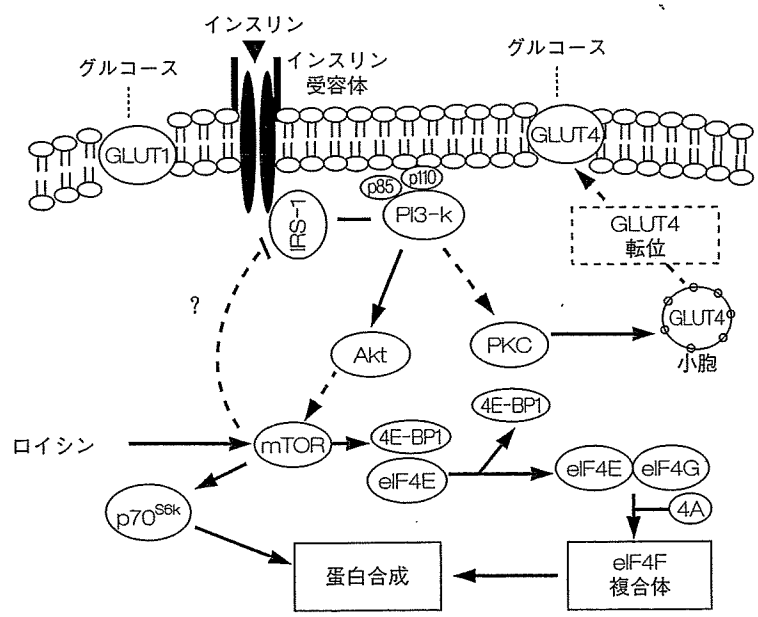


図❶ ロイシンの作用部位
(Layman DK *et al*, 2004[16]より引用)

NAFLDの治療の中心であることが確認されている．当科では行動療法としてのグラフ化体重日記を中心に治療をおこなっている[4]．しかし，体重の減量過程で挫折する患者も数多く，適切な減量については課題が残されている．減量の実行と継続の困難さに加え，減量に伴う代謝の変化がその一因とも考えられている[5][6]．減量を長期に維持するための戦略が必要である．

BCAAの生理活性

分岐鎖アミノ酸（branched chain amino acid：BCAA）の生理活性が注目されている．ロイシン，イソロイシン，バリンのBCAAは，肝硬変症における肝性脳症の改善や低アルブミン血症時のアルブミンの回復などの目的にすでに用いられている．最近では発癌を抑止するとの報告もみられる[7][8]．

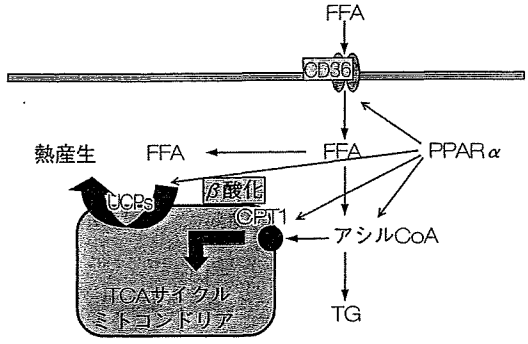
ロイシンは蛋白の基質としてのみならず，mammalian target of rapamycin（mTOR）を介して蛋白合成を促し，空腹時や飢餓時の筋肉の消耗を防ぐことが明らかになっている．筋肉ではインスリン/PI3キナーゼシグナリングのmodulators，プロテインキナーゼC(PKC)を介して糖の利用を調整することも明らかになった（図❶）[9]．

また，イソロイシンはロイシンと同様な生理作用とともに，mTORを介して血管内皮増殖因子（vascular endothelial growth factor：VEGF）を抑制し，転移性肝癌の増殖を抑制する作用も報告された[10]．バリンの生理活性は不明であったが，最近，肝硬変症で機能低下した樹状細胞の機能を回復させることが報告された[11]．

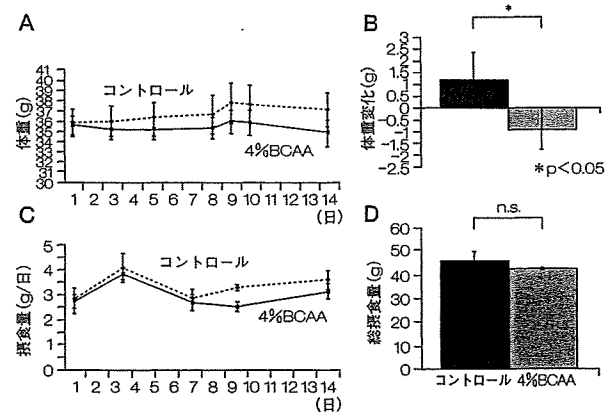
BCAAの生理活性はきわめて多様で，肝・脂肪組織における作用も報告されるようになった．BCAAの肝・骨格筋・脂肪組織での脂質代謝について最近の知見を述べる．

脂肪酸の代謝

NAFLDはメタボリックシンドロームの表現型である．病態として，栄養過剰，運動不足に伴う，内臓脂肪蓄積が背景にある．高脂肪食を用いたラットでは，栄養摂取に伴い，肝臓に脂肪が蓄積し，肝臓への脂肪蓄積から遅れて，血中のコレステロールやTGが上昇，インスリン抵抗性を惹起するとされている[12]．肝臓の脂肪蓄積がインスリン抵抗性発症にきわめて重要である．肝・骨格筋の余分な脂肪蓄積は臓器でのインスリン感受性の低下を伴い，高インスリン血症をもたらす．高インスリン血症は肝臓におけるinsulin receptor substrate-2（IRS-2）



図❷ 細胞内の脂肪酸代謝



図❸ 体重の推移と摂食量
A）体重の推移
B）体重変化（2週間の増減）
C，D）摂食量
摂食量は両群で変化ないが，BCAA投与群で体重の減少を認めた．

の発現・リン酸化の低下を招き，肝臓のインスリン抵抗性を悪化させる．また，肝・脂肪組織では脂肪合成の核内転写因子であるsterol regulatory element binding protein 1c（SREBP1c）の発現・活性化を促進することで脂肪蓄積を促す．

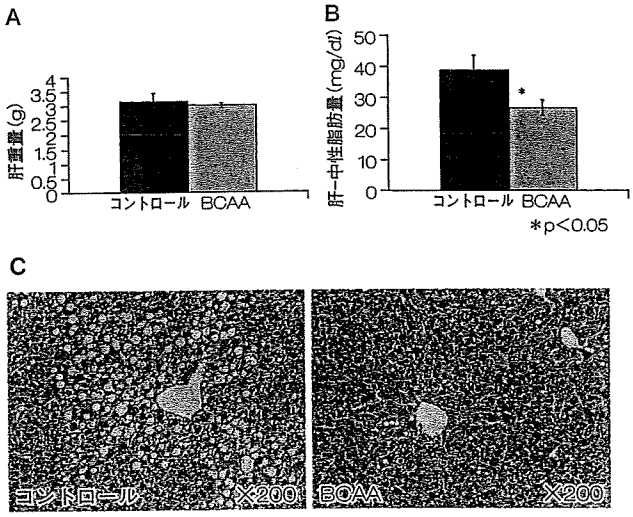
血中や脂肪組織のTGは，加水分解を受け，遊離脂肪酸（free fatty acid：FFA）となり，肝細胞に取り込まれる．また，糖質からFFAが供給される経路もある．脂肪酸はアシルCoAへと変換され，ミトコンドリア内でβ酸化を受けアセチルCoAへと変換される．アシルCoAは一部TGとなり，リン脂質やアポリポプロテインB（apoB）の修飾を受けVLDLとなり血中へと分泌される（図❷）．

CD36/FATは脂肪酸やVLDLの取り込みに関係する膜の糖蛋白で，肝臓ではperoxisome proliferator-activated receptor（PPAR）α，脂肪組織ではPPARγに制御されている[13]．Carnitin palmitoyltransferase 1（CPT1）はミトコンドリアのβ酸化のkey酵素でPPARαに制御されている．

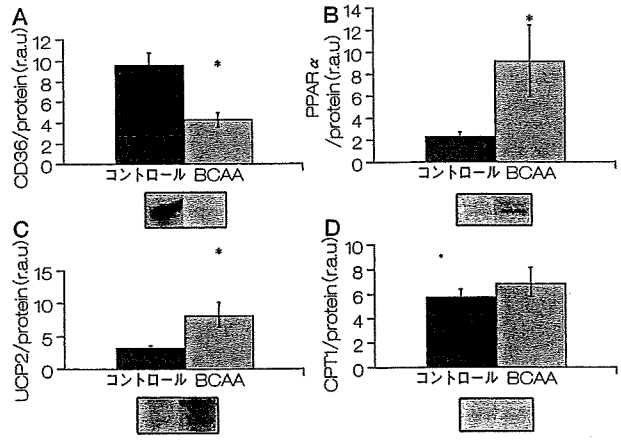
BCAAの肝・骨格筋・脂肪組織における脂質代謝への影響

ロイシンが食事誘導性の肥満糖尿病モデルマウスで，脂肪量を減らすのみならず，安静時のエネルギー消費を減少させ，さらに糖代謝やコレステロール代謝も改善することが報告された[14]．骨格筋・脂肪組織でのuncoupling protein（UCP）3の発現を亢進させ，エネルギー消費を促進する[14]．われわれもこの点に注目し同様の実験をおこなった．8週齢のC57BL/6Jを用い，45% high fat diet（HFD）を4週間摂食させたのち，4% BCAAを加え，2週間自由に摂食させた．BCAA添加群と非添加群において以下の項目で比較検討した．評価項目としては投与終了後の血清グルコース（blood sugar：BS），インスリン値（immunoreactive insulin：IRI），TG，FFA，脂肪酸代謝の取り込みの指標としてのCD36/FAT，ミトコンドリアにおけるβ酸化の評価として，CPT1および脱共役蛋白であるUCPs，脂肪酸代謝をつかさどるPPARαをウエスタンブロット法で評価した．その結果，両群で摂食量に有意差はなかった．体重はBCAA投与群で有意に低下していた（図❸）．肝・骨格筋・脂肪組織で脂肪の減少が誘導された（図❹）．肝組織中の脂肪は，病理組織学的な検討で肝細胞内の脂肪滴は明らかに改善し，組織含有TG量もBCAA投与群で明らかに減少していた．血清BS，TG，FFAは両群に有意な差はなかった．しかし，血清IRIはBCAA投与群で有意に減少していた．メタボリックシンドロームに関連するアディポネクチン[15]，レプチンは両群で有意な差はなかった．

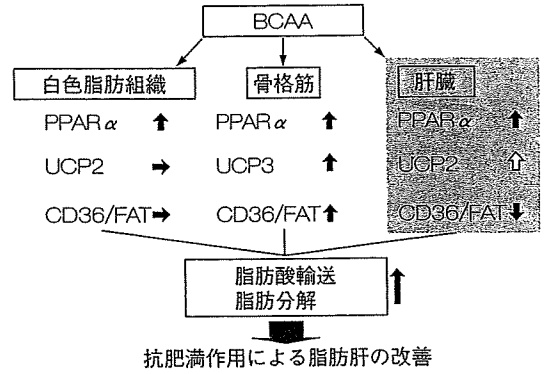
肝臓ではCD36/FATの発現が減少していた．CPT1に両群で有意な差はなかった．PPARα，UCP2は有意に発現が増加していた（図❺）．骨格筋ではBCAA投与群



図❹ BCAAによる肝脂肪化改善効果
A）肝重量
B）中性脂肪量
C）肝組織像（HE染色）
BCAA群では肝臓の中性脂肪蓄積が抑制された．



図❺ 肝臓における脂質代謝マーカー
r.a.u：relative arbitrary unit



図❻ BCAAの各臓器における脂肪酸代謝改善作用の機序

で，組織中のTG量は減少していた．また，CD36/FATはBCAA投与群で発現が増強しており，PPARα，UCP3の発現も同様に増加していた．CPT1に両群で有意な差はなかった．白色脂肪組織においてもTG量はBCAA投与群で減少していた．CD36/FATやUCP2では有意な差はなかったがPPARαはBCAA群で発現が増加していた．まとめると，BCAAは骨格筋や脂肪組織ではCD36/FATを介し脂肪酸の細胞内への取り込みを亢進させ，UCPを介した脂肪酸の燃焼をおこなうことが明らかにされた（図❻）．肝臓では脂肪酸の燃焼がPPARα-UCP2を介して亢進しており，各臓器における脂肪酸の動態は，NAFLDの戦略を考えるうえで有用である．

さらにBCAAのなかではイソロイシンに最も強い作用が確認された．

おわりに

NAFLDの治療戦略はこれまで，肝臓への脂肪酸の供給とβ酸化のバランスで考えられていた．NASHではさらに酸化ストレスの抑止としてさまざまな薬剤が検討されてきた．とくにBCAAの多彩な生理活性を追及することにより，肝・骨格筋・脂肪組織など統合した治療戦略へのきっかけになりうる．肝臓での変化のみならず，他臓器での代謝の変化もあわせ，治療戦略を練る必要がある．とくに，BCAAは食事療法における，高蛋白食・低炭水化物食による減量[16]や運動療法時の筋肉の回復に有用である．さらに本稿で述べた糖質・脂質代謝の薬理作用などからエネルギー効率の向上，熱産生を促し，蛋白の消失を防ぎ，糖代謝を改善するため，飽食の時代に適していると考えられる．最近のメタボリックシンドロームに対する，治療戦略すべてに，関連する特異な治療薬・サプリメントとして存在することとなる．実際の診療での減量の継続は困難であることも多く，食事療法，運動療法，薬物療法を支援する位置づけとしても考えてみたい．

文 献

1) Day CP, James OF：Steatohepatitis：a tale of two "hits"? *Gastroenterology* **114**：842-845, 1998
2) Dixon JB, Bhathal PS, Hughes NR *et al*：Nonalcoholic fatty liver disease：Improvement in liver histological analysis with weight loss. *Hepatology* **39**：1647-1654, 2004
3) Huang MA, Greenson JK, Chao C *et al*：One-year intense nutritional counseling results in histological improvement in patients with non-alcoholic steatohepatitis：a pilot study. *Am J Gastroenterol* **100**：1072-1081, 2005
4) Fujimoto K, Sakata T, Etou H *et al*：Charting of daily weight pattern reinforces maintenance of weight reduction in moderately obese paients. *Am J Med Sci* **303**：145-150, 1992
5) Leibel RL, Rosenbaum M, Hirsch J：Changes in energy expenditure resulting from altered body weight. *N Engl J Med* **332**：621-628, 1995
6) Rosenbaum M, Goldsmith R, Bloomfield D *et al*：Low-dose leptin reverses skeletal muscle, autonomic, and neuroendocrine adaptations to maintenance of reduced weight. *J Clin Invest* **115**：3579-3586, 2005
7) Muto Y, Sato S, Watanabe A *et al*：Overweight and obesity increase the risk for liver cancer in patients with liver cirrhosis and long-term oral supplementation with branched-chain amino acid granules inhibits liver carcinogenesis in heavier patients with liver cirrhosis. *Hepatol Res* **35**：204-214, 2006
8) Kobayashi M, Ikeda K, Arase Y *et al*：Inhibitory effect of branched-chain amino acid granules on progression of compensated liver cirrhosis due to hepatitis C virus. *J Gastroenterol* **43**：63-70, 2008
9) Nishitani S, Takehana K, Fujitani S *et al*：Branched-chain amino acids improve glucose metabolism in rats with liver cirrhosis. *Am J Physiol Gastrointest Liver Physiol* **288**：G1292-G1300, 2005
10) Murata K, Moriyama M：Isoleucine, an essential amino acid, prevents liver metastases of colon cancer by antiangiogenesis. *Cancer Res* **67**：3263-3268, 2007
11) Kakazu M, Kanno N, Ueno Y *et al*：Extracellular branched-chain amino acids, especially valine, regulate maturation and function of monocyte-derived dendritic cells. *J Immunol* **179**：7137-7146, 2007
12) Fukuchi S, Hamaguchi K, Seike M *et al*：Role of fatty acid composition in the development of metabolic disorders in sucrose-induced obese rats. *Exp Biol Med*（Maywood）**229**：486-493, 2004
13) Endo M, Masaki T, Seike M *et al*：TNF-α induces hepatic steatosis in mice by enhancing gene expression of sterol regulatory element binding protein-1c（SREBP-1c）. *Exp Biol Med*（Maywood）**232**：614-621, 2007
14) Zhang Y, Guo K, LeBlanc RE *et al*：Increasing dietary leucine intake reduces diet-induced obesity and improves glucose and cholesterol metabolism in mice via multimechanisms. *Dibetes* **56**：1647-1654, 2007
15) Masaki T, Chiba S, Tatsukawa H *et al*：Adiponectin protects LPS-induced liver injury through modulation of TNF-α in KK-Ay obese mice. *Hepatology* **40**：177-184, 2004
16) Layman DK, Baum JI：Dietary protein impact on glycemic control during weight loss. *J Nutr* **134**：968S-973S, 2004

せいけ・まさたか

清家正隆　大分大学医学部附属病院肝疾患相談センター

大分県生まれ.
専門は肝臓病・肝癌治療.
研究テーマは脂肪性肝炎の病態解明.

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Case report

Sustained virological response in a patient with chronic hepatitis C treated by monotherapy with the NS3-4A protease inhibitor telaprevir

Fumitaka Suzuki[a,*], Yoshiyuki Suzuki[a], Norio Akuta[a], Hitomi Sezaki[a], Hiromi Yatsuji[a], Yasuji Arase[a], Miharu Hirakawa[a], Yusuke Kawamura[a], Tetsuya Hosaka[a], Masahiro Kobayashi[a], Satoshi Saito[a], Kenji Ikeda[a], Mariko Kobayashi[b], Sachiyo Watahiki[b], Rie Mineta[b], Satomi Iwasaki[b], Hiromitsu Kumada[a]

[a] Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan
[b] Research Institute for Hepatology, Toranomon Branch Hospital, Kawasaki, Japan

ARTICLE INFO

Article history:
Received 22 April 2009
Received in revised form 10 July 2009
Accepted 25 September 2009

Keywords:
Hepatitis C virus
Protease inhibitor
Telaprevir
Sustained virological response

ABSTRACT

Here, we describe for the first time a case of sustained virological response (SVR) achieved in a patient with chronic hepatitis C (CH-C) by monotherapy with a NS3-4A protease inhibitor, telaprevir, without interferon therapy. A 59-year-old treatment-naïve Japanese man was enrolled in a phase II trial of telaprevir by repeat oral administration at a dose of 750 mg every 8 h for 24 weeks. At the start of treatment, he exhibited a low-level viremia with genotype 1b of the hepatitis C virus (HCV). After the first week of treatment with telaprevir, serum HCV RNA was undetectable, and negativity remained until the end of treatment. Moreover, he was evaluated as having a SVR after the post-treatment 24-week follow-up program. Two characteristics may explain the strong antiviral activity of telaprevir in the present case. First, although pre-treatment PCR-direct sequencing and cloning for the N-terminal in the NS3 region showed a protease inhibitor-resistant variant (T54A) in 1 of 22 independent clones, the T54A substitution has only a low-level resistance to protease inhibitors and his viral load was low. Second, when compared to a consequence sequence of 35 treatment-naïve patients with HCV genotype 1b, R130K and Q195K substitutions were unique to the present case. Although it is presently unknown whether the R130K and Q195K substitutions are related to SVR, this case suggests that long-term telaprevir monotherapy may be effective in CH-C patients with genotype 1 and a low viral load.

1. Introduction

The goals of antiviral treatment in patients with chronic hepatitis C (CH-C) are long-lasting eradication of the virus and a decrease in disease-related hepatic mortality. Standard treatment uses a combination of pegylated interferon and ribavirin (PEG-IFN-RBV), which provides a sustained virological response (SVR) rate of over 50%.[1,2] In Japan, approximately 70% of patients with CH-C are infected with genotype 1b, and those with a high titer of genotype 1b (≥100 KIU/mL [Amplicor; Roche Diagnostics K.K. Tokyo, Japan]) have lower rates of SVR (<50%), even on 48 weeks of PEG-IFN-RBV combination therapy.[3] Further, although treatment for CH-C is currently based on interferon (IFN), use of this agent is associated with serious adverse effects in some patients, such as mental disorders, apathy, and laboratory abnormalities.[1,2,4] Moreover, most CH-C patients in Japan over 70 years of age cannot receive IFN therapy due to either or both co-morbidities and the risk of adverse effects. For these reasons, a new treatment strategy is needed for patients with CH-C that displays high SVR rates and a favorable side-effect profile.

One recently introduced treatment strategy for CH-C is inhibition of the NS3-4A protease in the HCV polyprotein. Potential inhibitors include telaprevir (VX-950; MP-424; Mitsubishi Tanabe Pharma Co., Osaka, Japan), which has been selected as a clinical therapy candidate for the treatment of CH-C.[5] In some patients with genotype 1 and a high viral load, however, the efficacy of telaprevir monotherapy was limited, and combination therapy of telaprevir plus PEG-IFN-RBV is now standard.[6–10] On this background, we therefore report here for the first time a patient with CH-C who achieved a SVR following monotherapy with telaprevir.

2. Case report

A 59-year-old Japanese man was admitted to Toranomon Hospital, Tokyo in July 2007 following a positive result for HCV RNA at general check-up. Laboratory tests before treatment showed mild

* Corresponding author. Tel.: +81 44 877 5111; fax: +81 44 860 1623.
E-mail address: fumitakas@toranomon.gr.jp (F. Suzuki).

1386-6532/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jcv.2009.09.029

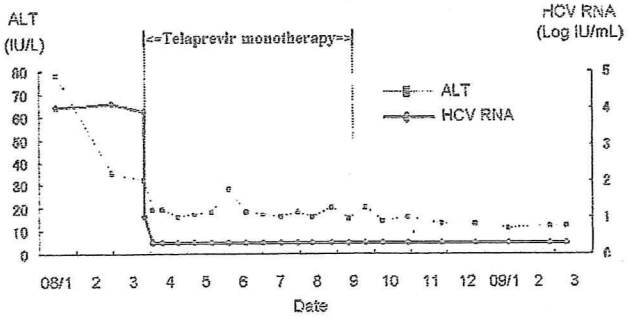


Fig. 1. Clinical course during and after 24 weeks of telaprevir monotherapy.

elevation of ALT (46 IU/L), and persistent HCV infection with genotype 1b and low-level viremia (<5 Log IU/mL [COBAS TaqMan HCV test, Roche Diagnostics K.K. Tokyo]) that continued to remain low until the start of treatment. He was diagnosed with CH-C by peritoneoscopy and liver biopsy (mild hepatitis [A1] and moderate fibrosis [F2]) at our hospital in February 2008. He had not received IFN therapy or any other antiviral drugs, and was enrolled in a phase II trial of telaprevir. Written informed consent was obtained, and the study was conducted in compliance with Good Clinical Practice and the Declaration of Helsinki. Treatment with telaprevir was started in March 2008, at which time serum HCV RNA was 3.9 Log IU/mL. Treatment was by repeat oral administration at a dose of 750 mg every 8 h for 24 weeks. Serum HCV RNA was undetectable after the first week and remained negative until the end of treatment (September 2008), and moreover remains undetectable as of March 2009. He was evaluated as having a SVR after the post-treatment 24-week follow-up program (Fig. 1).

The genome sequence for the N-terminal 609 nucleotides (203 amino acids) in the NS3 region of HCV isolates from the patient was analyzed before treatment with telaprevir. HCV RNA was extracted from 100 μL of serum and the nucleotide sequences were determined by direct sequencing and cloning. The primers used to amplify the NS3 region were NS3-F1 (5′-ACACCGCCGCCGTGTGGGGACAT-3′; nucleotides 3295–3316) and NS3-AS2 (5′-GCTCTTGCCGCTGCCAGTGGGA-3′; nucleotides 4040–4019) as the first (outer) primer pair and NS3-F3 (5′-CAGGGGTGGCGGCTCCTT-3′; nucleotides 3390–3407) and NS3-AS2 as the second (inner) primer pair.[11] Thirty-five cycles of first and second amplifications were performed as follows: denaturation for 30 s at 95 °C, annealing of primers for 1 min at 63 °C, extension for 1 min at 72 °C, and final extension was performed at 72 °C for 7 min. PCR-amplified DNA was purified after agarose gel electrophoresis and amplification products of the second-round PCR were ligated with plasmid and transformed in *Escherichia coli* in a cloning kit (TA Cloning; Invitrogen, Carlsbad, CA). Dideoxynucleotide termination sequencing was performed with the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems Japan, Tokyo). Sequences of 32 independent clones from the sample were determined and analyzed. The pre-treatment analyses by PCR-cloning showed a variant (T54A) resistant to protease inhibitors in 1 of the 32 clones.

We also made a consensus sequence of the NS3 region from the PCR-direct sequences of 35 treatment-naïve Japanese patients with HCV genotype 1b in our hospital (Fig. 2). Compared to the consensus sequence, there were a total of 5 identical substitution variants (V48I, P89S, S122G, R130K, Q195K) within the 32 independent clones from this patient, among which R130K and Q195K were unique to this patient.

3. Discussion

Previous studies showed that telaprevir monotherapy for HCV patients with genotype 1 and a high viral load demonstrated substantial antiviral activity, and the median maximum change was 4.77 Log IU/mL with administration at 750 mg every 8 h for 2 weeks.[6,7] In Reesink et al., HCV RNA decreased below the limit of

```
                1       10        20        30        40        50
CONSENSUS       APITAYSQQT RGLLGCIITS LTGRDKNQVE GEVQVVSTAT QSFLATCVNG
Case clone1     ---------- ---------- ---------- ---------- -------I--
Case clone2     ---------- ---------- ---------- ---------- -------I--
Case clone3     -----A---- ---------- ---------- ---------- -------I--
Case clone4     ---------- ---------- ---------- ---------- -------I--
Case clone5     ---------- ---------- ---------- ---------- -------I--
                51                                                100
CONSENSUS       VCNTVVHGAG SKTLAGPKSF ITQNYTNVDQ DLVGWQAPPG ARSLTPCTCG
Case clone1     ---------- ---------- ---------- ---------- ----------
Case clone2     -----S---- ---------- ---------- --------S- ----------
Case clone3     ---A------ ---------- ---------- --------S- ----------
Case clone4     ---------- ---------- ---------- --------S- -----L----
Case clone5     -----S---- ---------- ---------- --------S- ----------
                101                       130                 150
CONSENSUS       SSDLYLVTRH ADVIPVRRRG DSRGSLLSPR PVSYLKGSSG GPLLCPSGHA
Case clone1     ---------- ---------- -G-------K ---------- ----------
Case clone2     ---------- ---------- -G-------K ---------- ----------
Case clone3     ---------- ---------- -G-------K ---------- ----------
Case clone4     ---------- ---------- -G-------K ---------- ----------
Case clone5     ---------- ---------- -G-------K ---------- ----------
                151                                     195  200
CONSENSUS       VGIFRAAVCT RGVAKAVDFI PVESMETTMR SPVFTDNSSP PAVPQTFQVA
Case clone1     ---------- ---------- ---------- ---------- ----K-----  15
Case clone2     ---------- ---------- ---------- ---------- ----K-----  14
Case clone3     ---------- ---------- ---------- ---------- ----K-----   1
Case clone4     ---------- ---------- ---------- ---------- ----K-----   1
Case clone5     ---------- ---------- ---------- ---------- ----K----V   1
```

Fig. 2. Evolution of the HCV NS3 gene sequence at the start of telaprevir monotherapy. Consensus sequence was made from the HCV RNA of 35 treatment-naïve Japanese patients with genotype 1b in our hospital. The number of clones within each sample of identical amino acid sequences is given on the right at the end of each sequence. Dashes indicate identical amino acid sequences.

detection (10 IU/mL) for 2 patients in the group receiving 750 mg every 8 h.[6] In some patients, however, HCV RNA levels increased between days 7 and 14, and mutations that confer resistance to telaprevir were detected. This trial of telaprevir monotherapy was therefore terminated after 2 weeks, and combination therapy of telaprevir plus PEG-IFN-RBV is now used in the USA and Europe.[8–10] Our case may therefore represent an unusual and possibly serendipitous response to long-term telaprevir monotherapy, and the efficacy of monotherapy remains unclear.

To our knowledge, this is the first report of a patient with CH-C achieving SVR by telaprevir monotherapy, without the use of IFN. Three treatment-naïve Japanese patients were enrolled in our hospital for this phase II trial of telaprevir monotherapy over 24 weeks. Before treatment, the 2 non-SVR patients had a high HCV RNA viral load (>5 Log IU/mL), while the viral load in the SVR patient remained low. Further, while HCV RNA decreased below the limit of detection (10 IU/mL) and negativity of HCV RNA remained until the end of treatment in 2 patients, HCV RNA in the other non-SVR patient reappeared after treatment cessation.

The development of drug resistance has been a challenge for treatment strategies in many viral infections. The high replication rate and the error-prone nature of viral RNA polymerases generate a viral quasi-species from which variants resistant to viral inhibitors can be selected. Recently, Kuntzen et al. reported that viral loads were high in the majority of treatment-naïve patients carrying mutations of protease and polymerase inhibitors.[12] Low viral load may therefore be an important factor for achieving SVR by telaprevir monotherapy.

It has recently been reported that CH-C patients never treated with an NS3-4A protease inhibitor may nevertheless possess variants resistant to protease inhibitors involving the HCV RNA NS3 region.[12–14] While there was a resistant variant (T54A) in this case, this mutation exhibits only low-level resistance,[7] and the number of mutant variants may have been few along with substantial suppression of HCV replication by telaprevir. This may also help to explain the effectiveness of telaprevir in this case.

Moreover, two amino acid substitutions (R130K and Q195K) were unique to this patient. We therefore checked the nucleotide sequence data in the DDBJ/EMBL/GenBank databases and found a previous report by Franco et al. on the R130K substitution (EF013801, EF013863, EF013867, EF013869).[15] Interestingly, although only a minor clone (4% of total) in that study, the viral load of the patient with the R130K substitution was also low (2364 IU/mL). To date, however, the Q195K substitution has not been reported. Their presence in this case may indicate that telaprevir has a stronger antiviral activity against HCV with these substitutions.

The NS3-4A protease targeted by protease inhibitors is required for viral polyprotein processing, an essential step in viral replication, but is also responsible for disrupting IFN responses to the infection.[16] Previous studies have shown that high concentrations of protease inhibitors may restore retinoic acid-inducible gene I (RIG-I) signaling in HCV replicon cells,[16–18] and Liang et al. also recently reported that protease inhibitors could restore interferon regulatory factor 3 (IRF-3) signaling in HCV-infected cells.[19] In our patient, telaprevir may have therefore rescued the NS3-4A-mediated blockade of IRF-3 signaling *in vivo*.

Further studies are required, such as sequencing analyses of the HCV NS3 region, and research into the rescue of IFN-β signaling through the RIG-I pathway. It is foreseeable in the future for CH-C patients to be treated by one or a combination of two or more oral drugs with high efficacy and genetic barriers to resistance and low side-effect profiles. Telaprevir may hold promise for being one of these drugs, even if only within a subset of patients, and further studies into telaprevir monotherapy or combination therapy with other oral drugs is therefore warranted. Although still an isolated response, based on our current molecular understanding of HCV infection and pharmacotherapy, this case suggests that long-term telaprevir monotherapy may be effective in other CH-C patients with genotype 1 and a low viral load.

Conflict of interest

The authors have no commercial or other associations that may pose a conflict of interest.

Acknowledgments

This study was supported in part by a grant-in-aid from the Ministry of Health, Labor and Welfare, Japan.

References

1. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001;358:958–65.
2. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçales Jr FL, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002;347:975–82.
3. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. *J Hepatol* 2007;46:403–10.
4. Hadziyannis SJ, Sette Jr H, Morgan TR, Balan V, Diago M, Marcellin P, et al. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004;140:346–55.
5. Lin C, Kwong AD, Perni RB. Discovery and development of VX-950, a novel covalent, and reversible inhibitor of hepatitis C virus NS3/4A serine protease. *Infect Disord Drug Targets* 2006;6:3–16.
6. Reesink HW, Zeuzem S, Weegink CJ, Forestier N, Vigerust D, Purdy S, et al. Rapid decline of viral RNA in hepatitis C patients treated with VX-950, a phase IB, placebo-controlled, randomized study. *Gastroenterology* 2006;131:997–1002.
7. Sarrazin C, Kieffer TL, Bartels D, Hanzelka B, Muh U, Welker MW, et al. Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir. *Gastroenterology* 2007;132:1767–77.
8. Lawitz E, Rodriguez-Torres M, Muir AJ, Shafiro H, Alcorn H, Khunvichai K, et al. Antiviral effects and safety of telaprevir, peginterferon alfa-2a, and ribavirin for 28 days in hepatitis C patients. *J Hepatol* 2008;49:163–9.
9. McHutchison JG, Everson GT, Gordon SC, Jacobson IM, Sulkowski M, Kauffman R, et al. Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. *N Engl J Med* 2009;360:1827–38.
10. Hezode C, Forestier N, Dusheiko G, Ferenci P, Pol S, Goeser T, et al. Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. *N Engl J Med* 2009;360:1839–50.
11. Igata S, Pawlotsky JM, Nagano-Fujii M, Hidajat R, Deng L, Ito T, et al. Identification of hepatitis C virus (HCV) subtype 1b strains that are highly or only weakly associated with hepatocellular carcinoma on the basis of the secondary structure of an amino-terminal portion of the HCV NS5 protein. *Nucleic Acids Res* 2003;31:2825–41.
12. Kuntzen T, Timm J, Berical A, Lennon N, Berlin AM, Young SK, et al. Naturally occurring dominant resistance mutations to hepatitis C virus protease and polymerase inhibitors in treatment-naive patients. *Hepatology* 2008;48:1769–78.
13. Bartels DJ, Zhou Y, Zhang EZ, Marcial M, Byrn RA, Pfeiffer T, et al. Natural prevalence of hepatitis C virus variants with decreased sensitivity to NS3-4A protease inhibitors in treatment-naive subjects. *JID* 2008;198:800–7.
14. Cubero M, Esteban JI, Otero T, Sauleda S, Bes M, Esteban R, et al. Naturally occurring NS3-protease-inhibitor resistant mutant A156T in the liver of an untreated chronic hepatitis C patient. *Virology* 2008;370:237–45.
15. Franco S, Parera M, Aparicio E, Clotet B, Martinez MA. Generic and catalytic efficiency structure of an HCV protease quasispecies. *Hepatology* 2007;45:899–910.
16. Foy E, Li K, Wang C, Sumpter Jr R, Ikeda M, Lemon SM, et al. Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. *Science* 2003;300:1145–8.
17. Johnson CL, Owen DM, Gale Jr M. Functional and therapeutic analysis of hepatitis C virus NS3-4A protease control of antiviral immune defense. *J Biol Chem* 2007;282:10792–803.
18. Loo YM, Owen DM, Li K, Erickson AK, Johnson CL, Fish PM, et al. Viral and therapeutic control of IFN-β promoter stimulator 1 during hepatitis C virus infection. *Proc Natl Acad Sci USA* 2006;103:6001–6.
19. Liang Y, Ishida H, Lenz O, Lin TI, Nyanguile O, Simmen K, et al. Antiviral suppression vs restoration of RIG-I signaling by hepatitis C protease and polymerase inhibitors. *Gastroenterology* 2008;135:1710–8.

Influence of Amino-Acid Polymorphism in the Core Protein on Progression of Liver Disease in Patients Infected With Hepatitis C Virus Genotype 1b

Mariko Kobayashi,[1]* Norio Akuta,[2] Fumitaka Suzuki,[2] Tetsuya Hosaka,[2] Hitomi Sezaki,[2] Masahiro Kobayashi,[2] Yoshiyuki Suzuki,[2] Yasuji Arase,[2] Kenji Ikeda,[2] Sachiyo Watahiki,[1] Rie Mineta,[1] Satomi Iwasaki,[1] Yuzo Miyakawa,[3] and Hiromitsu Kumada[2]

[1]*Research Institute for Hepatology, Toranomon Hospital, Tokyo, Japan*
[2]*Department of Hepatology, Toranomon Hospital, Tokyo, Japan*
[3]*Miyakawa Memorial Research Foundation, Tokyo, Japan*

The substitution of amino acid (aa) 70 of arginine for glutamine and/or that of aa91 of leucine for methionine in the core protein in patients infected with hepatitis C virus (HCV) genotype 1b is associated with a poor response to pegylated interferon and ribavirin. Factors influencing these substitutions were sought in 1,097 patients infected with HCV-1b who had not received antiviral treatment. HCV variants with Arg70 and Leu91 (wild-type) decreased, while those with Gln70 and/or Met91 (mutant types) increased with age ($P < 0.001$). Of the 1,097 patients, 464 (42.3%) were infected with the Gln70 variant and the remaining 633 patients with the Arg70 variant. The proportion of patients with the Gln70 variant increased with the severity of liver disease ($P < 0.001$), elevated γ-glutamyl transpeptidase (γ-GTP) levels ($P < 0.001$) and a decrease in platelet count ($P = 0.008$). In univariate analysis patients with hepatocellular carcinoma, elevated aspartate aminotransferase (AST ≥ 58 IU/L) and γ-GTP (≥61 IU/L), and decreased albumin levels (<3.9 g/dl) were more frequent in the patients with the Gln70 variant than the Arg70 variant ($P = 0.003$, 0.005, <0.001, and 0.031, respectively). In multivariate analysis HCC (odds ratio 1.829 [95% confidence interval 1.147–2.917]) and γ-GTP ≥61 IU/L (1.647 [1.268–2.139]) increased the risk for the Gln70 variant. In conclusion, the substitution of amino aa70 of Arg for Gln in patients infected with HCV-1b increases with age, and it is associated with severe liver disease accompanied by elevated AST and γ-GTP levels, as well as the development of hepatocellular carinoma. ***J. Med. Virol. 82:41–48, 2010.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: cirrhosis; core protein; hepatitis C; hepatocellular carcinoma; interferon; ribavirin

INTRODUCTION

Worldwide, an estimated 170 million people are infected with hepatitis C virus (HCV) persistently [Cohen, 1999]. Decompensated cirrhosis and hepatocellular carcinoma (HCC) can develop in about 30% of patients infected with HCV [Alberti et al., 1999; Seeff, 2002]. HCV has six major genotypes and dozens of subgenotypes, and they have distinct geographic distributions and are associated with the progression of liver disease [Simmonds, 1995]. Host and virological factors can influence the severity of liver disease and the response to antiviral treatment. HCV infection in the childhood and women runs a milder course than that in adulthood and men, and the intake of alcohol accelerates the progression of liver disease [Poynard et al., 1997; Kenny-Walsh, 1999; Vogt et al., 1999; Wiese et al., 2000]. Genotypes 1 and 4 aggravate liver disease and decrease the response to antiviral treatment, in comparison with genotypes 2, 3, and 6 [Tsubota et al., 1994; Hui et al., 2003; Hadziyannis et al., 2004; Legrand-Abravanel et al., 2005; Yuen and Lai, 2006]. High levels of HCV RNA in the serum can induce severe liver disease and decrease treatment response [Tsubota et al., 1994].

In Japan, genotype 1b in a high viral load (>100 KIU/ml) accounts for >70% of HCV infection, and decreases the treatment response in patients with chronic hepatitis C [Kumada et al., 2006]. Even with pegylated interferon (PEG-IFN) combined with ribavirin, the sustained virological response for longer than 24 weeks after the withdrawal of treatment is achieved merely in

Grant sponsor: Ministry of Health, Labour and Welfare of Japan.

*Correspondence to: Mariko Kobayashi, BS, Research Institute for Hepatology, Toranomon Hospital, 1-3-1, Kajigaya, Takatsu-ku, Kawasaki City 213-8587, Japan.
E-mail: vj7m-kbys@asahi-net.or.jp

Accepted 18 July 2009
DOI 10.1002/jmv.21629
Published online in Wiley InterScience (www.interscience.wiley.com)

50% of the patients with HCV-1b in high levels [Manns et al., 2001; Fried et al., 2002]. It is necessary to predict the response to PEG-IFN/ribavirin before the start of antiviral therapy, to avoid severe side-effects in the patients who will barely gain sustained virological response.

The core protein of HCV is coded for by the C gene, and consists of 191 amino acids (aa) [Rosenberg, 2001]. Although the core protein is conserved better than the other structural and non-structural proteins of HCV, polymorphisms of core protein are known, and they influence the response to antiviral treatment. In patients infected with HCV-1b, for example, the substitution of arginine at position 70 (Arg70) for glutamine (Gln70) and that of leucine at position 91 (Leu91) for methionine (Met70) decrease sustained virological response in the patients with chronic hepatitis C who are treated with PEG-IFN/ribavirin and increase the development of HCC [Akuta et al., 2007a,b,d, 2008].

In the Department of Hepatology at the Toranomon Hospital in Metropolitan Tokyo, the amino-acid sequence of the core-protein was determined in 1,079 patients infected with HCV-1b who had not received antiviral treatment. The substitution of Arg70 for Gln70 and that of Leu91 or Met 91 were correlated with the age at presentation, liver function tests and the severity of liver disease. Based on the results obtained, Gln70 would contribute to the progression of chronic hepatitis C.

MATERIALS AND METHODS

Patients

During 1966–2008, 1,097 patients infected with HCV-1b visited the Department of Hepatology at the Toranomon Hospital in Metropolitan Tokyo. They were: (1) negative for hepatitis B surface antigen by radioimmunoassay (Dainabot, Tokyo, Japan) or antibody to human immunodeficiency virus type-1; (2) positive for anti-HCV by a third-generation enzyme immunoassay (Chiron Corp., Emeryville, CA) and HCV RNA by the polymerase chain reaction (PCR) (Cobas Amplicor HCV Monitor ver.2.0, Roche Diagnostics, Tokyo, Japan); (3) infected with HCV genotype 1b but not with other genotypes; (4) without previous antiviral treatment; (5) without other forms of hepatitis, including hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease and autoimmune liver disease; and (6) had serum samples stored at −80°C. Of the 1,097 patients, 778 (70.9%) had chronic hepatitis, 221 (20.1%) cirrhosis, and 98 (8.9%) HCC. Amino acids in the core protein at positions 70 and 91 were determined, and were correlated with liver disease and biochemical and virological markers. Informed consent was obtained from each patient in this study, and the protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected by approval by Ethic Committee of the institution.

Histopathological Diagnoses of Liver Disease

Liver biopsy was performed under laparoscopy by a modified Vim Silverman needle (Tohoku University style, Kakinuma Factory, Tokyo). The sample was fixed in 10% formalin, and was stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff. It contained at least six portal areas. The pathological diagnosis was made by one of the authors (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on the scoring system of Desmet et al. [1994]. Cirrhosis was diagnosed by imaging on ultrasonography (US), computed tomography (CT), or magnetic resonance imaging (MRI). HCC was diagnosed by US and/or CT. Angiography was performed when HCC was strongly suspected by US, CT, MRI, or liver biopsy. An increasing trend of tumor markers was taken into consideration for the diagnosis of HCC.

Determination of Amino-Acid Substitutions in the Core Protein

Amino acid (aa) at position 70 of Arg or Gln and aa91 of Leu or Met were determined by PCR with primers specific for each of them [Okamoto et al., 2007]. It is highly reproducible, and has a sensitivity of 94.4% in the determination of aa70 or aa91 in samples with HCV RNA titers >10 KIU/ml. The concordance of the results of this method with those of direct sequencing reached 97.1%. Amino acids at positions 70 and 91 were confirmed by direct sequencing of most samples [Akuta et al., 2005].

Statistical Analysis

Changes of Arg70/Leu91 (wild-type) and Gln70 and/or Met91 (mutant types) with age were analyzed by the Cochran–Armitage trend test (SAS version 9.1.3; SAS Institute, Inc., Cary, NC). Frequencies were compared between groups by the Kruskal–Wallis test and Fisher's exact test. Univariate and multivariate logistic regression analyses were used for the evaluation of factors independently associated with the substitution of aa70. They included the following ten variables: age, sex, liver disease, platelet count, hemoglobin, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), and substitution of aa at position 91 in the core protein. Each variable was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. Variables that achieved statistical significance on univariate analysis were tested by the multivariate Cox proportional hazard model to identify independent factors. Statistical comparisons were performed using SPSS ver.11.0 (SPSS, Inc., Chicago, IL). A P-value <0.05 by the two-tailed test was considered significant.

RESULTS

Clinical and Virological Characteristics of the 1,097 Patients Who Were Infected With HCV-1b

Table I lists the baseline characteristics of the 1,097 patients who were infected with HCV-1b and had not received antiviral treatment. They had the median age of 60 years and included 590 (53.8%) men. The median transaminase levels were elevated, and alpha-fetoprotein was within the normal limit (<10 μg/L). The majority of the patients (70.9%) had chronic hepatitis, while HCC had developed in 8.9% of the patients. Amino acids at positions 70 and 91 in the core protein were both the wild-type (Arg70 and Leu91) in 37.6% of them, and both mutant types (Gln70 and Met91) in 16.4%. The Gln70 variant was detected in 464 of the 1,097 (42.3%) patients.

The Prevalence of Amino-Acid Substitutions Stratified by Age and Sex

The 1,097 patients infected with HCV-1b were classified into three age groups, and the prevalence of Arg70/Leu91 (wild-type) and that of Gln70 and/or Met91 (mutant types) were compared (Fig. 1). Ag70/Leu91 decreased with age by trend analysis, from 63.6% in the patients aged ≤30 years to 36.6% in those ≥41 years ($P < 0.001$ by the Cochran–Armitage trend test). Table II lists the prevalence of the Gln70 variant in men and women stratified by the age. There were no sex differences in the prevalence of the Gln70 variant.

The Prevalence of the Gln70 Variant in Patients With Different Liver Diseases

Figure 2 compares the prevalence of the Gln70 variant among patients infected with HCV-1b who presented with different liver diseases at the baseline. The prevalence of the Gln70 variant increased with the progression of liver disease from chronic hepatitis (32.6%) to cirrhosis (43.0%) and HCC (53.1%) ($P < 0.001$ by the Kruskal–Wallis test). In patients with cirrhosis, the 126 patients with the Arg70 variant were aged with the mean of 62 years (range: 32–78 years) in comparison with the 95 patients with the Gln70 variant who were aged 59 years (25–80). In patients with HCC, the 47 patients with the Arg70 variant were aged with the mean of 66 years (range: 37–81 years) in comparison with the 51 patients with the Gln70 variant who were aged 66 years (46–78).

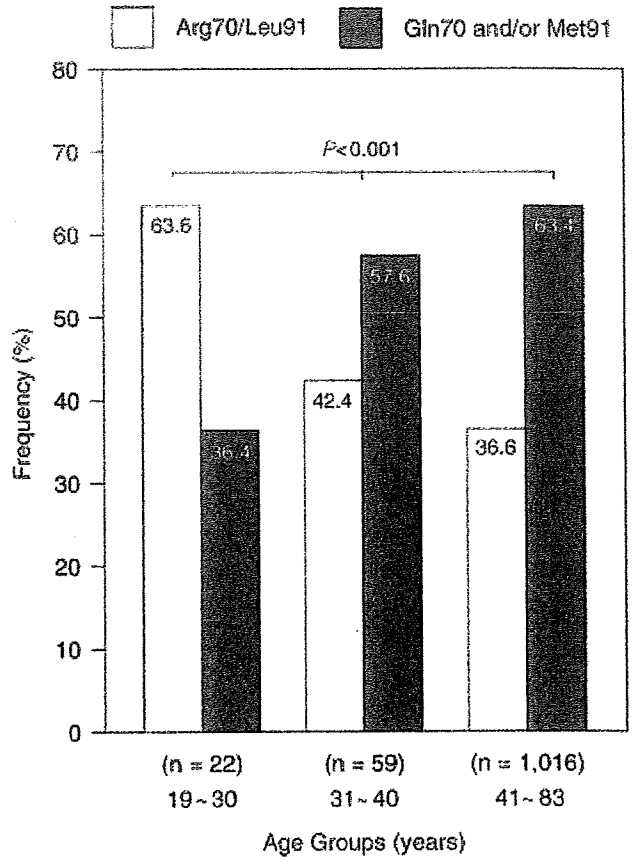


Fig. 1. The age-specific prevalence of Gln70 in treatment-naive patients infected with HCV-1b.

TABLE I. Clinical and Virological Characteristics of the 1,097 Patients Who Were Infected With Hepatitis C Virus of Genotype 1b

Age (years)	60 (19–83)
Men	590 (53.8%)
Follow-up period (years)	8 (3–28)
Hemoglobin (g/dl)	14.0 (4.5–26.8)
Platelets ($\times 10^3/mm^3$)	15.4 (2.0–34.1)
Aspartate aminotransferase (IU/L)	58 (8–617)
Alanine aminotransferase (IU/L)	69 (6–776)
Alpha-fetoprotein (μg/L)	6 (2–65,700)
Liver disease	
Chronic hepatitis	778 (70.9%)
Cirrhosis	221 (20.1%)
Hepatocellular carcinoma	98 (8.9%)
Amino acids in the core protein	
Arg70/Leu91 (double wild-type)	412 (37.6%)
Gln70/Leu91 (mutant type)	284 (25.9%)
Arg70/Met91 (mutant type)	221 (20.1%)
Gln70/Met91 (double mutant type)	180 (16.4%)

Values are the median with range in parentheses or the number with percentage in parentheses.

TABLE II. Frequency of Gln70 in the Core Protein in Patients Infected With HCV-1b Stratified by Age and Sex

Age (years)	Men	Women	Differences
19–30	23.5% (4/17)	20% (1/5)	1.0
31–40	34.1% (14/41)	38.9% (7/18)	0.773
41–50	37.2% (45/121)	40% (14/35)	0.763
51–60	39.1% (72/184)	40.1% (63/157)	0.912
61–70	36.0% (62/172)	30.1% (74/246)	0.205
70–83	45.5% (25/55)	43.5% (20/46)	0.842
Total	37.6% (222/590)	35.3% (179/507)	0.451

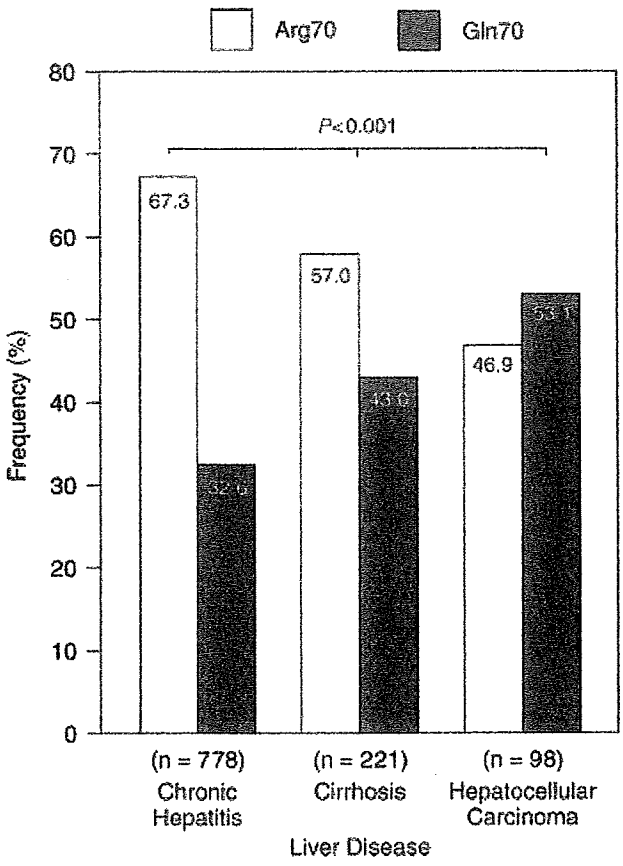


Fig. 2. The prevalence of the Gln70 variant among patients with chronic hepatitis, cirrhosis and hepatocellular carcinoma.

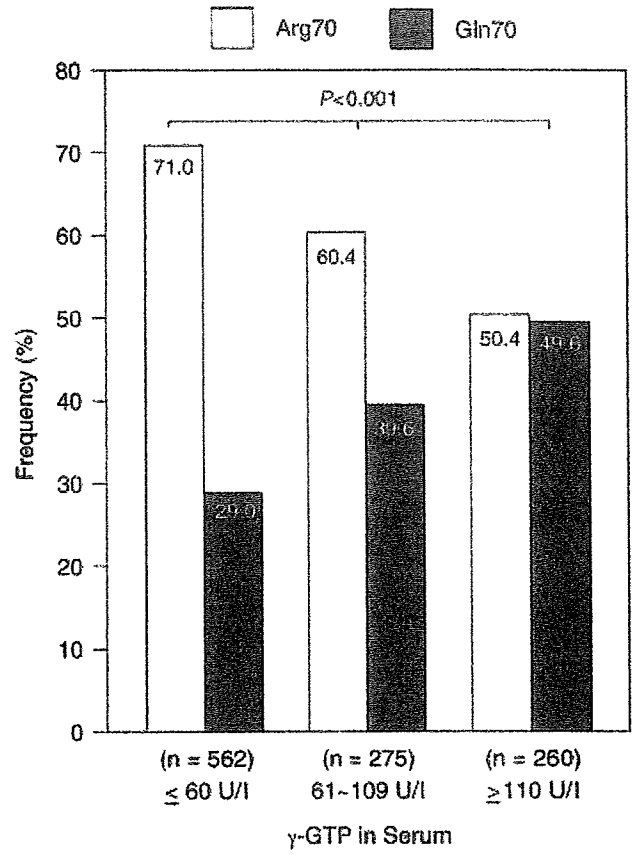


Fig. 3. The prevalence of the Gln70 variant among patients with different γ-GTP levels.

The Influence of γ-GTP Levels on the Prevalence of the Gln70 Variant

The prevalence of Gln70 was compared among patients with different γ-GTP levels at the baseline (Fig. 3). The prevalence of the Gln70 variant increased in parallel with the γ-GTP levels from 29.0% to 49.6% ($P < 0.001$ by the Kruskal–Wallis test).

The Influence of Platelet Count on the Prevalence of the Gln70 Variant

The prevalence of the Gln70 variant was compared among three groups of patients with various platelet counts at the baseline (Fig. 4). The prevalence of the Gln70 variant increased as the platelet count decreased ($P = 0.008$ by the Kruskal–Wallis test).

Factors Associated With the Gln70 Variant in Patients Infected with HCV-1b

Since the Gln70 variant, in comparison with the Arg70 variant, aggravated liver disease in patients infected with HCV-1b (Figs. 2–4), ten factors were evaluated for the association with the Gln70 variant by the univariate analysis (Table III); the cut-off value was set at the median of studied patients. Among them, HCC, elevated levels of AST (≥58 IU/L) and γ-GTP (>61 U/L), as well as decreased albumin concentration (<3.9 g/dl), were associated with the Gln70 variant ($P = 0.003$, 0.005, <0.001, and 0.031, respectively). A similar analysis was performed for the substitution of Leu91 for Met91 (Table IV). Except for the association with the substitution of Arg70 for Gln70, the Met91 variant had no influence on any variable examined.

Two factors associated independently with the Gln70 variant were identified by the multivariate analysis (Table V). The risk for the Gln70 variant was increased by HCC (odds ratio 1.829 [95% confidence interval 1.147–2.917], $P = 0.011$) and γ-GTP ≥61 IU/L (1.647 [1.268–2.139], $P < 0.001$).

DISCUSSION

The response to PEG-IFN and ribavirin is influenced by genotypes and viral load, and is poorest in patients with HCV-1b in high HCV RNA levels [Manns et al., 2001; Fried et al., 2002; Hadziyannis et al., 2004]. The prediction of sustained virological response would circumvent side-effects and costs in non-responders. Amino-acid substitutions in the core protein are useful

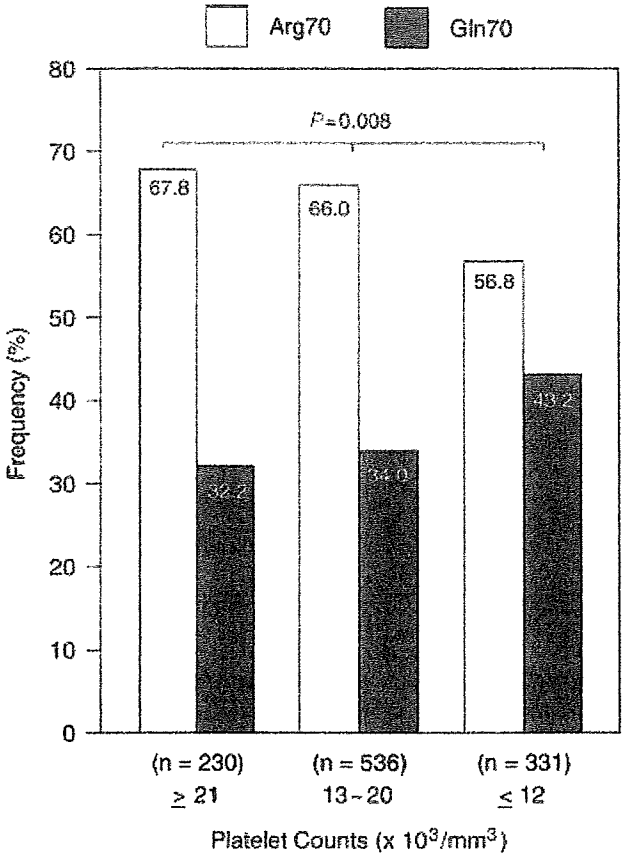


Fig. 4. The prevalence of the Gln70 among three groups of patients with different platelet counts.

for predicting the non-response in patients infected with HCV-1b. The substitution of Arg70 for Gln70 in the prototype sequence of HCV-1b [Kato et al., 1990] and/or that of Leu91 for Met91 can predict the non-response to IFN-based treatment [Akuta et al., 2005, 2006, 2007c,d]. It has been beyond the scope of previous studies, however, whether or not these amino-acid substitutions influence the progression of hepatitis C in the patients who have not received antiviral treatment. The availability of pre-treatment sera from many patients with chronic hepatitis C permitted the evaluation of the influence of aa substitutions in the core protein on the progression of liver disease without therapeutic intervention.

First, the prevalence of the Gln70 variant increased with the age of patients until they had reached 50 years (Fig. 1). It is not certain if HCV-1b with Arg70 underwent a point mutation for Gln70 (G-to-A at nucleotide 209), or these amino-acid residues were present in HCV-1b strains prevalent at the time of infection. Follow-up of patients for aa substitutions will resolve this issue. Another possibility for this difference would be a selection bias. If the patients with the Arg90 variant fare better than those with the Gln70 variant, they would not develop liver disease severe enough to visit hospital.

Secondly, the patients infected with HCV-1b with Gln70 increased in parallel with γ-GTP levels and the severity of liver disease from chronic hepatitis to cirrhosis and HCC, as well as with a decrease in platelet count (Figs. 2–4). Since the Met91 variant did not make such difference, the aggravation of liver disease would have been due to the Gln70 variant, but not to the Met91 variant. Increases in the γ-GTP level may have been related to the development of HCC; γ-GTP has been proposed as a sensitive marker of cirrhosis and HCC [Penn and Worthington, 1983]. Decreased platelet counts have been associated with HCC [Ikeda et al., 2001; Lu et al., 2006; Kumada et al., 2009]. Although the proportion of the Gln70 variant increases with the severity of liver disease (Fig. 2), the median age of patients with cirrhosis or HCC did not differ between the patients with the Arg70 variant and Gln70 variant who

TABLE III. Factors Associated With the Substitution of aa70 of Arginine for Glutamine in the Core Protein in 1,097 Patients Infected With HCV Genotype1b by Univariate Analysis

Factor	Category	Gln70	*P*-value
Sex	1: Male	38.6% (228/590)	0.663
	2: Female	37.3% (189/507)	
Age (years)	1: <60	40.6% (219/540)	0.093
	2: ≥60	35.5% (198/557)	
AST (IU/L)	1: <58	33.9% (184/543)	0.005
	2: ≥58	42.2% (234/554)	
ALT (IU/L)	1: <75	36.9% (213/578)	0.376
	2: ≥75	39.3% (204/519)	
Albumin (g/dl)	1: <3.9	42.5% (194/457)	0.031
	2: ≥3.9	35.8% (229/640)	
γ-GTP (IU/L)	1: <61	29.0% (163/562)	<0.001
	2: ≥ 61	44.4% (238/535)	
Hemoglobin (g/dl)	1: <14	35.1% (176/501)	0.083
	2: ≥14	40.4% (241/596)	
Platelet count (×10³/mm³)	1: <150	39.9% (207/519)	0.253
	2: ≥150	36.3% (210/578)	
Hepatocellular carcinoma	1: No	36.6% (366/999)	0.003
	2: Yes	53.1% (52/ 98)	
Substitutions of core aa91	1: Leucine	35.6% (227/638)	0.051
	2: Methionine	41.4% (190/459)	

TABLE IV. Factors Associated With the Substitution of aa91 of Leucine for Methionine in the Core Protein in 1,097 Patients Infected With HCV Genotype1b by Univariate Analysis

Factor	Category	Met91	*P*-value
Sex	1: Male	40.8% (241/590)	0.500
	2: Female	43.0% (218/507)	
Age (years)	1: <60	43.5% (235/540)	0.271
	2: ≥60	40.2% (220/517)	
AST (IU/L)	1: <58	43.6% (234/537)	0.196
	2: ≥58	39.7% (217/547)	
ALT (IU/L)	1: <75	42.4% (238/561)	0.618
	2: ≥75	40.8% (205/502)	
Albumin (g/dl)	1: <3.9	42.0% (177/421)	0.797
	2: ≥3.9	41.2% (249/604)	
γ-GTP (IU/L)	1: <61	40.4% (237/586)	0.327
	2: ≥61	43.4% (222/511)	
Hemoglobin (g/dl)	1: <14	40.8% (193/473)	0.658
	2: ≥14	42.3% (240/567)	
Platelet count ($\times 10^3/mm^3$)	1: <150	40.5% (202/499)	0.454
	2: ≥150	42.9% (240/559)	
Hepatocellular carcinoma	1: No	42.3% (423/999)	0.334
	2: Yes	36.7% (36/ 98)	
Substitutions of core aa71	1: Arginine	49.0% (269/680)	0.051
	2: Glutamine	45.6% (190/417)	

had cirrhosis (62 years vs. 59 years) of HCC (66 years vs. 66 years). This would indicate a possibility that the Gln70 variant would be a factor for the aggravation of liver disease that might be independent of age.

The distinct capacity of Gln70 and Met91 for decreasing the response to combined treatment in patients infected with HCV-1b was proposed in a recent study [Okanoue et al., 2008]. The Gln70 variant decreased sustained virological response, while the Met91 variant did not, although the Met91 variant reduced the rate of rapid virological response within 4 weeks after the start of therapy. The role of the Gln70 variant greater than that of the Met91 variant in the progression of liver disease has been confirmed in this study (Tables III and IV). In the multivariate analysis, the risk for Gln70 was increased by HCC (odds ratio 1.829 [95% confidence interval 1.147–2.917]) and γ-GTP ≥61 U/L (1.647 [1.268–2.139]). The Gln70 variant would aggravate liver disease toward the development of HCC in patients infected with HCV-1b who have not received antiviral treatment.

It would be a matter of conjecture how the Gln70 variant influences the severity of liver disease. Previous suggestions for a reduced response of patients with the Gln70 variant were confined to interaction of the core protein with IFN receptors and IFN-signaling pathways [Alexander, 2002; Blindenbacher et al., 2003; Bode et al., 2003]; these studies were restricted to patients receiving IFN-based treatments [Akuta et al., 2007a,b,d, 2008]. The ability of the Gln70 variant for accelerating the progression of liver disease, in the absence of exogenous IFN, has changed this issue into a wider perspective. There still remains a possibility, however, that the Gln70 variant would interact with the endogenous IFN induced by HCV infection, and aggravate liver disease.

Another possibility may be the cytotoxic T-cell (CTL) response, as has been demonstrated for the pathogenesis of chronic hepatitis B [Chisari and Ferrari, 1995]. Since both hepatitis B virus (HBV) and HCV do not have a cytopathic capacity, hepatitis B and C would be mediated by immune responses directed at viral proteins. Amino-acid sequences bearing a CTL epitope restricted by the MHC class-I are demonstrated in the HBV core protein [Bertoletti et al., 1993; Bertoletti and Gehring, 2006], and are implicated in liver disease in the patients with the HLA-2 phenotype [Penna et al., 1991; Bertoletti et al., 1994]. It is tempting to speculate that the substitution of Arg70 for Gln70 might generate a CTL epitope and stimulate cytotoxic lymphocytes toward inflammation of the liver [Kita et al., 1993; Jackson et al., 1999].

In conclusion, amino-acid substitutions in the core protein influence the progression of liver disease, and the Gln70 variant aggravates hepatic inflammation and increases the risk for HCC in the patients who have not received antiviral treatment. The ability of the Gln70

TABLE V. Factors Associated with the Substitution of aa70 of Arginine for Glutamine in the Core Protein in 1,097 Patients Infected with HCV Genotype1b by Multivariate Analysis

Factor	Category	Odds ratio (95%CI)	*P*-value
Hepatocellular carcinoma	1: No	1	0.011
	2: Yes	1.829 (1.147–2.917)	
γ-GTP (IU/L)	1: <61	1	<0.001
	2: ≥61	1.647 (1.268–2.139)	

variant to aggravate liver disease, in the absence of exogenous IFN, would lend further support on its capacity of predicting sustained virological response before the start of therapy. It is possible that mechanisms other than the resistance to IFN, such as cytotoxic T-cell responses, might be involved in an increased pathogenetic potential of HCV-1b with Gln70.

REFERENCES

Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Watahiki S, Sato J, Matsuda M, Arase Y, Ikeda K, Kumada H. 2005. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 48:372–380.

Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Watahiki S, Sato J, Arase Y, Ikeda K, Kumada H. 2006. Predictive factors of virological non-response to interferon-ribavirin combination therapy for patients infected with hepatitis C virus of genotype 1b and high viral load. J Med Virol 78:83–90.

Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2007a. Amino acid substitutions in the hepatitis C virus core region are the important predictor of hepatocarcinogenesis. Hepatology 46:1357–1364.

Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2007b. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: Amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol 46:403–410.

Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2007c. Predictors of viral kinetics to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b. J Med Virol 79:1686–1695.

Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Arase Y, Ikeda K, Miyakawa Y, Kumada H. 2007d. Prediction of response to pegylated interferon and ribavirin in hepatitis C by polymorphisms in the viral core protein and very early dynamics of viremia. Intervirology 50:361–368.

Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2008. Substitution of amino acid 70 in the hepatitis C virus core region of genotype 1b is an important predictor of elevated alpha-fetoprotein in patients without hepatocellular carcinoma. J Med Virol 80:1354–1362.

Alberti A, Chemello L, Benvegnu L. 1999. Natural history of hepatitis C. J Hepatol 31:S17–S24.

2002. Suppressors of cytokine signalling (SOCS) in the immune system. Nat Rev Immunol 2:410–416.

Bertoletti A, Gehring AJ. 2006. The immune response during hepatitis B virus infection. J Gen Virol 87:1439–1449.

Bertoletti A, Chisari FV, Penna A, Guilhot S, Galati L, Missale G, Fowler P, Schlicht HJ, Vitiello A, Chesnut RC, Fiaccardori F, Ferrari C. 1993. Definition of a minimal optimal cytotoxic T-cell epitope within the hepatitis B virus nucleocapsid protein. J Virol 67:2376–2380.

Bertoletti A, Costanzo A, Chisari FV, Levrero M, Artini M, Sette A, Penna A, Giuberti T, Fiaccadori F, Ferrari C. 1994. Cytotoxic T lymphocyte response to a wild type hepatitis B virus epitope in patients chronically infected by variant viruses carrying substitutions within the epitope. J Exp Med 180:933–943.

Blindenbacher A, Duong FH, Hunziker L, Stutvoet ST, Wang X, Terracciano L, Moradpour D, Blum HE, Alonzi T, Tripodi M, La Monica N, Heim MH. 2003. Expression of hepatitis c virus proteins inhibits interferon alpha signaling in the liver of transgenic mice. Gastroenterology 124:1465–1475.

Bode JG, Ludwig S, Ehrhardt C, Albrecht U, Erhardt A, Schaper F, Heinrich PC, Haussinger D. 2003. IFN-alpha antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. FASEB J 17:488–490.

Chisari FV, Ferrari C. 1995. Hepatitis B virus immunopathogenesis. Annu Rev Immunol 13:29–60.

Cohen J. 1999. The scientific challenge of hepatitis C. Science 285:26–30.

Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. 1994. Classification of chronic hepatitis: Diagnosis, grading and staging. Hepatology 19:1513–1520.

Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL, Jr., Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347:975–982.

Hadziyannis SJ, Sette H, Jr., Morgan TR, Balan V, Diago M, Marcellin P, Ramadori G, Bodenheimer H, Jr., Bernstein D, Rizzetto M, Zeuzem S, Pockros PJ, Lin A, Ackrill AM. 2004. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: A randomized study of treatment duration and ribavirin dose. Ann Intern Med 140:346–355.

Hui CK, Yuen MF, Sablon E, Chan AO, Wong BC, Lai CL. 2003. Interferon and ribavirin therapy for chronic hepatitis C virus genotype 6: A comparison with genotype 1. J Infect Dis 187:1071–1074.

Ikeda K, Saitoh S, Kobayashi M, Suzuki Y, Suzuki F, Tsubota A, Arase Y, Murashima N, Chayama K, Kumada H. 2001. Long-term interferon therapy for 1 year or longer reduces the hepatocellular carcinogenesis rate in patients with liver cirrhosis caused by hepatitis C virus: A pilot study. J Gastroenterol Hepatol 16:406–415.

Jackson M, Smith B, Bevitt DJ, Steward M, Toms GL, Bassendine MF, Diamond AG. 1999. Comparison of cytotoxic T-lymphocyte responses to hepatitis C virus core protein in uninfected and infected individuals. J Med Virol 58:239–246.

Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, Shimotohno K. 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci USA 87:9524–9528.

Kenny-Walsh E. 1999. Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. Irish Hepatology Research Group. N Engl J Med 340:1228–1233.

Kita H, Moriyama T, Kaneko T, Harase I, Nomura M, Miura H, Nakamura I, Yazaki Y, Imawari M. 1993. HLA B44-restricted cytotoxic T lymphocytes recognizing an epitope on hepatitis C virus nucleocapsid protein. Hepatology 18:1039–1044.

Kumada T, Toyoda H, Honda T, Kuzuya T, Katano Y, Nakano I, Goto H. 2006. Treatment of chronic hepatitis C with interferon alone or combined with ribavirin in Japan. Intervirology 49:112–118.

Kumada T, Toyoda H, Kiriyama S, Sone Y, Tanikawa M, Hisanaga Y, Kanamori A, Atsumi H, Takagi M, Nakano S, Arakawa T, Fujimori M. 2009. Incidence of hepatocellular carcinoma in hepatitis C carriers with normal alanine aminotransferase levels. J Hepatol 50:729–735.

Legrand-Abravanel F, Nicot F, Boulestin A, Sandres-Saune K, Vinel JP, Alric L, Izopet J. 2005. Pegylated interferon and ribavirin therapy for chronic hepatitis C virus genotype 4 infection. J Med Virol 77:66–69.

Lu SN, Wang JH, Liu SL, Hung CH, Chen CH, Tung HD, Chen TM, Huang WS, Lee CM, Chen CC, Changchien CS. 2006. Thrombocytopenia as a surrogate for cirrhosis and a marker for the identification of patients at high-risk for hepatocellular carcinoma. Cancer 107:2212–2222.

Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: A randomised trial. Lancet 358:958–965.

Okamoto K, Akuta N, Kumada H, Kobayashi M, Matsuo Y, Tazawa H. 2007. A nucleotide sequence variation detection system for the core region of hepatitis C virus-1b. J Virol Methods 141:1–6.

Okanoue T, Itoh Y, Yotsuyanagi H, Tanaka E, Yoshioka K, Izumi N, Kumada H. 2008. Substitution of core amino acid 91 lowers rapid virological response and substitution of amino acid 70 lowers sustained virological response to peginterferon alfa-2b plus ribabirin in chrnoic hepatitis C patients with genotype 1b—Nationwide Study (Abstract). Hepatology 48:868A.

Penn R, Worthington DJ. 1983. Is serum gamma-glutamyltransferase a misleading test? Br Med J 286:531–535.

Penna A, Chisari FV, Bertoletti A, Missale G, Fowler P, Giuberti T, Fiaccadori F, Ferrari C. 1991. Cytotoxic T lymphocytes recognize an

HLA-A2-restricted epitope within the hepatitis B virus nucleocapsid antigen. J Exp Med 174:1565–1570.

Poynard T, Bedossa P, Opolon P. 1997. Natural history of liver fibrosis progression in patients with chronic hepatitis C. Lancet 349:825–832.

Rosenberg S. 2001. Recent advances in the molecular biology of hepatitis C virus. J Mol Biol 313:451–464.

Seeff LB. 2002. Natural history of chronic hepatitis C. Hepatology 36:S35–S46.

Simmonds P. 1995. Variability of hepatitis C virus. Hepatology 21:570–583.

Tsubota A, Chayama K, Ikeda K, Yasuji A, Koida I, Saitoh S, Hashimoto M, Iwasaki S, Kobayashi M, Hiromitsu K. 1994. Factors predictive of response to interferon-alpha therapy in hepatitis C virus infection. Hepatology 19:1088–1094.

Vogt M, Lang T, Frosner G, Klingler C, Sendl AF, Zeller A, Wiebecke B, Langer B, Meisner H, Hess J. 1999. Prevalence and clinical outcome of hepatitis C infection in children who underwent cardiac surgery before the implementation of blood-donor screening. N Engl J Med 341:866–870.

Wiese M, Berr F, Lafrenz M, Porst H, Oesen U. 2000. Low frequency of cirrhosis in a hepatitis C (genotype 1b) single-source outbreak in Germany: A 20-year multicenter study. Hepatology 32:91–96.

Yuen MF, Lai CL. 2006. Response to combined interferon and ribavirin is better in patients infected with hepatitis C virus genotype 6 than genotype 1 in Hong Kong. Intervirology 49:96–98.

Hepatology Research 2009 doi: 10.1111/j.1872-034X.2009.00582.x

Original Article

Development of HCC in patients receiving adefovir dipivoxil for lamivudine-resistant hepatitis B virus mutants

Tetsuya Hosaka,[1] Fumitaka Suzuki,[1] Masahiro Kobayashi,[1] Miharu Hirakawa,[1] Yusuke Kawamura,[1] Hiromi Yastuji,[1] Hitomi Sezaki,[1] Norio Akuta,[1] Yoshiyuki Suzuki,[1] Satoshi Saitoh,[1] Yasuji Arase,[1] Kenji Ikeda,[1] Yuzo Miyakawa[2] and Hiromitsu Kumada[1]

[1]*Department of Hepatology, Toranomon Hospital, and* [2]*Miyakawa Memorial Research Foundation, Tokyo, Japan*

Aim: To identify factors for the development of hepatocellular carcinoma (HCC) in the patients who receive adefovir add-on lamivudine for treatment of lamivudine-resistant hepatitis B virus (HBV) mutants.

Methods: A total of 247 patients who developed lamivudine-resistant HBV mutants, with an increase of HBV DNA ≥ 1 log copies/mL, received adefovir dipivoxil 10 mg add-on lamivudine 100 mg daily during a median of 115 weeks (range: 25–282 weeks). They were followed for the development of HCC by imaging modalities every 3–6 months.

Results: HCC developed in 18 of the 247 (7.3%) patients. Eight factors were in significant association with the development of HCC by the univariate analysis. They included age, cirrhosis, platelet counts, levels of bilirubin, aspartate aminotransferase (AST), alanine aminotransferase and α-fetoprotein, as well as YMDD mutants at the start of adefovir dipivoxil. By the multivariate analysis, AST levels, YIDD mutants, cirrhosis and age were independent factors for the development of HCC. By the Kaplan-Meier analysis, AST levels ≥ 70 IU/L, YIDD mutants, cirrhosis and age ≥ 50 years increased the risk of HCC ($P = 0.018$, $P = 0.035$, $P = 0.002$ and $P = 0.014$, respectively). HCC developed more frequently in the patients with than without cirrhosis at the start of adefovir (10/59 [16.9%] vs. 8/188 [4.3%], $P = 0.002$).

Conclusion: HCC can develop in cirrhotic patients receiving adefovir add-on lamivudine. Hence, the patients with baseline AST ≥ 70 IU/L and YIDD mutants would need to be monitored closely for HCC.

Key words: adefovir dipivoxil, chronic hepatitis B, hepatitis B virus, hepatocellular carcinoma, lamivudine, rescue therapy

INTRODUCTION

WORLDWIDE, AN ESTIMATED 400 million people are infected with hepatitis B virus (HBV) persistently, and one million die of decompensated cirrhosis and/or hepatocellular carcinoma (HCC) annually.[1,2] Interferon (IFN) was introduced for treatment of chronic hepatitis B, and it has been replaced for pegylated-IFN.[3] Due to substantial side-effects and requirement for injection, however, IFN-based therapies are not favored.

In 1998, lamivudine was approved as the first nucleot(s)ide analogue for treatment of chronic hepatitis B,[4] and then adefovir in 2002.[5] Due to its lower costs and safety records, lamivdine has gained a wide popularity for treatment of chronic hepatitis B. However, drug-resistant mutants arise in parallel with the duration of lamivudine, in 12.5% after 1 year, in 43.8% after 3 years, and 62.5–70.2% after 5 years.[6,7] For preventing breakthrough hepatitis induced by lamivudine-resistant HBV mutants, additional adefovir dipivoxil 10 mg daily has been recommended;[8,9] it is more effective than switching to adefovir monotherapy and has fewer chances of developing drug-resistant mutants.[10,11]

Since 1995, 930 patients with chronic hepatitis have been treated with lamivudine in the Department of Hepatology at the Toranomon Hospital in Metropolitan Tokyo.[12] HBV mutants with mutations in the thyrosine-methionine-aspartic acid-aspartic acid (YMDD) motif elicited in the 247 (26.5%) patients, and they started to receive additional adefovir since December, 2002.[13,14] However, HCC developed in 18 (7.3%) of them during the combination therapy for 25–282 weeks; HCC has

Correspondence: Dr Tetsuya Hosaka, Department of Hepatology, Toranomon Hospital, 1-3-1, Kajigaya, Takatsu-ku, Kawasaki 213-8587, Japan. Email: hosa-p@toranomon.gr.jp
Received 25 April 2009; revision 6 June 2009; accepted 9 June 2009.

not been reported in any of the patients who have received adefovir add-on lamivudine for 5 years.[15–17] Hence, factors for the development of HCC in the patients receiving adefovir add-on lamivudine were sought for in a retrospective study.

METHODS

Patients

OVER A PERIOD of 13 years, from September 1995 to September 2007, 930 patients with chronic hepatitis B received long-term lamivudine treatment at the Department of Hepatology at the Toranomon Hospital in Metropolitan Tokyo. Drug-resistant YMDD mutants developed in 247 (26.5%) of them, accompanied by an increase in HBV DNA ≥ 1 log copies/mL, and they received adefovir 10 mg in addition to lamivudine 100 mg daily during the median of 115 weeks (range: 25–282 weeks). They have been followed for liver function and virological markers of HBV infection monthly, as well as blood counts and tumor makers including alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist-II (PIVKA-II). Cirrhosis was diagnosed by laparoscopy or liver biopsy, and in the patients who had not received them, by clinical data, imaging modalities and portal hypertension. HCC was diagnosed by hypervascularity on angiography and/or histological examination, characteristic features of computed tomography, magnetic resonance imaging and ultrasonography. An informed consent was obtained from each patient in this study, and the protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a *priori* approval by the institution's human research committee.

Markers of HBV infection

Hepatitis B e antigen (HBeAg) was determined by enzyme-linked immunosorbent assay (ELISA) with commercial kits (HBeAg EIA, Institute of Immunology, Tokyo). HBV DNA was quantitated by the Amplicor monitor assay (Roche Diagnostics, Tokyo) with a dynamic range over 2.6–7.6 log copies/mL. Genotypes of HBV were determined serologically by the combination of epitopes expressed on the pre-S2 region product, which is specific for each of the seven major genotypes (A–G),[18,19] with use of commercial kits (HBV Genotype EIA, Institute of Immunology).

Detection of YMDD mutants

YMDD mutants were determined by polymerase chain reaction (PCR)-based enzyme-linked mini-sequence assay (PCR-ELIMA) with commercial kits (Genome Science Laboratories, Tokyo).

Statistical analyses

Categorial variables were compared between groups by the χ^2 test, and non-categorical variables by the Mann–Whitney *U*-test. A *P*-value < 0.05 was considered significant. Factors associated with HCC by univariate analysis were evaluated by the multivariate analysis by the stepwise Cox proportional hazard model. Development of HCC with time was analyzed by the Kaplan–Meier method, and differences were evaluated by the log-rank test. Data were analyzed by the SPSS software, version 11.0 (Chicago, IL).

RESULTS

Baseline characteristics of the patients who did and who did not develop hepatocellular carcinoma during adefovir add-on lamivudine treatment

TABLE 1 COMPARES characteristics at the start of adefovir between the 18 patients who developed HCC and the 229 who did not. Eight factors were associated with the development of HCC by the univariate analysis. They included age, cirrhosis, platelet counts, bilirubin, AST, alanine aminotransferease (ALT) and α-fetoprotein (AFP) levels, as well as YMDD mutants. HCC developed more frequently in the patients with than without cirrhosis at the start of adefovir (10/59 [16.9%] vs. 8/188 [4.3%], $P = 0.002$). There were 61 (26.6%) patients who had cirrhosis at the start of adefovir. Of them, one of the 18 (2.2%) with HCC and 18 of the 229 (2.2%) without HCC presented with decompensation; no patients developed decompensation after the start of adefovir.

Rates of HBV DNA disappearance from serum (< 2.6 log copies/mL) were: 55% (113/207) at 1 year, 71% (119/168) at 2 years, 77% (78/101) at 3 years and 85% (35/41) at 4 years. Rates of AST nomarlization (< 38 IU/L) were: 87% (179/207) at 1 year, 90% (151/168) at 2 years, 92% (93/101) at 3 years and 95% (39/41) at 4 years; and those of ALT normalization (< 50 IU/L) were: 88% (183/207) at 1 year, 91% (153/168) at 2 years, 93% (94/101) at 3 years and 98% (40/41) at 4 years. There were no differences in the rate of HBV DNA disappearance from serum between the patients with and without HCC: 57% (8/14) vs. 54% (105/193) at 1 year ($P = 1.0$); 86% (12/14) vs. 70% (107/154) at 2 years ($P = 0.229$); and 89% (8/9) vs.

Table 1 Characteristics of patients who did and did not develop hepatocellular carcinoma (HCC) at the start of adefovir†

	HCC developed ($n = 18$)	HCC did not develop ($n = 229$)	Differences *P*-value
Duration of lamivudine before the start of adefovir	128 (31–346)	144 (13–617)	0.321
Age (years)	52 (35–75)	45 (26–75)	0.008
Men	15 (83%)	183 (80%)	1.000
Cirrhosis	10 (56%)	51 (22%)	0.004
Platelets ($\times 10^3/mm^3$)	12.0 (4.6–19.7)	16.3 (3.1–31.9)	0.001
Albumin (g/dL)	3.6 (2.3–4.7)	3.9 (2.8–4.7)	0.073
Bilirubin (mg/dL)	0.8 (0.5–15.5)	0.7 (0.2–6.0)	0.046
Creatinine (mg/dL)	0.8 (0.5–1.0)	0.8 (0.4–1.6)	0.950
AST (IU/L)	119 (55–248)	66 (14–1413)	0.003
ALT (IU/L)	151 (61–576)	104 (13–1563)	0.035
AFP (ng/dL)	8 (2–130)	4 (1–282)	0.026
HBV genotypes			0.228
C	18 (100%)	189 (87%)	
Others	0	27 (13%)	
HBeAg	8 (44%)	132 (58%)	0.323
HBV DNA (log copies/mL)	7.1 (4.4–>7.6)	7.1 (<2.6–>7.6)	0.623
YMDD mutants			0.041
YIDD	13 (72%)	109 (45%)	
YVDD	5 (28%)	62 (25%)	
YI/VDD	0	56 (23%)	

†Values are the median with the range in parentheses or *n* with percent in parentheses.
AFP, alpha-fetoprotein; ALT, alaine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus.

92% (85/92) at 3 years ($P = 0.555$). Rates of normalized AST levels in the patients with and without HCC were: 50% (7/14) vs. 90% (173/193) at 1 year ($P < 0.001$); 79% (11/14) vs. 91% (140/154) at 2 year ($P = 0.166$); and 67% (6/9) vs. 95% (87/92) at 3 year ($P = 0.037$). Rates of ALT normalization in the patients with and without HCC were: 71% (10/14) vs. 90% (174/193) at 1 year ($P = 0.037$); 79% (11/14) vs. 90% (139/154) at 2 year ($P = 0.189$); and 56% (5/9) vs. 92% (85/92) at 3 year ($P = 0.015$). Thus, normalization of AST and ALT was less frequent in the patients with than without HCC.

Characteristics of the 18 patients who developed HCC are compared between the baseline and at the development of HCC (Table 2). At the start of adefovir, 10 (56%) of them had developed cirrhosis and 16 (89%) had AST levels ≥ 70 IU/L. HBV DNA was not detectable in 10 (56%) of them at the development of HCC. Of the eight patients with detectable HBV DNA levels (≥ 2.6 log copies/mL), five (63%) developed HCC within 1 year after the start of adefovir. AST was elevated (> 38 IU/L) in eight patients, including four (50%) without detectable HBV DNA levels.

Factors independently associated with the development of hepatocellular carcinoma

Eight factors associated with the development of HCC by the univariate analysis, including age, cirrhosis, platelet counts, bilirubin, AST, ALT and AFP levels, as well as YMDD mutants (Table 1), were evaluated by the multivariate analysis. AST ≥ 70 IU/L, YIDD mutants, age ≥ 50 years and cirrhosis at the baseline were independent risk factors for the development of HCC (Table 3). There were no differences in the distribution of YIDD, YVDD and the mixture thereof among the patients with distinct AST, ALT or HBV DNA levels or between those with and without cirrhosis at the start of adefovir. HBV mutants with mutations resistant to adefovir (rtA181T/S, rtN236T) occurred in two of the 247 (0.8%) patients; none of them developed HCC.

The median time between the elevation of HBV DNA > 5.0 log copies/mL and the administration of adefovir was 124 (range: 0–815) days for the 13 patients who developed HCC and 147 (0–3268) days for the 166 patients who did not ($P = 0.605$). The median time between the elevation of ALT > 43 IU/L and the start of

Table 2 Characteristics of the 18 patients at commencement of adefovir (ADV) and development of hepatocellular carcinoma (HCC)

Patient no.	Age (years)	Sex	At the commencement of ADV					YMDD mutant	Period of ADV (years)	At the development of HCC		
			Liver disease	AST (IU/L)	ALT (IU/L)	HBeAg	HBV DNA (log copies/mL)			AST (IU/L)	ALT (IU/L)	HBV DNA (log copies/mL)
1	50	M	CH	248	576	−	6.9	I	4.5	26	27	< 2.6
2	35	M	LC	217	164	+	7.5	I	1.6	54	34	< 2.6
3	50	M	LC	192	272	+	> 7.6	I	1.2	68	89	< 2.6
4	61	M	CH	192	332	−	6.9	I	2.8	22	23	< 2.6
5	65	M	CH	174	219	−	5.2	V	0.1	30	43	< 2.6
6	58	M	CH	160	216	−	6.5	V	2.2	41	32	< 2.6
7	53	M	LC	127	97	+	> 7.6	I	0.5	55	41	3.2
8	75	M	LC	119	209	+	> 7.6	V	1.1	121	125	2.6
9	58	F	CH	118	214	+	4.4	I	3.3	21	13	< 2.6
10	48	M	CH	116	99	+	> 7.6	I	3.3	32	36	< 2.6
11	51	F	LC	111	130	−	5.3	I	0.9	88	95	< 2.6
12	47	M	CH	85	138	+	> 7.6	I	1.3	28	29	3.1
13	61	M	LC	81	65	−	5.6	I	0.2	32	27	2.9
14	59	F	LC	80	132	−	> 7.6	V	0.1	32	41	3.2
15	40	M	LC	75	124	−	6.3	I	3.8	21	24	< 2.6
16	48	M	CH	71	61	−	6.6	I	0.6	48	26	3.7
17	55	M	LC	55	76	+	7.3	I	0.2	50	64	5.4
18	43	M	LC	27	21	−	5.4	V	1.6	30	23	3.7

ALT, alaine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; I, YIDD mutant; LC, cirrhosis; V, YVDD mutant.

Table 3 Independent risk factors influencing the development of hepatocellular carcinoma

Factors	Category	Hazard ratio (95% CI†)	*P*-value
AST (IU/l)	1: < 70	1	0.016
	2: ≥ 70	6.21 (1.40–27.5)	
YMDD mutants	1: YVDD or YV/IDD	1	0.012
	2: YIDD	3.97 (1.36–11.6)	
Age (years)	1: < 50	1	0.023
	2: ≥ 50	3.24 (1.17–8.95)	
Cirrhosis	1: Absent	1	0.030
	2: Present	1.42 (1.04–1.96)	

†Confidence interval.

adefovir was 59 (0–896) days for the patients who developed HCC and 54 (0–3240) days for those who did not ($P = 0.330$). Hence, exacerbation of hepatitis was not a risk factor for the development of HCC.

Age-specific risk factors for the development of HCC were evaluated by the multivariate analysis. In the patients < 50 years, platelet counts < $13 \times 10^3/mm^3$ was the only significant risk factor for HCC (hazard ratio 6.88 [95% confidence interval; 1.26–37.6]), while AST levels ≥ 70 IU/L was that in those ≥ 50 years (hazard ratio: 9.50 [95% confidence interval 1.20–74.9]).

Factors increasing the cumulative incidence of hepatocellular carcinoma

AST levels ≥ 70 IU/L at the start of adefovir increased the development of HCC during follow-ups ranging to 5 years (Fig. 1). HCC developed more frequently in the patients with YIDD mutants than in those with YVDD or the mixture of YVDD and YIDD mutants (Fig. 2). The cumulative incidence of HCC in the patients with YIDD mutants alone was: 4% at 1 year, 10% at 3 years and 43% at 5 years. In contrast, HCC never developed in the patients with the mixture of YIDD and YVDD mutants through 5 years of follow-up. HCC developed more frequently in the patients with cirrhosis and those aged ≥ 50 years (Figs 3,4, respectively).

DISCUSSION

HCC DEVELOPED IN 18 of the 247 (7.3%) patients who had received adefovir add-on lamivudine during a long-term ranging to 5 years. There were some differences in the characteristics at the start of adefovir dipivoxil between the patients who did and who did not develop HCC. The patients who developed HCC were older, more frequently had signs of early cirrhosis with less platelet counts, as well as higher levels of AST, ALT and AFP, than those who did not develop HCC. By multivariate analysis, AST ≥ 70 IU/L, YIDD mutants in

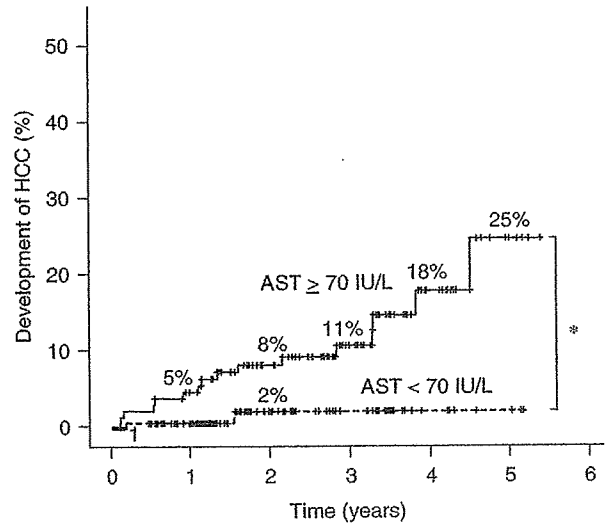


Figure 1 Kaplan–Meier life-table for the cumulative incidence of hepatocellular carcinoma (HCC) during adefovir add-on lamivudine in the patients with different baseline aspartate aminotransferase (AST) levels. *$P = 0.009$.

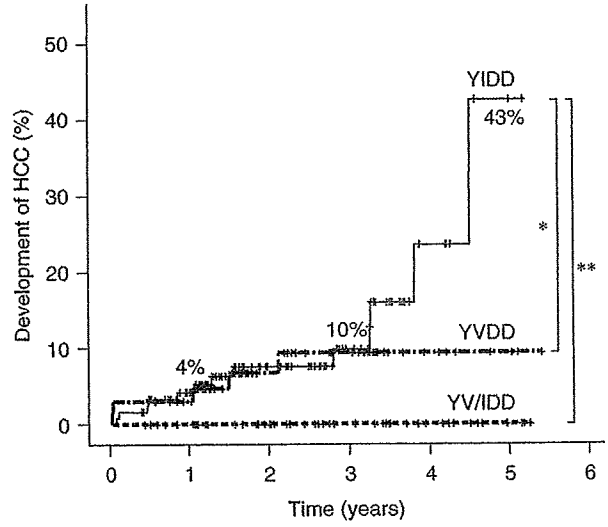


Figure 2 Kaplan–Meier life-table for the cumulative incidence of hepatocellular carcinoma (HCC) during adeforvir add-on lamivudine in the patients with distinct YMDD mutants.*$P = 0.035$; **$P = 0.003$.