmission. However, we did not find any conclusive link between liver function, such as NH3 levels, and the frequency of RLS. In fact, some reports are also saying that there is no clear relationship between the severity of insomnia and liver disease.[41] Therefore, sleeplessness and RLS might be present from the early stages of liver disease.

We showed there were no significant differences in the morbidity rate of some diseases that could cause secondary RLS, such as anemia, HCV infection, diabetes, collagen disease and Parkinson's disease. However, it cannot be assured that all causal factors for secondary RLS were excluded, because so many conditions have been reported as causes of secondary RLS. However, in the present study, it was clear that RLS cases were highly complicated with Japanese CLD patients. It should be necessary to pay attention to the possible existence of RLS in CLD patients because many successful therapeutic agents for RLS have recently been reported, such as pramipexole, ropinirole, rotigotine (dopamine D2 agonist), gabapentin (voltage-dependent calcium channel $\alpha 2\delta$ subunit ligand).[38,42,43] In addition, these patients might benefit from such treatments. Future studies will be needed to clarify the mechanisms of the apparent association between RLS and liver disease.

In conclusion, the prevalence of RLS in Japanese CLD patients is much more frequent than that of the general population. In addition, RLS increases the incidence of poor sleep and decreases the quality of the life in CLD patients. This is the first report that clarifies the prevalence of RLS in Japanese CLD patients.

REFERENCES

1 Allen RP, Picchietti D, Hening WA, Trenkwalder C, Walters AS, Montplaisi J. Restless legs syndrome: diagnostic criteria, special considerations, and epidemiology. A report from the restless legs syndrome diagnosis and epidemiology workshop at the National Institutes of Health. *Sleep Med* 2003; 4: 101–19.

2 Berger K, Luedemann J, Trenkwalder C, John U, Kessler C. Sex and the risk of restless legs syndrome in the general population. *Arch Intern Med* 2004; **164**: 196–202.

3 Tison F, Crochard A, Leger D, Bouee S, Lainey E, El Hasnaoui A. Epidemiology of restless legs syndrome in French adults: a nationwide survey: the INSTANT Study. *Neurology* 2005; **65**: 239–46.

4 Lee HB, Hening WA, Allen RP, Earley CJ, Eaton WW, Lyketsos CG. Race and restless legs syndrome symptoms in an adult community sample in east Baltimore. *Sleep Med* 2006; 7: 642–5.

5 Winkelman JW, Finn L, Young T. Prevalence and correlates of restless legs syndrome symptoms in the Wisconsin Sleep Cohort. *Sleep Med* 2006; 7: 545–52.

6 Bjorvatn B, Leissner L, Ulfberg J *et al.* Prevalence, severity and risk factors of restless legs syndrome in the general adult population in two Scandinavian countries. *Sleep Med* 2005; 6: 307–12.

7 Nomura T, Inoue Y, Kusumi M, Uemura Y, Nakashima K. Prevalence of restless legs syndrome in a rural community in Japan. *Mov Disord* 2008; 23: 2363–9.

8 Tan EK, Seah A, See SJ, Lim E, Wong MC, Koh KK. Restless legs syndrome in an Asian population: a study in Singapore. *Mov Disord* 2001; **16**: 577–9.

9 Tsuboi Y, Imamura A, Sugimura M, Nakano S, Shirakawa S, Yamada T. Prevalence of restless legs syndrome in a Japanese elderly population. *Parkinsonism Relat Disord* 2009; **15**: 598–601.

10 Winkelman JW, Redline S, Baldwin CM, Resnick HE, Newman AB, Gottlieb DJ. Polysomnographic and health-related quality of life correlates of restless legs syndrome in the Sleep Heart Health Study. *Sleep* 2009; 32: 772–8.

11 Hornyak M, Feige B, Voderholzer U, Philipsen A, Riemann D. Polysomnography findings in patients with restless legs syndrome and in healthy controls: a comparative observational study. *Sleep* 2007; 30: 861–5.

12 Parrino L, Boselli M, Buccino GP, Spaggiari MC, Di Giovanni G, Terzano MG. The cyclic alternating pattern plays a gate-control on periodic limb movements during non-rapid eye movement sleep. *J Clin Neurophysiol* 1996; **13**: 314–23.

13 Karadeniz D, Ondze B, Besset A, Billiard M. EEG arousals and awakenings in relation with periodic leg movements during sleep. *J Sleep Res* 2000; 9: 273–7.

14 Sforza E, Juony C, Ibanez V. Time-dependent variation in cerebral and autonomic activity during periodic leg movements in sleep: implications for arousal mechanisms. *Clin Neurophysiol* 2002; **113**: 883–91.

15 Guggisberg AG, Hess CW, Mathis J. The significance of the sympathetic nervous system in the pathophysiology of periodic leg movements in sleep. *Sleep* 2007; **30**: 755–66.

16 Kutner NG, Bliwise DL. Restless legs complaint in African-American and Caucasian hemodialysis patients. *Sleep Med* 2002; 3: 497–500.

17 Gigli GL, Adorati M, Dolso P *et al.* Restless legs syndrome in end-stage renal disease. *Sleep Med* 2004; 5: 309–15.

18 Bhowmik D, Bhatia M, Gupta S, Agarwal SK, Tiwari SC, Dash SC. Restless legs syndrome in hemodialysis patients in India: a case controlled study. *Sleep Med* 2003; 4: 143–6.

19 Takaki J, Nishi T, Nangaku M *et al.* Clinical and psychological aspects of restless legs syndrome in uremic patients on hemodialysis. *Am J Kidney Dis* 2003; **41**: 833–9.

20 Taylor-Gjevre RM, Gjevre JA, Skomro R, Nair B. Restless legs syndrome in a rheumatoid arthritis patient cohort. *J Clin Rheumatol* 2009; **15**: 12–15.

21 Merlino G, Fratticci L, Valente M *et al.* Association of restless legs syndrome in type 2 diabetes: a case-control study. *Sleep* 2007; 30: 866–71.
22 Weinstock LB, Bosworth BP, Scherl EJ *et al.* Crohn's disease is associated with restless legs syndrome. *Inflamm Bowel Dis* 2010; 16: 275–9.
23 Franco RA, Ashwathnarayan R, Deshpandee A *et al.* The high prevalence of restless legs syndrome symptoms in liver disease in an academic-based hepatology practice. *J Clin Sleep Med* 2008; 4: 45–9.
24 Cordoba J, Cabrera J, Lataif L, Penev P, Zee P, Blei AT. High prevalence of sleep disturbance in cirrhosis. *Hepatology* 1998; 27: 339–45.
25 Cho SJ, Hong JP, Hahm BJ *et al.* Restless legs syndrome in a community sample of Korean adults: prevalence, impact on quality of life, and association with DSM-IV psychiatric disorders. *Sleep* 2009; 32: 1069–76.
26 Pugh RN, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg* 1973; 60: 646–9.
27 DeMaeyer EM, Dallman P, Gurney JM, Hallberg L, Sood S, Srikantia S. *Preventing and Controlling Iron Deficiency Anaemia through Primary Health Care: A Guide for Health Administrators and Programme Managers; Preventing and Controlling Iron Deficiency Anaemia through Primary Health Care: A Guide for Health Administrators and Programme Managers.* Geneva: World Health Organization, 1989.
28 Doi Y, Minowa M, Uchiyama M *et al.* Psychometric assessment of subjective sleep quality using the Japanese version of the Pittsburgh Sleep Quality Index (PSQI-J) in psychiatric disordered and control subjects. *Psychiatry Res* 2000; 97: 165–72.
29 Buysse DJ, Reynolds CF, 3rd, Monk TH, Berman SR, Kupfer DJ. The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. *Psychiatry Res* 1989; 28: 193–213.
30 Fukuhara S, Ware JE, Jr, Kosinski M, Wada S, Gandek B. Psychometric and clinical tests of validity of the Japanese SF-36 Health Survey. *J Clin Epidemiol* 1998; 51: 1045–53.
31 McHorney CA, Ware JE, Jr, Raczek AE. The MOS 36-Item Short-Form Health Survey (SF-36): II. Psychometric and clinical tests of validity in measuring physical and mental health constructs. *Med Care* 1993; 31: 247–63.
32 LaRochelle JS, Karp BI. Restless legs syndrome due to interferon-alpha. *Mov Disord* 2004; 19: 730–1.
33 Bhowmik D, Bhatia M, Tiwari S *et al.* Low prevalence of restless legs syndrome in patients with advanced chronic renal failure in the Indian population: a case controlled study. *Ren Fail* 2004; 26: 69–72.
34 Goffredo Filho GS, Gorini CC, Purysko AS, Silva HC, Elias IE. Restless legs syndrome in patients on chronic hemodialysis in a Brazilian city: frequency, biochemical findings and comorbidities. *Arq Neuropsiquiatr* 2003; 61: 723–7.
35 Crespo J, Cifrian J, Pinto JA, Jimenez-Gomez A, Pons-Romero F. Sleep apnea obstructive syndrome: a new complication previously undescribed in cirrhotic patients with ascites. *Am J Gastroenterol* 2003; 98: 2815–16.
36 Cervenka S, Palhagen SE, Comley RA *et al.* Support for dopaminergic hypoactivity in restless legs syndrome: a PET study on D2-receptor binding. *Brain* 2006; 129: 2017–28.
37 Partinen M, Hirvonen K, Jama L *et al.* Efficacy and safety of pramipexole in idiopathic restless legs syndrome: a polysomnographic dose-finding study–the PRELUDE study. *Sleep Med* 2006; 7: 407–17.
38 Trenkwalder C, Benes H, Poewe W *et al.* Efficacy of rotigotine for treatment of moderate-to-severe restless legs syndrome: a randomised, double-blind, placebo-controlled trial. *Lancet Neurol* 2008; 7: 595–604.
39 Burkhard PR, Delavelle J, Du Pasquier R, Spahr L. Chronic parkinsonism associated with cirrhosis: a distinct subset of acquired hepatocerebral degeneration. *Arch Neurol* 2003; 60: 521–8.
40 Kim JM, Kim JS, Jeong SH *et al.* Dopaminergic neuronal integrity in parkinsonism associated with liver cirrhosis. *Neurotoxicology* 2010; 31: 351–5.
41 Mostacci B, Ferlisi M, Baldi Antognini A *et al.* Sleep disturbance and daytime sleepiness in patients with cirrhosis: a case control study. *Neurol Sci* 2008; 29: 237–40.
42 Thorpe AJ, Clair A, Hochman S, Clemens S. Possible sites of therapeutic action in restless legs syndrome: focus on dopamine and alpha2delta ligands. *Eur Neurol* 2011; 66: 18–29.
43 Jacques Montplaisir RPA, Walters A, Ferini-Strambi L. Restless Legs Syndrome and Periodic Limb Movements during Sleep. *Principles and Practice of Sleep Medicine.* 5 edn 2010; 1026–37.

ARTICLE
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Branched-Chain Amino Acid Deficiency Stabilizes Insulin-Induced Vascular Endothelial Growth Factor mRNA in Hepatocellular Carcinoma Cells

Satoshi Miuma,[1*] Tatsuki Ichikawa,[1] Kazuhiko Arima,[2] Shigeyuki Takeshita,[1] Toru Muraoka,[1] Toshihisa Matsuzaki,[1] Masashi Ootani,[1] Hidetaka Shibata,[1] Motohisa Akiyama,[1] Eisuke Ozawa,[1] Hisamitsu Miyaaki,[1] Naota Taura,[1] Fuminao Takeshima,[1] and Kazuhiko Nakao[1]

[1]*Department of Gastroenterology and Hepatology, Nagasaki University, Sakamoto 1-7-1, Nagasaki 852-8501, Japan*
[2]*Medical Gene Technology Graduate School of Biomedical Sciences, Nagasaki University, Sakamoto 1-7-1, Nagasaki 852-8501, Japan*

ABSTRACT

Abnormal sugar metabolism is closely related to chronic liver diseases, including hepatocellular carcinoma (HCC). We previously reported that fasting hyperinsulinemia is a poor prognostic factor for HCC patients. A recent large-scale study has shown that long-term administration of branched chain amino acids (BCAA) reduces the risk of HCC development in obese cirrhotic patients who have been diagnosed with diabetes mellitus, although the mechanism by which it does so is unclear. In this study, we analyzed the expression of vascular endothelial growth factor (VEGF) in HepG2 cells under high-insulin culture conditions, and examined the effect of BCAA on VEGF expression. VEGF secretion was significantly increased by 200 nM of insulin under BCAA deficient conditions, but it was repressed by the addition of BCAA. BCAA activated the mTOR pathway and increase HIF-1α expression under high-insulin culture conditions, however quantitative PCR analysis showed that insulin-induced expression of VEGF mRNAs (VEGF121 and VEGF165) decreased 2 h after the addition of BCAA. The half-lives of both VEGF121 and 165 mRNAs were shortened in the presence of BCAA compared to the absence of BCAA. Therefore it is thought that BCAA regulate VEGF expression mainly at the post-transcriptional level. We also examined which of the Valine, Leucine, and Isoleucine components of BCAA were essential for VEGF mRNA degradation. All three BCAA components were required for acceleration of insulin-induced VEGF mRNA degradation. These results suggest that administration of BCAA may downregulate VEGF expression in patients who have hyperinsulinemia and are in the process of developing HCC. J. Cell. Biochem. 113: 3113–3121, 2012.

KEY WORDS: BCAA; HCC; VEGF; HYPERINSULINEMIA; DECAY; STABILITY

Hepatocellular carcinoma (HCC) is the fifth most frequent malignant neoplasm in the world [Bosch et al., 2004]. The rising incidence of HCC has been extensively reported in the United States [El-Serag and Mason, 2000], Japan, and several other countries [Yu and Yuan, 2004]. In recent years, much interest has centered on the relationship between abnormal sugar metabolism and liver disease including HCC, because of its association with non-alcoholic fatty liver disease (NAFLD) including its severe form, non-alcoholic steatohepatitis (NASH) [Marchesini et al., 1999]. We have reported that the development of liver fibrosis is closely associated

Abbreviations: HCC, hepatocellular carcinoma; BCAA, branched chain amino acids; VEGF, vascular endothelial growth factor; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; HIF, hypoxia inducible factor; PI-3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; FBS, fetal bovine serum; ELISA, enzyme linked immunosorbent assay; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; mTOR, mammalian target of rapamycin; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; aa, amino acid.

Additional supporting information may be found in the online version of this article.

*Correspondence to: Satoshi Miuma, MD, Department of Gastroenterology and Hepatology Graduate School of Biomedical Sciences, Nagasaki University, Sakamoto 1-7-1, Nagasaki 852-8501, Japan.
E-mail: miuma1002@gmail.com

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with insulin resistance in HCV infected patients [Taura et al., 2006]. The combined data indicate that it is likely that insulin resistance in chronic liver disease triggers hyperinsulinemia and may modulate the biological characteristics of HCC cells. Indeed, Komura et al. [2007] have reported that insulin therapy for coexisting diabetes mellitus is an independent risk factor for HCC recurrence after a curative resection. To examine the relevance of hyperinsulinemia to the progression of HCC, we retrospectively studied a total of 140 patients, who were newly diagnosed with HCC at the Department of Gastroenterology and Hepatology in Nagasaki University Hospital [Miuma et al., 2009]. In that study, we reported that fasting hyperinsulinemia is a risk factor that is associated with a poor prognosis at the early stage of HCC and with a high-recurrence rate at the curative stage of HCC. Hyperinsulinemia alone is also thought to be a risk and a poor prognosis factor for patients with HCC.

A recent large-scale study has reported that administration of branched chain amino acids (BCAA) improves glucose intolerance and hyperinsulinemia in cirrhotic patients [Muto et al., 2005]. It has also been reported that in obese cirrhotic patients, who have been diagnosed with diabetes mellitus, the risk of developing HCC is significantly reduced following long-term administration of BCAA [Muto et al., 2006]. This report is very interesting, because it shows that a close association exists between insulin resistance due to hyperinsulinemia and BCAA, and that this association contributes to the progression of HCC in cirrhotic patients. There have been only a few reports to date regarding the suppression of liver cancer progression by BCAA. Murata and Moriyama [2007] showed that isoleucine prevents tumor growth in a mouse liver metastatic model of colon cancer through inhibition of vascular endothelial growth factor (VEGF). Yoshiji et al. [2010] reported that BCAA exerts a chemopreventive effect against HCC, which is associated with the suppression of VEGF expression and hepatic neovascularization in obese diabetic rats. Both of these reports suggest an anti-angiogenesis activity of BCAA or Isoleucine through suppression of VEGF expression. However, the mechanism by which BCAA administration suppresses VEGF expression remains unclear.

Angiogenesis is a necessary event for tumor growth and metastasis [Folkman et al., 1989; Weidner et al., 1991]. VEGF is one of the most potent of the angiogenic factors that have been identified [Ahmed et al., 2004; Underiner et al., 2004]. Furthermore previous studies [Ng et al., 2001; Poon et al., 2001] have reported that VEGF is a potent angiogenic factor leading to HCC invasiveness and metastasis. One trigger of VEGF expression is hypoxia caused by an imbalance in oxygen supply and consumption [Knighton et al., 1983; Shweiki et al., 1992]. Hypoxia-induced upregulation of VEGF is considered to be mediated primarily through hypoxia inducible factor (HIF), which is a heterodimeric basic helix–loop–helix transcription factor composed of two subunits, HIF-1α and HIF-1β [Wang et al., 1995; Sharp and Bernaudin, 2004]. Under hypoxic conditions, HIF-1α binds to HIF-1β and forms the HIF complex, which recognizes a consensus hypoxia response element in the VEGF promoter as well as in the promoters of a broad range of other HIF target genes [Hirota and Semenza, 2005]. Some reports suggest that the HIF-1 system is also induced by growth factors such as insulin under non-hypoxic conditions [Jiang et al., 2001; Laughner et al., 2001; Stiehl et al., 2002]. It has been reported that insulin induction of VEGF through HIF-1α mainly occurs through activation of the PI-3K pathway. However, VEGF induction by HIF-1α in HCC cells also involves a MAPK pathway and ROS production [Fukuda et al., 2002; Biswas et al., 2007].

Our aim was to determine the impact of BCAA on the development or progression of HCC in patients with hyperinsulinemia from the aspect of angiogenesis. Therefore, in the present study, we examined the effect of BCAA on VEGF expression in HCC cells cultured under high-insulin conditions.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

Dulbecco's essential medium, fetal bovine serum (FBS) and a solution of human Insulin were obtained from Sigma Chemical Co. (St. Louis, MO). Each of the BCAA components; Valine, Leucine, and Isoleucine, as well as BCAA-free medium, which contained all amino acids except for the BCAA components, were obtained from Ajinomoto Pharmaceuticals Co. (Tokyo, Japan; Table I). Actinomycin D was obtained from Nacalai Tesque Co. (Kyoto, Japan). Human HCC cell lines, HepG2, Huh1, and Huh7 were obtained from the American Type Tissue Culture Collection (ATCC). They were maintained in Dulbecco's essential medium with low glucose containing 10% FBS, 100 mg/ml penicillin G, and 50 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO2.

QUANTIFICATION OF SECRETED VEGF PROTEIN BY ELISA

An equal number of HepG2, Huh-7, and Huh1 (5×10^4 cells per well) were plated in 96-well plates in DMEM containing 10% FBS. After the cells reached 70–80% confluency, the growth medium

TABLE I. Amino Acid Composition of BCAA Free Medium

Amino acid	Concentration (mM)	Amino acid	Concentration (mM)
Glycine	0.4	L-Asparagine	0.4
L-Alanine	0.4	L-Glutamic Acid	0.4
L-Serine	0.4	L-Aspartic Acid	0.4
L-Threonine	0.8	L-Valine	0
L-Cystine 2HCl	0.2	L-Leucine	0
L-Methionine	0.2	L-Isoleucine	0
L-Glutamine	4.0	L-Phenylalanine	0.4
L-Arginine-HCl	0.4	L-Tyrosine	0.4
L-Proline	0.4	L-Tryptophan	0.08
L-Lysine-HCl	0.8	L-HistidineHCl-H20	0.2

was removed and was replaced with fresh BCAA-free medium without FBS. The cells were then incubated for 20 h, following which the medium was replaced with media with or without 200 nM insulin for 48 h. If BCAA was added, it was added at the concentrations indicated in the text, 30 min before insulin treatment. The medium was then harvested, filtered, and used for measurement of secreted VEGF. VEGF present in the medium was measured using the Quantikine Human VEGF ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. In brief, the cells were incubated in a 96-well plate as describe above, and 20 μl of CellTiter96® AQ_{ueous} One Solution Reagent (Promega, Madison, WI,) were added to each well. Following incubation for 2 h at 37°C, the color reaction was recorded at 490 nm using an automated plate reader (Bio-Rad, Melville, NY).

WESTERN BLOTTING

HepG2 cells were seeded in a 60 mm-diameter dish and grown to 80% confluence. The culture medium was then changed to BCAA-free medium without FBS and the cells were grown for a further 20 h. The cells were then treated with or without insulin for various times. If the components of BCAA were added, they were added at the concentrations indicated in the text 30 min before insulin treatment. The cells were then lysed by the addition of lysis buffer for 10 min at 4°C, and insoluble material was removed by centrifugation at 14,000 rpm for 30 min at 4°C. The same amount of protein from each lysate (each with 30 μg total protein, 20 μl/well) was subjected to 15% SDS-polyacrylamide gel electrophoresis. The proteins were transferred onto nitrocellulose membranes, which were then blocked for 1 h using 5% non-fat dried milk in PBS containing 0.1% Tween-20 (PBS-T). The membranes were washed with PBS-T and incubated at 4°C overnight in the presence of individual primary antibodies. The membranes were washed with PBS-T and incubated with sheep anti-mouse IgG or donkey anti-rabbit IgG coupled with horseradish peroxidase (Amersham Biosciences, Piscataway, NJ) for 1 h. The enhanced chemiluminescence system (SuperSignal West Pico Chemiluminescent Substrate; Pierce Chemicals, Rockford, IL) was used for signal detection.

Rabbit polyclonal anti-human phospho-mTOR, rabbit polyclonal anti-human phospho-p70S6K, rabbit monoclonal anti-human phospho-eIF4EBP1, rabbit polyclonal anti-human HIF-1α, and rabbit polyclonal anti-human β-Actin were obtained from Cell signaling Technology (San Diego, CA). All western blotting was performed at least in duplicate.

QUANTITATIVE PCR

The mRNAs of VEGF variants were quantified using quantitative polymerase chain reaction (qPCR). Total RNA was isolated from cell lines using the GenElute (TM) Mammalian Total RNA Miniprep Kit (Sigma Chemical Co.) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of RNA using the QuantiTect Reverse Transcription kit (QIAGEN, Valencia, CA), and random hexamers. The cDNA was stored at −20°C until further analysis. Quantification of messenger RNA (mRNA) was performed using TaqMan or SYBR Green real-time PCR and the LightCycler (TM) 2.0 for Real-Time PCR system (Roche Applied Science, Indianapolis, IN). TaqMan qPCR of each VEGF isoform, without cross-reaction, was performed using the QuantiTect Probe PCR kit (QIAGEN). A common forward primer 5′-ATCTTCAAGCCATCCTGTGTGC-3′ and fluorescent hybridization probe 5′-AGTGTGTGCCCACTGAGGAGTCC-3′, both based on exon 3 sequences, were used. Each splice variant was amplified using specific reverse primers that spanned the variant specific exon boundaries: exon 5/8 for VEGF121 (5′-TGCGCTTGTCACATTTTTCTTG-3′), exon 5/7 for VEGF165 (5′-CAAGGCCCACAGGGATTTTC-3′), and exon 6/7 for VEGF189 (5′-CACAGGGAACGCTCCAGGAC-3′).

SYBR Green real-time PCR was performed for human GAPDH (forward primer 5′-TGAAGGTCGGAGTCAACGGATTTGGTCGTA-3′, reverse primer 5′-ATCTCGCTCCTGGAAGATGGTGATGGGATT-3′. To confirm specific amplification by the SYBR Green PCR, a dissociation curve analysis was performed for each primer pair, and both non-RT negative controls and water controls were used for these analyses. The amounts of loaded cDNA were normalized using GAPDH as an endogenous control. Differential gene expression was calculated by evaluation of the threshold cycle (Ct), and relative quantification was calculated using the comparative Ct method. All experiments were performed at least in duplicate.

RNA KINETICS

After HepG2 cells were treated with actinomycin-D (5 μg/ml) they were then incubated with or without 0.8 mM BCAA and 200 nM insulin. VEGF121 and VEGF165 mRNA were prepared at 0, 1, 2, 4, 6, and 8 h after actinomycin-D treatment and their mRNA levels were quantified using qPCR. The RNA quantities are expressed as a percentage of the mRNA level at the time point when actinomycin-D was added (0 h), and are referred to as the "percentage of RNA remaining." Degradation curves were estimated for these mRNAs using GraphPad Software (San Diego, CA).

STATISTICAL ANALYSIS

Values are expressed as means ± SD. Multiple comparisons were done using one-way ANOVA. Intergroup comparisons were done using Bonferroni's correction for multiple comparisons. A level of $P < 0.05$ was considered statistically significant.

RESULTS

BCAA SUPPRESSES INSULIN-INDUCED VEGF EXPRESSION IN HCC CELL LINES

HepG2, Huh7, and Huh1 cells were exposed to 200 nM of insulin in BCAA-free medium with or without 0.8 mM of BCAA for 48 h. VEGF secreted from the cells was then measured using an ELISA. In BCAA-free medium, insulin increased VEGF secretion in all HCC cell lines 1.4- to 2.3-fold compared to VEGF secretion in the absence of insulin (Fig. 1A). When 0.8 mM of BCAA was added to the BCAA-free medium, insulin-induced VEGF secretion was significantly suppressed in the HepG2 cells, but was only slightly suppressed in the Huh7 and Huh1 cells (Fig. 1A). We next assayed the effect of exposure of HepG2 cells to various concentrations of BCAA, in the presence or absence of insulin for 48 h, on VEGF secretion and on

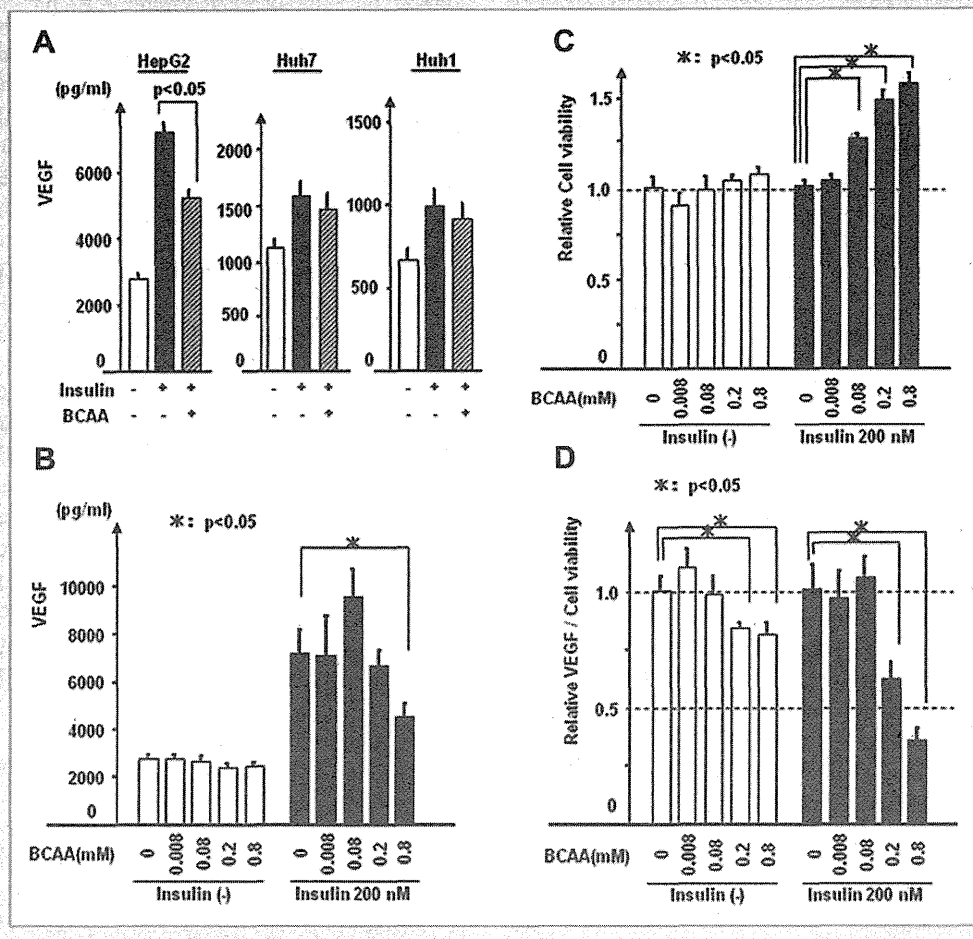

Fig. 1. Vascular endothelial growth factor (VEGF) secretion from HepG2, Huh-7, and Huh-1 cells after insulin treatment with or without BCAA. A: HepG2, Huh7, and Huh1 cells were exposed to BCAA-free medium with or without insulin (200 nM), or with insulin plus BCAA (0.8 mM) for 48 h, and VEGF secretion was analyzed by ELISA. Data represent the mean ± SD for four separate experiments. B,C: HepG2 cells were exposed to various BCAA concentrations with or without insulin for 48 h. VEGF secretion (B), and cell viability (C), were analyzed using an ELISA and an MTS assay, respectively. Data represent the mean ± SD for four separate experiments. D: The ratio of VEGF secretion to cell viability was then calculated. Data represent the ratio of VEGF secretion to cell viability over control value (the ratio in BCAA free medium), which was arbitrarily set to one with or without insulin, respectively, and represent the mean ± SD for four separate experiments.

cell viability. As the BCAA concentration was increased, insulin-induced VEGF secretion was significantly suppressed (Fig. 1B). In contrast, cell viability was significantly increased with increasing BCAA concentrations under high-insulin culture conditions (Fig. 1C). The ratio of VEGF secretion to cell viability was significantly decreased as the concentration of BCAA increased, either in the presence or absence of insulin. However, the decrease in this ratio was stronger in the presence of insulin (Fig. 1D).

BOTH BCAA AND INSULIN ACTIVATE THE mTOR PATHWAY AND INCREASE THE EXPRESSION OF HIF-1α

Previous reports showed that insulin-induced VEGF expression under non-hypoxic conditions involves activation of the PI-3K pathway, which is followed by activation of mTOR, P70S6K, and eIF4BP1 and results in the expression of HIF-1α [Fukuda et al., 2002; Stiehl et al., 2002]. It has also been shown that BCAA stimulates mTOR and activates signals that regulate protein translation and synthesis [Ijichi et al., 2003]. To determine the involvement of these signaling pathways in the above BCAA/insulin effects on HepG2 cells, we therefore examined the phosphorylation of PI-3K/m-TOR signaling proteins at 2 h, and the expression of HIF-1α at 6 h after BCAA and/or insulin treatment of HepG2 cells, using western blotting. As shown in Figure 2, the expression of phosphorylated mTOR, phosphorylated P70S6K, phosphorylated eIF4BP1, and HIF-1α was upregulated by BCAA or insulin treatment alone. Additionally, these changes were enhanced by combined treatment with both BCAA and insulin. These results indicate that BCAA and insulin synergistically activate intracellular signaling pathways that induce HIF-1α protein expression, although they had opposing effects on VEGF expression (Fig. 1).

A HIGH-INSULIN CONCENTRATION AUGMENTS, WHEREAS BCAA SUPPRESSES, THE EXPRESSION OF THREE MAJOR VARIANT VEGF mRNAs

We next analyzed the effect of BCAA and/or insulin treatment on the mRNA expression level of VEGF using qPCR. It is known that

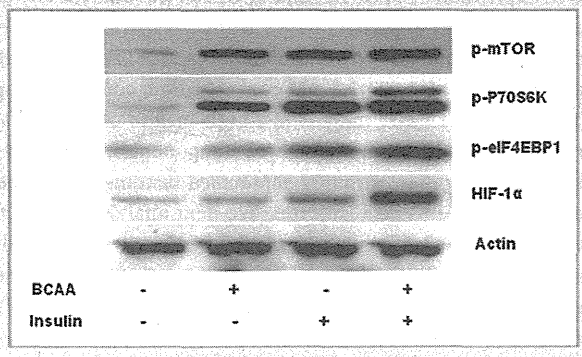

Fig. 2. Effects of BCAA and insulin on the expression of phosphorylated mTOR, phosphorylated p70S6K, phosphorylated eIF4EBP-1, and HIF-1α. HepG2 cells were exposed to BCAA-free medium with or without BCAA (0.8 mM) and/or insulin (200 nM). The expression of phosphorylated mTOR, phosphorylated p70S6K, and phosphorylated eIF4EBP-1 in cell lysates was analyzed after 2 h incubation by Western blotting using the appropriate antibodies. The expression of HIF-1α was similarly analyzed following 6 h incubation. Actin was blotted as a loading control.

there are many VEGF isoforms, that are derived from a single gene by alternative splicing. The three major isoforms of VEGF are VEGF121 (121 aa), VEGF165 (165 aa), and VEGF189 (189 aa) [Park et al., 1993; Neufeld et al., 1994; Takahashi and Shibuya, 2005]. It has been reported that the majority of HCC abundantly express VEGF121 and VEGF165, and that high-VEGF165 expression is related to poor prognosis of HCC patients [Jeng et al., 2004a]. Prior to analysis of VEGF mRNA expression in cells, we confirmed the reliability of the specific primer pairs and probes designed to assay VEGF121, VEGF165, and VEGF189 mRNA expression (Supplementary Figure). HepG2 cells were then incubated for various times with 200 nM of insulin in BCAA-free medium with or without 0.8 mM of BCAA, and the expression of VEGF mRNAs in the cells was then analyzed using qPCR. As shown in Figure 3, VEGF165 mRNA was the most abundant, and VEGF121 mRNA was the second-most abundant VEGF mRNA in HepG2 cells. VEGF189 mRNA was only weakly expressed. Insulin-induced expression of all three VEGF mRNAs decreased significantly 2h after BCAA treatment and remained low over the next 14 h. Since it takes 6 h to induce detectable levels of HIF-1α following BCAA treatment it is likely that the suppression of VEGF mRNA expression by BCAA is independent of the HIF-1 system.

BCAA DECREASE THE STABILITY OF INSULIN-INDUCED VEGF mRNA

The above data suggested that BCAA and insulin modulate intracellular signaling that regulates HIF-1α expression in a coordinated manner, but that BCAA antagonizes the effect of insulin on induction of the expression of VEGF mRNA. We therefore further analyzed the effects of BCAA and insulin on the post transcriptional regulation of VEGF mRNAs in these cells by determination of their effect on the half-life of VEGF mRNAs following inhibition of transcription using actinomycin-D. HepG2 cells were treated with actinomycin-D (5 μg/ml), and were incubated with insulin (200 nM) with or without BCAA (0.8 mM) for 0, 1, 2, 4, 6, and 8 h. The level of VEGF121 and VEGF165 mRNAs at these time points (termed "the remaining mRNA") was analyzed using q-PCR. Insulin treatment increased the stability of VEGF121 and VEGF165 mRNA in BCAA-free medium, respectively (Fig. 4). The addition of BCAA significantly decreased the stability of VEGF121 or VEGF165

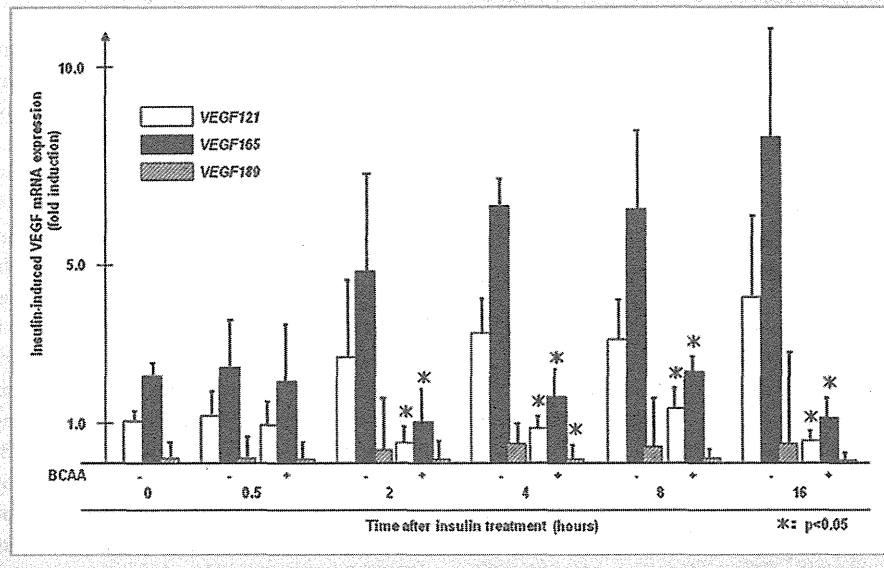

Fig. 3. Time course analysis of the mRNA expression of the three major isoforms of VEGF after insulin treatment with or without BCAA. HepG2 cells were exposed to insulin (200 nM) in BCAA free medium with or without BCAA (0.8 mM). The mRNA expression of VEGF121, VEGF165, and VEGF189 in the cells was then analyzed at the indicated times using quantitative PCR. Data represent fold induction of VEGF mRNA expression over control value (VEGF121 mRNA at 0 h), which was arbitrarily set to one, and represent the mean ± SD for four separate experiments. (*$P < 0.05$ for VEGF121, VEGF165, and VEGF189 mRNA level of BCAA(+)Insulin(+) group compared with BCAA(−)Insulin(+) group at each time).