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＜短　報＞

C型慢性肝炎に対するペグインターフェロンとリバビリン併用療法における NS3-4Aプロテアーゼ阻害剤（Telaprevir）併用12週間治療のウイルス学的効果の検討

瀬崎ひとみ[*,$]　鈴木　文孝　芥田　憲夫　平川　美晴　川村　祐介
八辻　寛美　保坂　哲也　小林　正宏　鈴木　義之　斎藤　聡
荒瀬　康司　池田　健次　熊田　博光

緒言：現在，C型慢性肝炎に対する治療はペグインターフェロン（PEG-IFN）とリバビリンの併用療法が標準治療法となっているが，海外においては新規の抗HCV薬であるNS3-4A protease inhibitor（Telaprevir）の強力なHCV増殖抑制作用が報告され[1]，PEG-IFNとリバビリンとの3者併用療法により治療効果が飛躍的に改善することが明らかにされてきている．そこで今回我々は，genotype 1型，高ウイルス量のC型慢性肝炎患者に対してPEG-IFNα-2bとリバビリンの併用療法にTelaprevirを併用した3者併用12週間治療のウイルス学的効果を検討した．

対象と方法：対象は，genotype 1b，高ウイルス量の症例で，当院において2008年5月から2008年7月までにPEG-IFNα-2bとリバビリン治療にTelaprevirを併用する3者併用12週間治療を施行することに同意した初回治療例の10例である．男性4例，女性6例，年齢は36-64歳（中央値51歳）であった．Telaprevirは無作為に2群に分類され，A群は1回750 mg，B群は1回500 mgで8時間ごとに3回投与された．投与中のHCV RNAの陰性化をTaqMan PCR法にて評価し，さらに12週併用療法終了後24週経過観察した時点での完全著効（SVR）率を評価した．

結果：治療中および治療終了後の経過をFig. 1に示す．12週間の治療を完遂できたのは5例（50%）であった．4例はヘモグロビン値の低下，1例は倦怠感により治療中止となった．しかしながら，HCV RNAは全例で治療中に陰性化を認め，陰性化時期は2〜5週（中央値2週）と非常に早期であった．Case 1〜5は12週までに中止となったが，このうち2週目で陰性化した3例は5週目，7週目，10週目に治療を中止したにもかかわらずSVRとなった．Case 6〜10は12週間投与を完遂した症例であるが，5週目で陰性化した1例を除き，4例がSVRに至った．最終的なSVR率は全体で7/10例（70%）と高率であった．

Telaprevirの用量はA群6例，B群4例に割り付けられた．中止率は両群とも50%であり，SVR率はA群4/6例（66.7%），B群3/4例（75%）と両群間で治療効果，副作用に差は認めなかった．

男女別にみると，男性3/4例（75.0%），女性4/6例（66.7%）であり，50歳以上の女性のみでみても，3/3例（100%）と高率にSVRを得られた．

HCV core領域70番目のアミノ酸変異の有無から治療効果をみると，wild typeの症例は5/6例（83.3%），mutant typeでは2/4例（50%）がSVRに至った．

考察：NS3-4A protease inhibitor（Telaprevir）を用いたPEG-IFNとリバビリンとの3者併用療法は非常に抗ウイルス効果が高く，以前我々は，genotype 1b型の慢性肝炎症例に対する3者併用12週間投与における治療中のHCV RNA動態を検討し，2週目で50%，4週目で79%，8週目で94%，12週目で100%にHCV RNAの陰性化を認めたことを報告した[2]．今回は，この症例のうち初回治療例について24週間の経過観察終了後の最終的な治療成績を検討した．その結果，初回治療例に対しては12週間の治療でもSVRに至る症例が70%に達し，ウイルス排除を目的とした治療として有用であることが判明した．これは欧米のPROVE1[3]および

虎の門病院肝臓センター
*Corresponding author: hitomis@mx1.harmonix.ne.jp
$利益相反申告：瀬崎ひとみは株式会社田辺三菱製薬
＜受付日2010年2月17日＞＜採択日2010年5月18日＞

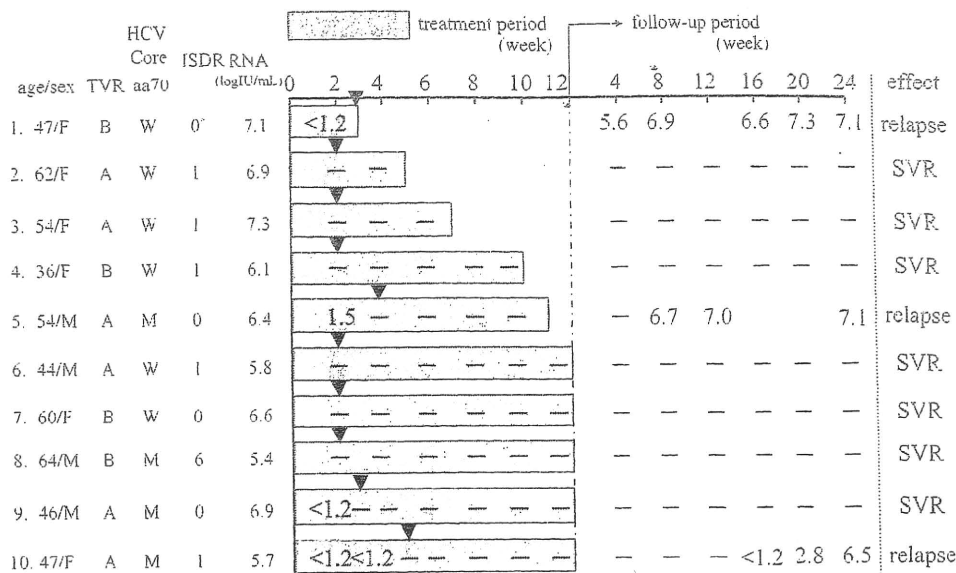

Fig. 1 Clinical course and dynamics of HCV RNA during and after 24 weeks of the triple treatment with telaprevir, pegylated interferon and ribavirin. TVR, telaprevir: A, 2250 mg/day; B, 1500 mg/day; W, wild type; M, mutant type; ISDR, interferon sensitivity determining resion; * Numbers of amino acids substitutions in ISDR is shown. Arrowheads show the time of HCV RNA loss from the serum.

PROVE2[4]における3剤12週間併用療法の成績（35%と60%）と比較しても良好な成績であるといえる．欧米ではgenotype 1a型の割合が高く，それぞれの対象症例はgenotype 1a型が53%と45%を占めているのが特徴であり，この点が当院のgenotype 1b型の成績と比較しSVR率が低い原因であると考えられる．また，今回の検討ではPEG-IFNとリバビリンの2剤併用療法では治療効果が低いとされる50歳以上の女性においても，全例がSVRに至っており，こういった難治と考えられる症例に対しても治療効果を改善できるものと期待される．

一方，HCV core領域の70番目のアミノ酸がmutant typeの場合，治療中のHCV RNAの陰性化率は良好であるが，12週間の治療ではwild typeに比較してSVR率が低い可能性が示唆された．以前の我々の検討より，3剤併用療法時の治療早期のHCV RNAの低下にcore領域のアミノ酸変異が関与していることを報告しており[5]，今回の検討から最終的な治療成績にもHCV core領域の変異の有無が関与する可能性が考えられる．

Telaprevirを併用した3剤併用療法中における注意点としては，海外からの報告[3][4]にもあるように，掻痒，皮疹の出現頻度が約40-50%と高い点である．当院でも，1例全身性の皮疹の出現により治療中止となった症例を経験した[2]．また，貧血の出現も2剤の併用療法時に比し多いと報告されており，当院でも今回の検討症例を含めた5例がヘモグロビン値8.5 g/dL以下となり，治療中止となったことを報告した．その内3例は5週以内と早期に中止となっており，3剤併用療法時にはヘモグロビンの低下についてより厳重な経過観察が必要であり，早期にリバビリンの減量を考慮する必要があると考えられる．

今回の検討により，genotype 1b型の初回治療例に対しては，Telaprevirを併用した3者併用療法は12週間でも治療効果が高いことが示唆された．Telaprevirの用量については，1日2250 mg群と1500 mg群とで治療効果に差を認めなかったが，最終的な適正用量については現在進行中の12週間の3者併用療法後さらに12週間PEG-IFNとリバビリンを投与する24週間治療の有効性，安全性の結果をもとに検討されるべきである．また24週併用することにより，50歳以上の女性，HCV core領域70番目のアミノ酸がmutant typeの症例あるいは前治療で無効であった症例など難治と考えられる症例でも治療効果を改善しうるか，さらに詳細な検討が必要であると思われる．

索引用語：C型慢性肝炎，リバビリン併用療法，
NS3-4Aプロテアーゼ阻害剤

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英文要旨

The efficacy of virological response in treatment-naïve patients with chronic hepatitis C treated by NS3-4A protease inhibitor (telaprevir), pegylated interferon and ribavirin for 12 weeks

Hitomi Sezaki*, Fumitaka Suzuki, Norio Akuta, Miharu Hirakawa, Yusuke Kawamura, Hiromi Yatsuji, Tetsuya Hosaka, Masahiro Kobayashi, Yoshiyuki Suzuki, Satoshi Saitoh, Yasuji Arase, Kenji Ikeda, Hiromitsu Kumada

We investigated the efficacy of the triple treatment with telaprevir, pegylated interferon (PEG-IFN) and ribavirin for 12 weeks in treatment-naïve patients infected with hepatitis C virus (HCV) genotype 1b and high baseline viral loads. All of 10 cases became HCV-RNA negative during treatment. SVR rate attained to a high rate, 70% (7/10). Especially, SVR rate of females over 50 years old attained 100% (3/3). HCV RNA was lost from serum rapidly in patients infected with HCV-1b in high viral loads, and SVR rate of the triple treatment for 12weeks was high. Our results suggested that triple treatment with telaprevir, PEG-IFN and ribavirin could improve the efficacy in treatment-naïve patients.

Key words: chronic hepatitis C, interferon plus ribavirin combination therapy, NS3-4A protease inhibitor

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Department of Hepatology, Toranomon Hospital, Tokyo, Japan
*Corresponding author: hitomis@mx1.harmonix.ne.jp

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＜短　報＞

核酸アナログ未使用のB型慢性肝炎症例へのエンテカビル治療中に rtA181T 変異ウイルスが増殖した1症例

八辻　寛美[1)*]　鈴木　文孝[1)]　平川　美晴[1)]　川村　祐介[1)]　瀬崎ひとみ[1)]
保坂　哲也[1)]　芥田　憲夫[1)]　小林　正宏[1)]　鈴木　義之[1)]　斉藤　聡[1)]
荒瀬　康司[1)]　池田　健次[1)]　岩崎　里美[2)]　峰田　理恵[2)]　綿引　祥予[2)]
小林万利子[2)]　熊田　博光[1)]

緒言：核酸アナログ未使用のB型慢性肝炎患者へのエンテカビル治療中に，既報のエンテカビル耐性ウイルスが出現していないにもかかわらず，viral rebound を生じた症例を経験したため，報告する．

症例：51歳女性．1978年にB型慢性肝炎と診断され，2008年6月よりエンテカビル（0.5 mg/日）治療を開始した．治療開始時 HBV-DNA 7.2 log copies/ml，HBeAg 陽性，genotype C であった．2009年2月 HBV-DNA 2.5 log copies/ml まで下がるも，その後2009年4月 HBV-DNA 6.0 log copies/ml，8月 8.2 log copies/ml と viral rebound が出現し，トランスアミナーゼの上昇も認めた（Fig. 1）．

治療開始時および治療中の HBV-DNA polymerase RT 領域のアミノ酸配列の比較検討：患者血清から抽出された HBV-DNA は PCR 法にて増幅したのち，direct sequence 法にて塩基配列を決定した．クローニング解析もあわせて行った．ダイレクトシークエンスでは核酸アナログ未使用であるにもかかわらず，エンテカビル開始時に rtA181T 変異のわずかな混在を認め，クローニング解析では8.5%（3/35クローン）に rtA181T 変異を確認した．また治療開始後15カ月ではダイレクトシークエンスにて rtA181T 変異の混在の割合が増加しており，クローニング解析にて rtA181T 変異は39.5%（17/43クローン）に増加していた．尚，エンテカビル開始時および治療中に rtA181 以外の既報のエンテカビル耐性に関与するアミノ酸（rtL180，T184，S202，M204，M250）に変異は認められなかった（Fig. 1）．

考察：今回我々は，エンテカビル投与にて rtA181T 変異が増殖した症例を経験した．本症例はエンテカビル投与中に viral rebound を生じ，その際既報のエンテカビル耐性ウイルスは出現せず，治療開始時よりわずかに認められていた rtA181T 変異ウイルスが増殖していた．クローニング解析にて rtA181T 変異ウイルスは治療開始時8.5%から治療開始15カ月後に39.5%に増加し，他に有意なアミノ酸変異を認めないことから，rtA181T 変異がエンテカビル耐性に関与している可能性が考えられた．しかし本症例で出現した rtA181T 変異ウイルスのエンテカビル耐性への関与を証明するためには，今後本症例の血清を使用した in vitro の実験にて評価する必要があると考える．また本症例では viral rebound と同時にトランスアミナーゼ上昇も認めたが，軽度上昇にとどまっているため，現在もエンテカビル治療を継続し厳重にフォローしている．

本症例は，核酸アナログ未使用のB型慢性肝炎症例であったにもかかわらず，エンテカビル治療開始前より rtA181T 変異が存在していた．核酸アナログ未使用症例にラミブジン耐性に関与する rtL180M，rtM204V 変異が存在するという報告はあるが，本症例のように rtA181T 変異が核酸アナログ使用前に存在したという報告は過去になく，初めての報告である．

rtA181T 変異は以前よりアデホビル耐性に関与するアミノ酸変異として知られていたが，最近ではラミブジンとアデホビルの交差耐性のある変異であることがわかっている[1)]．このため rtA181T 変異に対してエンテカビルの効果が期待されている．しかし海外からは，ラミブジン耐性ウイルスに対するアデホビル単独治療

1）虎の門病院肝臓センター
2）虎の門病院肝臓研究室
*Corresponding author: h-ooga@mx1.harmonix.ne.jp

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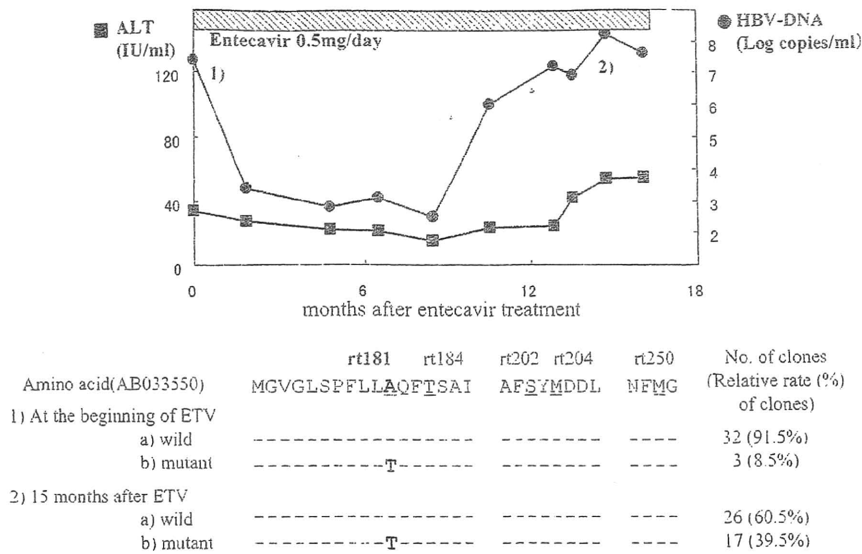

Amino acid(AB033550)	rt181 rt184 MGVGLSPFLLAQFTSAI	rt202 rt204 AFSYMDDL	rt250 NFMG	No. of clones (Relative rate (%) of clones)
1) At the beginning of ETV				
a) wild	-----------------	--------	----	32 (91.5%)
b) mutant	----------T------	--------	----	3 (8.5%)
2) 15 months after ETV				
a) wild	-----------------	--------	----	26 (60.5%)
b) mutant	----------T------	--------	----	17 (39.5%)

Fig. 1 Clinical course and clonal analysis of samples from patient with viral rebound during entecavir therapy

中に耐性ウイルス(rtA181T/V または N236T 変異ウイルス)が出現した症例は，ラミブジン耐性ウイルスのみの症例に比べ，エンテカビル治療におけるウイルス抑制効果が低いという報告があり[2]，また本症例のようにエンテカビル治療にて rtA181T 変異ウイルスが増加する症例も存在することから，今後 rtA181T 変異ウイルスに対する治療として，エンテカビル以外の核酸アナログ（テノフォビル，その他新規薬剤等）の有効性も検討していく必要があると考えられる．

索引用語：エンテカビル，耐性ウイルス，rtA181T

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英文要旨

Increase of rtA181T mutant strains during entecavir therapy for a patient with chronic hepatitis B virus infection

Hiromi Yatsuji[1)*], Fumitaka Suzuki[1)],
Miharu Hirakawa[1)], Yusuke Kawamura[1)],
Hitomi Sezaki[1)], Tetsuya Hosaka[1)],
Norio Akuta[1)], Masahiro Kobayashi[1)],
Yoshiyuki Suzuki[1)], Satoshi Saitoh[1)],
Yasuji Arase[1)], Kenji Ikeda[1)],
Satomi Iwasaki[2)], Rie Mineta[2)],
Sachiyo Watahiki[2)], Mariko Kobayashi[2)],
Hiromitsu Kumada[1)]

A 51-year-old Japanese woman with chronic hepatitis B who had never treated with nucleotide analogues was admitted to our hospital and treated with entecavir. In this patient, entecavir successfully reduced the HBV level, but viral and biochemical breakthrough was observed at 10 months after the beginning of therapy. The HBV viral load reached up to 8.2 log copies/ml, but direct sequence analysis showed no LAM and ETV resistant-related mutation (rtT184, S202, M204, M250). Comparison by clonal analysis of samples obtained before and after the viral breakthrough showed the increase of the rtA181T mutant strains (8.5% versus 39.5%). It was considered that the rtA181T mutant

strain in this case might be related to entecavir resistance.

Key words: entecavir, drug-resistant mutant, rtA181T

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1) Department of Hepatology, Toranomon Hospital
2) Department of Research Institute for Hepatology, Toranomon Branch Hospital, Kawasaki
*Corresponding author: h-ooga@mx1.harmonix.ne.jp

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＜速　報＞

IL28B と HCV Core aa70 置換との関連

小林万利子[1)*]　鈴木　文孝[2)]　芥田　憲夫[2)]　鈴木　義之[2)]　瀬崎ひとみ[2)]
八辻　寛美[2)]　保坂　哲也[2)]　小林　正宏[2)]　川村　裕介[2)]　平川　美晴[2)]
荒瀬　康司[2)]　池田　健次[2)]　峰田　理恵[1)]　岩崎　里美[1)]　綿引　祥予[1)]
中村　祐輔[3)]　茶山　一彰[4)]　熊田　博光[2)]

はじめに：C型慢性肝炎の治療法であるPEG-IFN/Rivabirin併用療法のHCV genotype 1bで高ウイルス量症例では，その排除率が50％台である．この難治症例の治療効果予測因子としてHepatitis C virus NS5A領域のInterferon sensitivity-determining regionやCore領域の70番目，91番目のアミノ酸置換が有用であることは周知のごとくであったが，近年アメリカ・日本から宿主側因子として*IL28B*のSNPsがPEG-IFN/Rivabirin併用療法の治療効果予測として有用であると報告[1)~5)]されている．今回我々は，C型慢性肝疾患患者のHCV Core aa70と*IL28B*を測定し性差との関連性を検討した．

対象と方法：1997年から2005年までに虎の門病院倫理委員会及びヒトゲノム委員会で承認された同意書を得た患者291人のchromosome 19上の*IL28B*近傍の2つのSNPs（rs8099917（T/G），rs12979860（C/T））とHCV Core領域aa70を測定したHCV genotype 1bとした．内訳は，男性177人（年齢：21-82（中央値56歳），女性114人（年齢：37-82（中央値61）歳）であった．

*IL28B*のSNPs（rs8099917，rs12979860）のタイピングはInvador assay，Taqman assayまたはdirect sequencing法にて決定した．rs8099917は290例，rs12979860は289例のタイピング可能であった．HCV Core領域aa70の測定は，PCR-direct sequence法にて測定した．性別とSNPの遺伝子型を検討した．

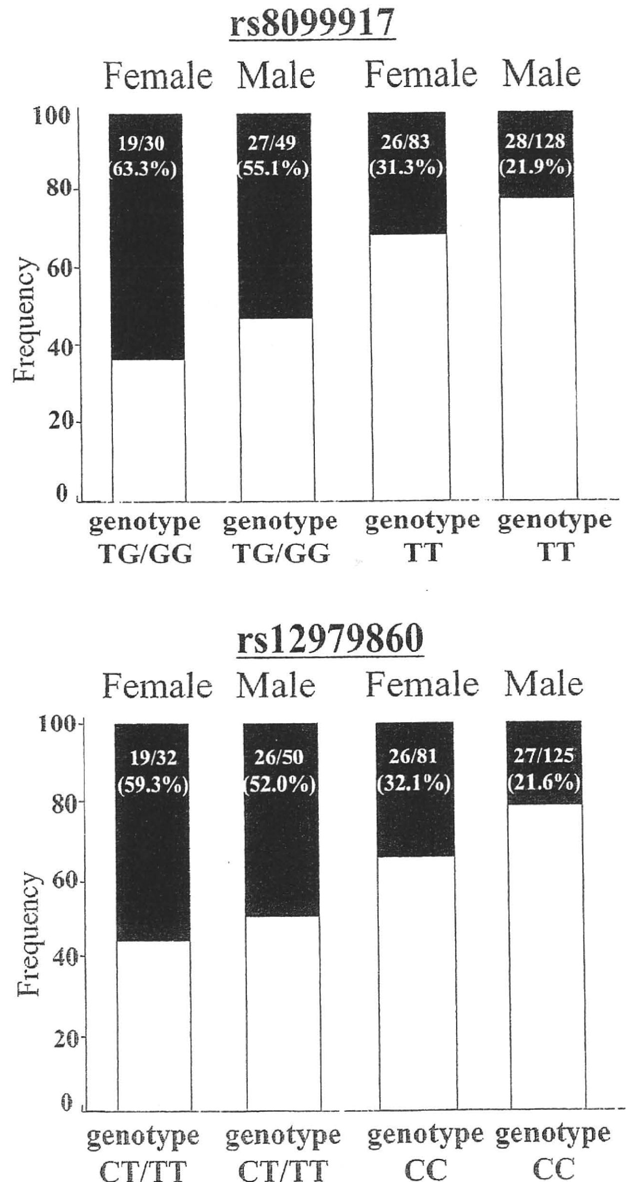

Fig. 1 Relationship between *IL28B* SNPs and amino acid substitution in hepatitis C virus core region in patients with chronic hepatitis C. Black bars represent aa70 mutant (Gln) while white bars represent aa70 wild (Arg)

1）虎の門病院肝臓研究室
2）虎の門病院肝臓センター
3）理化学研究所ゲノム医科学研究センター
4）広島大学大学院医歯薬学総合研究科分子病態制御内科学
*Corresponding author: vj7m-kbys@asahi-net.or.jp
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結果：Core aa70 置換からみた *IL28B* の SNP と性差の頻度

rs8099917 に関しては，Core aa70 の Mutant（Gln）がもっとも高頻度にみられたのは genotype TG/GG の女性で 19/30 例（63.3%），次いで男性の genotype TG/GG で 27/49 例（55.1%），女性の genotype TT で 26/83 例（31.3%）であり，最も低率であったのが男性の genotype TT で 28/128 例（21.9%）であった（Fig. 1）．

rs12979860 においても同様の傾向を認め，女性の genotype CT/TT で 19/32 例（59.3%），男性の genotype CT/TT で 26/50 例（52.0%）であり，女性の genotype CC で 26/81 例（32.1%），男性の genotype CC で 27/125 例（21.6%）であった（Fig. 1）．

考案：近年，*IL28B* 領域の SNPs が C 型肝炎ウイルスの自然排除[4]および慢性肝炎の PEG-IFN/Rivabirin 併用療法の治療効果と関連があることが報告された[1)~3)]．我々は，ウイルス側の予測因子である Core aa70 置換について性差を加味して SNP の遺伝子型別にその頻度を解析したところ 2 つの SNP で女性のマイナーアレルホモ接合体及びヘテロ接合体群において Core aa70（Gln）Mutant の頻度がいずれも 50% 台であった．このことは，高齢の女性は PEG-IFN/Rivabirin 併用療法の治療効果が低い傾向を示すことなんらかの関連が推測され，女性において Core aa70 は，経過観察中にメジャークローンとマイナークローンが入れ代わる可能性が示唆された．今後，治療効果予測として宿主側因子の一つである *IL28B* の SNPs と Core aa70 置換の組み合わせにより，より有効な治療効果予測が可能になると思われた．

索引用語：C 型慢性肝疾患，*IL28B*，コア領域

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英文要旨

Relationship between SNPs in the *IL28B* region and amino acid substitutions in HCV core region in Japanese patients with chronic hepatitis C

Mariko Kobayashi[1)*], Fumitaka Suzuki[2)], Norio Akuta[2)], Yoshiyuki Suzuki[2)], Hitomi Sezaki[2)], Hiromi Yatsuji[2)], Tetsuya Hosaka[2)], Masahiro Kobayashi[2)], Yusuke Kawamura[2)], Miharu Hirakawa[2)], Yasuji Arase[2)], Kenji Ikeda[2)], Rie Mineta[1)], Satomi Iwasaki[1)], Sachiyo Watahiki[1)], Yusuke Nakamura[3)], Kazuaki Chayama[4)], Hiromitsu Kumada[2)]

IL28 locus polymorphisms have been reported to affect PEG-IFN plus ribavirin combination therapy for patients with genotype 1b hepatitis C virus (HCV) infection. We examined a relationship between *IL28B* SNPs (rs8099917 and rs12979860) and amino acid substitutions in core region of HCV in patients with genotype 1b chronic hepatitis C. In each SNP, frequency of core aa 70 mutation was higher rate in female patients carrying minor allele than in male or female patients carrying no minor allele. Measurement of *IL28B* and Core aa70 before treatment is useful in PEG-IFN plus ribavirin therapy.

Key words: *IL28B*, HCV, core region

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1) Department of Research Institute for Hepatology, Toranomon Hospital, Kawasaki, Japan
2) Department of Hepatology, Toranomon Hospital, Tokyo, Japan
3) Laboratory for Molecular Medicine, Human Genome Center, The Institute of Medical Science, University of Tokyo, Tokyo, Japan
4) Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, Hiroshima, Japan
*Corresponding author: vj7m-kbys@asahi-net.or.jp

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Steatosis and hepatic expression of genes regulating lipid metabolism in Japanese patients infected with hepatitis C virus

Kohichiroh Yasui · Yuichi Harano · Hironori Mitsuyoshi · Kazuhiro Tsuji · Mio Endo · Tomoaki Nakajima · Masahito Minami · Yoshito Itoh · Yoh Zen · Yasuni Nakanuma · Toshikazu Yoshikawa · Takeshi Okanoue

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Abstract

Purpose Steatosis is a histological finding associated with the progression of chronic hepatitis C. The aims of this study were to elucidate risk factors associated with steatosis and to evaluate the association between steatosis and hepatic expression of genes regulating lipid metabolism.
Methods We analyzed 297 Japanese patients infected with hepatitis C virus and a subgroup of 100 patients who lack metabolic factors for steatosis. We determined intrahepatic mRNA levels of 18 genes regulating lipid metabolism in these 100 patients using real-time reverse transcription-polymerase chain reaction. Levels of peroxisome proliferator-activated receptor α and sterol regulatory element-binding protein 1 proteins were assessed by immunohistochemistry.
Results Steatosis was present in 171 (57%) of 297 patients. The presence of steatosis was independently associated with a higher body mass index, higher levels of γ-glutamyl transpeptidase and triglyceride, and a higher fibrosis stage. Steatosis was present in 43 (43%) of 100 patients lacking metabolic factors. Levels of mRNA and protein of peroxisome proliferator-activated receptor α, which regulates β-oxidation of fatty acid, were lower in patients with steatosis than in patients without steatosis.
Conclusions These findings indicate that impaired degradation of lipid may contribute to the development of hepatitis C virus-related steatosis.

Keywords Steatosis · Hepatitis C virus · Fibrosis · Gene expression · Peroxisome proliferator-activated receptor α

K. Yasui (✉) · Y. Harano · H. Mitsuyoshi · K. Tsuji · M. Endo · T. Nakajima · M. Minami · Y. Itoh · T. Yoshikawa · T. Okanoue
Department of Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kamigyo-ku, Kyoto 602-8566, Japan
e-mail: yasuik@koto.kpu-m.ac.jp

Y. Zen
Institute of Liver Studies, King's College Hospital, London, UK

Y. Nakanuma
Department of Human Pathology, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa 920-8640, Japan

T. Okanoue
Center of Gastroenterology and Hepatology, Saiseikai Suita Hospital, 1-2 Kawazono-cho, Suita 564-0013, Japan

Introduction

The prevalence of hepatic steatosis ranges from 40 to 86% (mean ~55%) in patients infected with hepatitis C virus (HCV) [1]. This range is higher than in the general population of adults in the Western world (20–30%) [2]. Steatosis appears to be associated with a more rapid progression of liver fibrosis and a lower response to interferon-α-based therapy [3–5].

Patients with HCV infection may have metabolic cofactors, such as obesity, diabetes, and alcohol abuse that contribute to the development of fatty liver. It is likely that two types of steatosis, viral and metabolic, coexist in patients with chronic hepatitis C [1, 3]. Known risk factors associated with steatosis include HCV genotype 3, a higher body mass index (BMI), diabetes, hyperlipidemia, ongoing alcohol abuse, older age, the presence of fibrosis, and

hepatic inflammation [1, 5]. However, different populations may have different risk factors for steatosis, and the distribution of HCV genotype differs from region to region. For example, HCV genotype 3, which is thought to be directly responsible for steatosis [6–8], is far less frequent in Japan than in Europe [7] or the United States [9].

Although the mechanisms of HCV-related steatosis are not well known, several viral and host factors appear to be involved [3]. In vitro studies [10] and a transgenic mouse models [11] have shown that HCV core protein can induce steatosis. HCV core protein, in turn, inhibits the activity of microsomal triglyceride transfer protein, which is essential for the assembly and secretion of very low density lipoproteins [12]. The intrahepatic levels of microsomal triglyceride transfer protein mRNA show an inverse correlation with the degree of steatosis in patients with chronic hepatitis C [13]. HCV infection and HCV core protein upregulates the expression of sterol regulatory element-binding protein 1 (SREBP1), a key transcriptional factor that activates the expression of genes involved in lipid synthesis [14, 15]. In addition, HCV core protein binds to retinoid X receptor α, a transcriptional regulator that controls many cellular functions including lipid metabolism [16]. HCV core protein also down-regulates the expression of peroxisome proliferator-activated receptor α (PPARα) and carnitine palmitoyl transferase 1 (CPT1) [17, 18], and the mRNA levels of PPARα and CPT1 are found to be reduced in patients with chronic HCV infection [19].

In the present study, we investigated the risk factors associated with steatosis in Japanese patients with chronic HCV infection. To elucidate the molecular mechanisms underlying HCV-related (i.e., viral) steatosis, we also systematically measured the intrahepatic expression levels of genes that regulate lipid degradation, secretion, synthesis, and uptake in patients who lack metabolic factors for steatosis.

Methods

Patients

The study included a total of 297 Japanese patients with chronic HCV infection who underwent liver biopsy between April 2004 and June 2006 at the Hospital of Kyoto Prefectural University of Medicine, Kyoto, Japan. To eliminate selection biases, the patients were recruited consecutively. Inclusion criteria were as follows: patients older than 18 years, positive for anti-HCV (third-generation enzyme immunoassay; Chiron, Emeryville, CA), and positive for serum HCV-RNA (Amplicor HCV assay; Roche Diagnostic Systems, Tokyo, Japan). Exclusion criteria were as follows: positive for hepatitis B virus surface antigen (radioimmunoassay; Dainabot, Tokyo, Japan); other types of liver diseases, including primary biliary cirrhosis, autoimmune hepatitis, alcoholic liver disease, Wilson's disease, or hemochromatosis; coinfection with human immunodeficiency virus; treated with antiviral or immunosuppressive agents within 6 months of enrollment; treated with drugs known to produce hepatic steatosis, including corticosteroids, high dose estrogen, methotrexate, or amiodarone within 6 months of enrollment; a history of gastrointestinal bypass surgery.

BMI was calculated using the following formula: weight in kilograms/(height in meters)^2. Obesity was defined as a BMI $\geq$25, according to the criteria of the Japan Society for the Study of Obesity [20]. Diabetes was defined as a fasting glucose level $\geq$126 mg/dl or by the use of insulin or oral hypoglycemic agents to control blood glucose. The ongoing alcohol intake per week recorded and converted to average grams per day. Significant alcohol intake was defined as consumption of >20 g/day.

The Ethics Committee of the Kyoto Prefectural University of Medicine approved this study. Informed consent was obtained from each patient in accordance with the Helsinki declaration.

Laboratory tests

Venous blood samples were taken in the morning after a 12-h overnight fast. The laboratory evaluation included a blood cell count and the measurement of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), total cholesterol, triglyceride, and fasting plasma glucose. These parameters were measured using the standard clinical chemistry techniques. The HCV genotype was determined according to the classification of Simmonds et al. [21]. The serum HCV-RNA level was quantified by Amplicor HCV monitor assay (version 2.0; Roche). These clinical and laboratory data were collected at the time of liver biopsy.

Histopathological examination

Liver biopsy specimens were obtained percutaneously from all patients for diagnostic purposes and divided into two parts. One part was fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin, Masson's trichrome, and silver impregnation. The sections were analyzed by an experienced hepatologist (T.O.) who was blinded to the laboratory parameters and clinical data. The degrees of inflammation and fibrosis were evaluated according to the criteria proposed by Desmet et al. [22]. Steatosis was graded based on percent of hepatocytes in the biopsy involved: none (0%), mild (<33%), moderate (33–66%), or severe (>66%) [23, 24]. The other part of the liver

biopsy was frozen immediately in liquid nitrogen and stored at −80°C for mRNA analysis.

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

We quantified mRNA by real-time fluorescence detection. Total RNA was obtained using an RNeasy Kit (Qiagen, Tokyo, Japan). Residual genomic DNA was removed and single-stranded complementary DNA was generated using a Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. Real-time quantitative RT-PCR experiments were performed with the LightCycler system using Faststart DNA Master Plus SYBR Green I (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's protocol. The 18 genes chosen for the current study, their protein products, and the primer sequences for amplifying them are listed in Table 1. The primers were designed using Primer3 version 0.4 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) on the basis of sequence data obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/). *ACTB* (β-actin gene) was used as an endogenous control.

Immunohistochemistry

Immunohistochemical staining for PPARα and SREBP1 was performed on formalin-fixed, paraffin-embedded sections from 100 liver biopsy specimens using rabbit polyclonal antibodies against human PPARα (clone H-98; Santa Cruz Biotechnology, Santa Cruz, CA) and SREBP1 (clone K-10; Santa Cruz Biotechnology), respectively. Deparaffinized sections were microwaved in a citrate buffer (pH 6.0) for 20 min. After blocking the endogenous peroxidase, the sections were incubated for 90 min at room temperature with 1:100 anti-PPARα or anti-SREBP1 antibodies. The sections were then incubated for 30 min at room temperature with peroxidase-labeled polymer-conjugated goat anti-rabbit immunoglobulin (Histofine Simple Stain Max-Po (Multi); Nichirei, Tokyo, Japan), followed by 3,3′-diaminobenzidine tetrahydrochloride as the chromogen. The sections were then lightly counterstained with hematoxylin. Negative controls were evaluated by substituting the primary antibody with nonimmunized rabbit serum. Immunoreactivity was scored according to the intensity of staining as follows: 1+, weak or absent; 2+, moderate; 3+, strong.

Table 1 Genes and primer sequences used for reverse transcription-polymerase chain reaction assays

Function/gene symbol	Alternate symbol	Protein product	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
Nuclear receptor				
PPARA	PPARα	Peroxisome proliferator-activative receptor α	ggaaagcccactctgccccct	agtcaccgaggaggggctcga
PPARG	PPARγ	Peroxisome proliferator-activative receptor γ	cattctggcccaccaactttgg	tggagatgcaggctccactttg
NR1H3	LXRα	Liver X receptor α	cgggcttccactacaatgtt	tcaggcggatctgttcttct
RXRA	RXRα	Retinoid X receptor α	tccttctcccaccgctccatc	cagctccgtcttgtccatctg
Fatty acid oxidation				
CPT1A	CPT1	Carnitine palmitoyltransferase 1	catcatcactggcgtgtacc	ttggcgtacatcgttgtcat
ACADS	SCAD	Short chain acyl-CoA dehydrogenase	ctcacgttggggaagaaaga	tgcgacagtcctcaaagatg
ACADM	MCAD	Medium chain acyl-CoA dehydrogenase	ttgagttcaccgaacagcag	agggggactggatattcacc
ACADL	LCAD	Long-chain acyl-CoA dehydrogenase	ttggcaaaacagttgctcac	ctcccacatgtatccccaac
ACADVL	VLCAD	Very long-chain acyl-CoA dehydrogenase	agccgtgaaggagaagatca	tgtgtttgaagccttgatgc
EHHADH	LBP	Enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase	cttcagccctggatgttgat	aaaagaagtgggtgccaatg
HADHA	LCHAD	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, alpha subunit	cacctctctgcctgttcctc	ggcaaagatgctgacacaga
ACOX1	AOX	Acly-CoA oxidase	tgatgcgaatgagtttctgc	agtgccacagctgagaggtt
CYP2E1	CYP2E	Cytochrome P450 CYP2E	cccaaaggatatcgacctca	agggtgtcctccacacactc
Intake of fatty acid				
SLC27A5	FATP5	Fatty acid transporor protein 5	acacactcggtgtccctttc	ctacagggcccactgtcatt
Transfer of trigyceride				
MTP	MTP	Microsomal triglyceride transfer protein	catctggcgaccctatcagt	ggccagctttcacaaaagag
Biosynthesis of fatty acid				
SREBF1	SREBP1	Sterol regulatory element-binding protein 1	tgcattttctgacacgcttc	ccaagctgtacaggctctcc
ACACA	ACC	Acetyl CoA carboxylase	gagaactgccctttctgcac	ccaagctccaggcttcatag
FASN	FAS	Fatty acid synthase	ttccgagattccatcctacg	tgtcatcaaaggtgctctcg

Table 2 Patient characteristics

Characteristic	
n	297
Age[a]	58 (20–78)
Male gender (%)	131 (44.9%)
BMI[a]	22.7 (15.6–35.1)
Obesity (%)	76 (25.6%)
Alcohol intake (%)	67 (22.6%)
Diabetes (%)	9 (3.0%)
HCV genotype (%)	
1	212 (71.4%)
2	76 (25.6%)
3	2 (0.7%)
Unknown	7 (2.3%)
HCV-RNA level (KIU/ml)[a]	1100 (5–9400)
Platelet count ($\times 10^4/\mu L$)[a]	17.6 (5.3–37.4)
AST (IU/L)[a]	47 (14–413)
ALT (IU/L)[a]	59 (9–537)
γ-GTP (IU/L)[a]	39 (10–490)
Fasting glucose (mg/dL)[a]	96 (68–223)
Total cholesterol (mg/dL)[a]	173 (19–318)
Triglyceride (mg/dL)[a]	91 (26–930)
Histological activity (%)	
0	3 (1.0%)
1	127 (42.8%)
2	120 (40.4%)
3	47 (15.8%)
Fibrosis (%)	
0	4 (1.3%)
1	100 (33.7%)
2	120 (40.4%)
3	62 (20.9%)
4	11 (3.7%)
Steatosis (%)	
None	126 (42.4%)
Mild (<33%)	163 (54.9%)
Moderate (33–66%)	7 (2.4%)
Severe (>66%)	1 (0.3%)

[a] Median (range)

Table 3 Univariate analysis of factors associated with steatosis

Factors	No steatosis ($n = 126$)	Steatosis ($n = 171$)	*P*
Age[a]	56 (20–78)	59 (27–75)	0.019
Male gender (%)	44 (34.9%)	87 (50.9%)	0.007
BMI[a]	21.8 (16.5–30.7)	23.9 (15.6–35.1)	<0.0001
Alcohol intake (%)	29 (23.0%)	38 (22.2%)	0.89
Diabetes (%)	4 (3.2%)	5 (2.9%)	1.00
HCV genotype (%)			
1	91 (72.2%)	121 (70.8%)	
2	31 (24.6%)	45 (26.3%)	
3	1 (0.8%)	1 (0.9%)	
Unknown	3 (2.4%)	4 (2.4%)	0.78
HCV-RNA level (KIU/ml)[a]	1257 (5–7030)	1063 (5–9400)	0.14
Platelet count ($\times 10^4/\mu L$)[a]	18.4 (5.9–32.7)	17.4 (5.3–37.4)	0.19
AST (IU/L)[a]	36 (15–413)	58 (14–339)	<0.0001
ALT (IU/L)[a]	40 (9–537)	73 (12–509)	<0.0001
γ-GTP (IU/L)[a]	25 (10–298)	56 (12–490)	<0.0001
Fasting glucose (mg/dL)[a]	95 (68–207)	97 (77–223)	0.002
Total cholesterol (mg/dL)[a]	179 (109–285)	171 (104–318)	0.13
Triglyceride (mg/dL)[a]	83 (26–214)	96 (32–930)	<0.0001
Histological activity (%)			
0	2 (1.6%)	1 (0.6%)	
1	72 (57.1%)	55 (32.2%)	
2	42 (33.3%)	78 (45.6%)	
3	10 (7.9%)	37 (21.6%)	<0.0001
Fibrosis (%)			
0	3 (2.4%)	1 (0.6%)	
1	62 (49.2%)	38 (22.2%)	
2	47 (37.3%)	73 (42.7%)	
3	11 (8.7%)	51 (29.8%)	
4	3 (2.4%)	8 (4.7%)	0.001

[a] Median (range)

Statistical analysis

Results are presented as numbers with percentages in parenthesis for qualitative data or as the medians and ranges for quantitative data. Univariate comparisons were made using a chi-square test for qualitative factors or a Mann–Whitney *U* test on ranks for quantitative factors with non-equal variance. Logistic regression analysis was used for multivariate analysis. *P* values below 0.05 by two-sided test were considered to be significant. Variables that achieved statistical significance on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors for steatosis. All statistical analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA).

Results

The characteristics of the 297 patients are summarized in Table 2. Steatosis was present in 171 (57.6%) patients. The grade of steatosis was mild in 163 (54.9%) patients, moderate in 7 (2.4%), and severe in 1 (0.3%).

Table 4 Multivariate analysis of factors independently associated with steatosis

Factors	Odds ratio	95% confidence interval	P
Age	1.02	1.00–1.05	0.05
Male gender	0.99	0.51–1.93	0.99
BMI	1.19	1.06–1.33	0.002
AST	1.00	0.98–1.02	0.54
ALT	0.99	0.98–1.00	0.37
γ-GTP	1.01	1.00–1.01	0.005
Fasting glucose	0.99	0.97–1.01	0.37
Triglyceride	1.01	1.00–1.01	0.007
Activity grade A2 or A3	1.81	0.94–3.51	0.07
Fibrosis stage F3 or F4	2.59	1.11–6.02	0.02

Data are from a total of 297 patients

Univariate correlations between variables and steatosis are shown in Table 3. Patients with steatosis, as compared to patients without steatosis, were older, more often male, had a higher BMI, higher AST, ALT, γ-GTP, fasting glucose, and triglyceride levels, a higher histological activity grade, and a higher fibrosis stage. Multivariate analysis revealed that the BMI, levels of γ-GTP and triglyceride, and fibrosis stage correlated independently with the presence of steatosis (Table 4).

To determine whether HCV has a direct effect on steatosis, we next analyzed a subgroup of patients lacking known metabolic causes of steatosis. Patients with obesity, diabetes, or ongoing alcohol intake were excluded. From the remaining 173 patients, we selected 100 patients whose liver RNA was available for gene expression analyses. There was no difference in clinicopathological characteristics between these 100 patients and the remaining 73 patients whose liver RNA was not available (data not shown). Steatosis was present in 43 (43%) of these 100 patients (Table 5). The presence of steatosis was associated with higher levels of AST, ALT, and γ-GTP, higher fasting glucose levels, and a higher fibrosis stage (Table 5).

To investigate the molecular mechanisms underlying HCV-related steatosis, we examined the expression of 18 genes regulating lipid metabolism in the liver (Table 1) using liver tissues derived from the 100 patients without obesity, diabetes, or ongoing alcohol intake. Real-time quantitative RT-PCR revealed that the expression of 10 genes (*PPARA*, *NR1H3*, *ACADS*, *ACADL*, *EHHADH*, *HADHA*, *ACOX1*, *CYP2E1*, *SLC27A5*, and *ACACA*) were significantly lower in patients with steatosis than in patients without steatosis (Fig. 1). There was no difference in the expression of the other 8 genes, including *SREBF1*, between the two groups.

To determine whether the protein levels corresponded with the mRNA levels, we performed immunohistochemistry for PPARα (encoded by *PPARA*) and SREBP1 (*SREBF1*) proteins in liver biopsy tissues from the same 100 patients. We chose these two proteins because they are key regulators of lipid degradation and lipid synthesis, respectively. The results are summarized in Table 6, and representative images are shown in Fig. 2a. PPARα was expressed in hepatocytes. Its expression was mainly observed in the nuclei. SREBP1 was expressed in the cytoplasm of hepatocytes. Levels of PPARα and SREBP1 proteins tended to correlate with levels of *PPARA* and *SREBF1* mRNA, respectively (Fig. 2b). As shown in Table 6, the expression of the PPARα protein was significantly lower in patients with steatosis than in patients without steatosis

Table 5 Univariate analysis of factors associated with steatosis in patients without obesity, diabetes, or alcohol intake

Factors	No steatosis ($n = 57$)	Steatosis ($n = 43$)	P
Age[a]	56 (30–77)	60 (27–73)	0.12
Male gender (%)	15 (26.3%)	12 (27.9%)	0.86
BMI[a]	21.4 (17.0–24.8)	22.0 (17.8–24.9)	0.34
HCV genotype (%)			
1	39 (68.4%)	30 (69.8%)	
2	18 (31.6%)	13 (30.2%)	
3	0 (0%)	0 (0%)	
Unknown	0 (0%)	0 (0%)	0.89
HCV-RNA level (KIU/mL)[a]	1510 (5–7030)	1110 (5–5100)	0.60
Platelet count (× 10^4/μL)[a]	19.8 (9.8–31.1)	17.3 (5.9–32.7)	0.06
AST (IU/L)[a]	31 (15–138)	61 (15–131)	<0.0001
ALT (IU/L)[a]	32 (12–175)	73 (14–290)	<0.0001
γ-GTP (IU/L)[a]	22 (10–137)	47 (12–151)	<0.0001
Fasting glucose (mg/dL)[a]	95 (75–112)	99 (79–121)	0.029
Total cholesterol (mg/dL)[a]	180 (120–281)	171 (119–300)	0.76
Triglyceride (mg/dL)[a]	86 (26–209)	88 (44–178)	0.23
Histological activity (%)			
0	1 (1.7%)	1 (2.3%)	
1	33 (58.0%)	14 (32.6%)	
2	19 (33.3%)	20 (46.5%)	
3	4 (7.0%)	8 (18.6%)	0.06
Fibrosis (%)			
0	1 (1.8%)	1 (2.3%)	
1	30 (52.6%)	10 (23.3%)	
2	20 (35.1%)	18 (41.9%)	
3	6 (10.5%)	13 (30.2%)	
4	0 (0%)	1 (2.3%)	0.018

[a] Median (range)

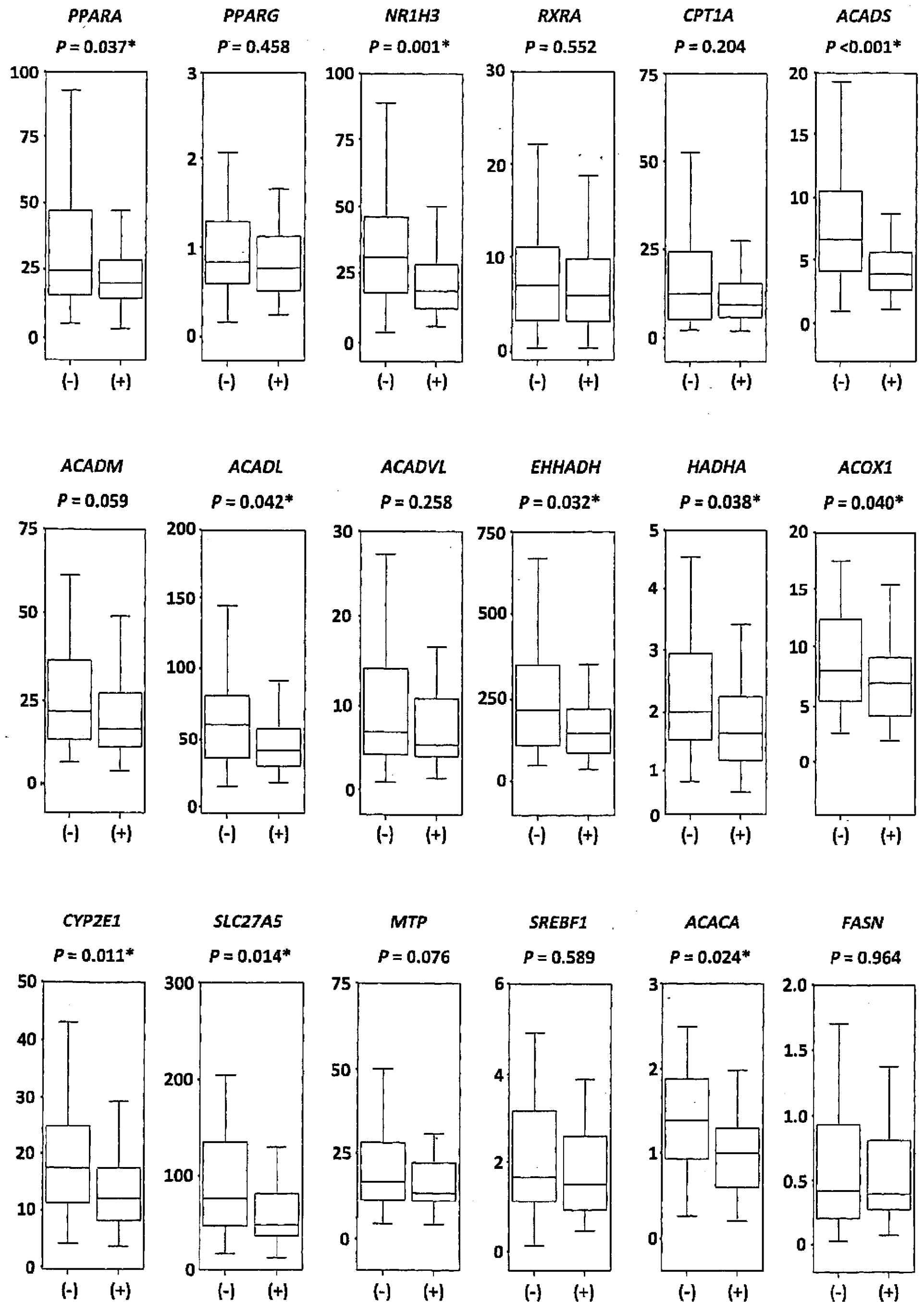

Fig. 1 Relative expression levels of 18 genes (see Table 1) in liver tissues from 57 patients without steatosis (−) and 43 patients with steatosis (+). Gene expression was evaluated by real-time quantitative RT-PCR. Results are presented relative to the expression of a reference gene (*ACTB*) to correct for variation in the amount of RNA in the RT-PCR. The box contains the values between the 25th and 75th percentiles, and the horizontal line is the median; the error bars stretch from the 10th to 90th percentiles. Differences between groups were analyzed using the Mann–Whitney *U* test. Asterisks indicate that the differences were statistically significant

($P = 0.017$). On the other hand, the presence of the SREPB1 protein was not associated with the steatosis. These findings agree with those from our analyses of *PPARA* and *SREBF1* mRNA levels. We also examined the relationship between the levels of PPARα and SREBP1 proteins and the degree of fibrosis (Table 6). The level of the PPARα protein was not associated with the degree of fibrosis. The expression of the SREBP1 protein tended to be higher in patients who had a higher fibrosis stage, although the association was not statistically significant.

Discussion

Our results demonstrated a high prevalence (57.6%) of steatosis among patients with chronic HCV infection in Japan, which confirms previous reports in Europe and the United States [1, 25–28]. The prevalence of steatosis was high (43.0%) even when known factors of steatosis, such as obesity, diabetes, or ongoing alcohol intake, were excluded. Consistent with previous reports [1, 29], the grade of steatosis was mild in most cases.

Table 6 Relationship between the presence of steatosis or the degree of fibrosis and levels of PPARα and SREBP1 proteins in liver tissues from patients without obesity, diabetes, or alcohol intake

	Steatosis			Fibrosis		
	Absent ($n = 57$)	Present ($n = 43$)	P	F1/F2 ($n = 80$)	F3/F4 ($n = 20$)	P
PPARα protein expression						
1+; mild or absent	9	17		20	6	
2+; moderate	38	23		49	12	
3+; strong	10	3	0.017	11	2	0.85
SREBP1 protein expression						
1+; mild or absent	16	6		17	5	
2+; moderate	31	29		52	8	
3+; strong	10	8	0.23	11	7	0.055

Multivariate analysis on the 297 patients with steatosis, including those with metabolic cofactors, revealed that a higher BMI, higher levels of γ-GTP and triglyceride, and a higher fibrosis stage correlate independently with steatosis. Previous studies have also observed an association between these clinicopathological factors and steatosis [1]. A recent meta-analysis of patients with chronic HCV infection in Europe, Australia, and the United States showed that steatosis is associated independently with HCV genotype 3, the presence of fibrosis, diabetes, hepatic inflammation, ongoing alcohol intake, a higher BMI, and an older age [5]. Although several studies have shown a significant and independent association between HCV genotype 3 and the presence of steatosis [1], we did not observe this association. This is due to the much lower prevalence of genotype 3 in Japan (<1%) than in Europe (24%) [7] and the United States (14%) [9]. There is some controversy with regard to the influence of steatosis on the progression of fibrosis [1, 3]. Some investigators suggest that steatosis accelerates fibrosis only in genotype 3-infected patients [7, 29, 30], whereas others suggest that there is an association in patients infected with genotype 1 [5, 31]. An analysis using paired liver biopsies revealed that steatosis was the only independent factor predictive of progression of fibrosis [32]. In agreement with a previous study [33], we also found that patients with steatosis had a higher γ-GTP. An increase in serum γ-GTP is associated with hepatic steatosis, central obesity and insulin resistance, and is a marker of metabolic and cardiovascular risk [34–36]. Elevated values of γ-GTP are caused by damage to cellular membranes, cellular regeneration or by enhanced synthesis as a result of induction of the biotransformation enzyme system. However, the mechanisms that explain the contribution of γ-GTP to steatosis have not been fully elucidated.

We analyzed the intrahepatic expression of genes that regulate (i) lipid degradation, (ii) lipid secretion, (iii) lipid synthesis, and (iv) lipid uptake. We then investigated the relationship between these levels and the presence of steatosis. Our experiments included more candidate genes than previous studies [13, 18, 19, 37]. The expression of *PPARA*, *ACADS*, *ACADL*, *EHHADH*, *HADHA*, *ACOX1*, and *CYP2E1* were lower in patients with steatosis. Immunohistochemistry confirmed that the expression of the PPARα protein was significantly lower in patients with steatosis than in patients without steatosis. PPARα, one of the proteins involved in lipid degradation, is a nuclear receptor that controls fatty acid metabolism by regulating the expression of genes encoding enzymes involved in mitochondrial and peroxisomal β-oxidation of fatty acids [38]. Short chain acyl-CoA dehydrogenase (encoded by *ACADS*), long-chain acyl-CoA dehydrogenase (*ACADL*), enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (*EHHADH*), hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, alpha subunit (*HADHA*), and acyl-CoA oxidase (*ACOX1*) are involved in fatty acid β-oxidation. *CYP2E1* encodes a member of the cytochrome P450 superfamily of enzymes that is involved in microsomal ω-oxidation. Acyl-CoA oxidase is the rate-limiting enzymes of peroxisomal β-oxidation. Also, *EHHADH*, *HADHA* and *ACOX1* are known to be a direct transcriptional target of PPARα [38]. The reduced expression of *PPARA*, *ACADS*, *ACADL*, *EHHADH*, *HADHA*, and *ACOX1* may lead to steatosis through down-regulation of fatty acid β-oxidation. However, not all of the genes regulating β-oxidation were down-regulated in patients with steatosis. For example, carnitine palmitoyl transferase 1 (encoded by *CPT1A*) is the rate-limiting enzymes of mitochondrial β-oxidation, and although *CPT1A* is a transcriptional target of PPARα [38], their expression was not significantly reduced in patients with steatosis.

In agreement with a previous study [13], we also found that the expression of *MTP*, a gene involved in lipid secretion, tended to be lower in patients with steatosis, although the association was not statistically significant. *MTP* is a transcriptional target of PPARα [38]. Because

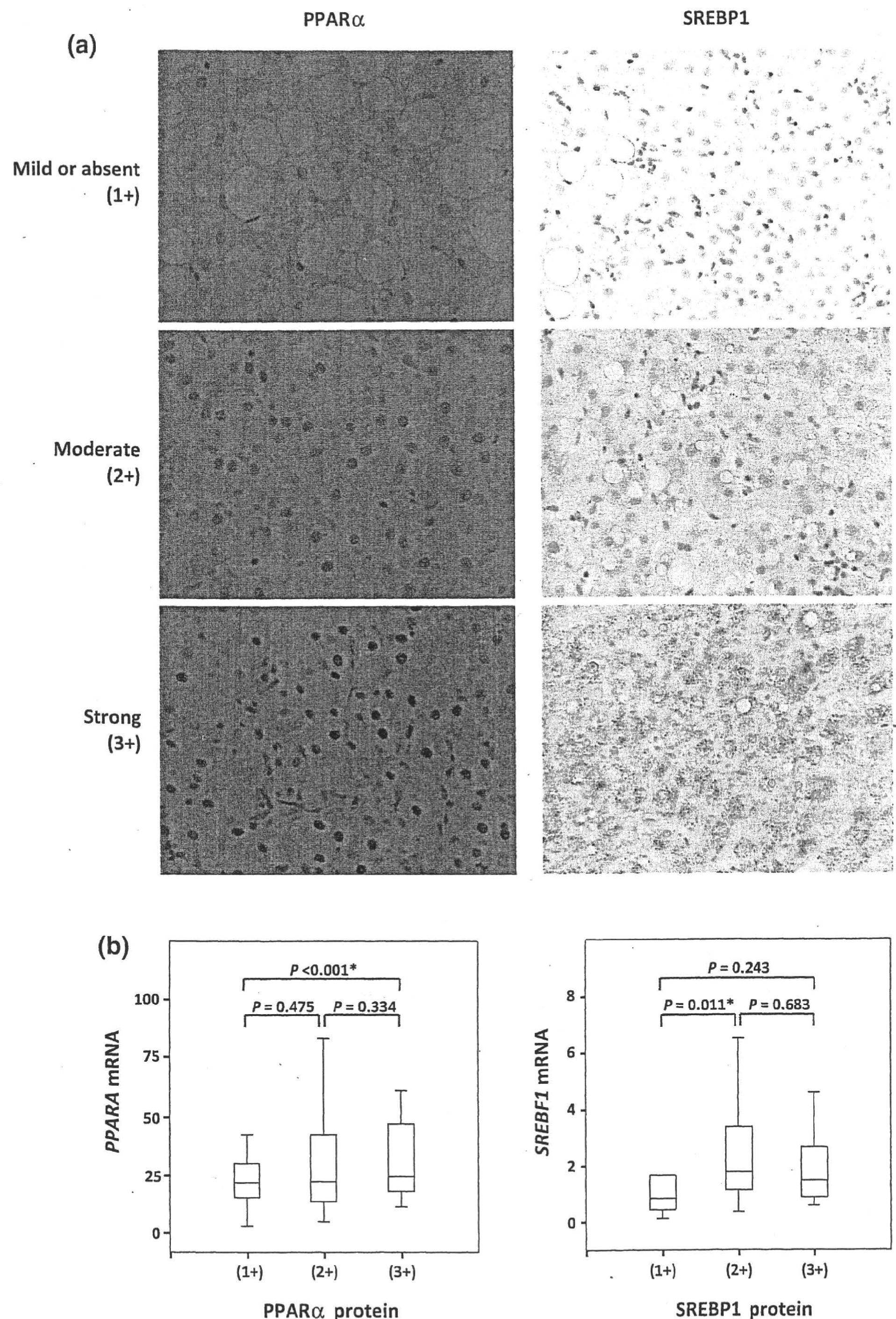

Fig. 2 Immunohistochemistry for PPARα and SREBP1 proteins. **a** Representative images from immunostaining for PPARα and SREBP1 proteins in liver tissues from patients with chronic hepatitis C. Shown are weak or absent staining (1+), moderate staining (2+), and strong staining (3+). Original magnification, ×400. **b** Relationship between relative levels of *PPARA* and *SREBF1* mRNA and proteins. *PPARA* and *SREBF1* mRNA levels were determined as described in Fig. 1. Levels of PPARα and SREBP1 proteins were evaluated as described in **a**. Differences between groups were analyzed using the Mann–Whitney *U* test. Asterisks indicate that the differences were statistically significant

microsomal triglyceride transfer protein plays a pivotal role in assembly and secretion of very low density lipoproteins, its reduced expression is expected to result in the increased accumulation of triglycerides (i.e., steatosis).

The nuclear receptor liver X receptor α (encoded by *NR1H3*) is known to promote hepatic lipogenesis by activating SREBP1. SREBP1 increases the transcription of genes involved in hepatic fatty acid synthesis, such as *FASN* (encoding fatty acid synthase) and *ACACA* (acetyl CoA carboxylase), and induces steatosis through increased accumulation of triglyceride. Unexpectedly, the levels of both *SREBF1* mRNA and protein and of *FASN* mRNA were not up-regulated in patients with steatosis. In addition, the expression of *NR1H3* and *ACACA* were lower in patients with steatosis. These findings contradict the idea that the increased expression of genes involved in synthesis of fatty acids leads to steatosis. One possible explanation is that the decreased expression of *NR1H3* and