ウイルス肝炎の臨床像と遺伝子多型

加藤直也* 室山良介*

索引用語：B型肝炎，C型肝炎，GWAS，SNP，インターロイキン-28B

1 はじめに

B型肝炎ウイルス(hepatitis B virus: HBV)，C型肝炎ウイルス(hepatitis C virus: HCV)は，わが国における慢性肝炎・肝硬変・肝細胞癌の最大の原因である．しかしながら，その臨床像には著しい個人差がある．これら臨床像の個人差にはウイルス側要因のみならず，宿主側要因が大きく寄与していることが明らかになりつつある．ヒトゲノムプロジェクトによりヒトの全遺伝子配列が明らかにされたこともあり，疾患と遺伝子多型との関連の解析が盛んに行われるようになった．技術的な進歩も相まって，疾患関連遺伝子多型解析も候補遺伝子解析のみならず，ゲノムワイド関連解析(Genome Wide Association Study : GWAS)が行われるようになってきており，重要な疾患関連遺伝子多型が発見されつつある．特に，最近になり複数のグループから報告されたIII型インターフェロン-λ3をコードするインターロイキン(IL) -28Bの1塩基多型(single nucleotide polymorphism: SNP)がペグインターフェロン，リバビリン併用療法の治療効果を規定するのみならず，HCVの持続感染に関連していることは，そのメカニズム解明も含め，C型肝炎の最大のトピックとなっている．

本稿では，以下のウイルス肝炎の臨床像の違いについての遺伝子多型につき，われわれのデータを交えつつ概説したい．1)肝炎ウイルス感染後の持続感染成立に関わる遺伝子多型，2)慢性感染による病態進展に関わる遺伝子多型，3)発癌に関わる遺伝子多型，4)治療効果に関わる遺伝子多型．

2 肝炎ウイルス持続感染と遺伝子多型

1．HBV

HBVに慢性的に感染している人(キャリア)は世界中で約4億人おり，B型肝炎はいまだ世界の死亡原因の第7位を占める疾患である．HBVキャリアの約10％が慢性肝炎であり，B型慢性肝炎は，肝硬変や肝細胞癌へしばしば進行する．しかしながら，その臨床

Naoya KATO et al : Clinical features of viral hepatitis and genetic polymorphisms
*東京大学医科学研究所疾患制御ゲノム医学ユニット［〒108-8639 東京都港区白金台4-6-1］

経過は患者によって大きな差がある．

Kamataniらは，日本人のB型慢性肝炎患者786人と健常者2,201人を用いて，2段階のゲノムワイド関連解析を行い，HLA-DPA1およびHLA-DPB1を含む領域に，B型慢性肝炎と有意な関連を示す11カ所のSNPを同定した[1]．HLA（human lymphocyte antigen，ヒト白血球型抗原）はヒトの主要組織適合性複合　体（major histocompatibility complex: MHC）で，基本的には自他認識のマーカー分子として機能していて，他人どうしの移植をすると拒絶反応が起こるもとになるものである．さらに，初めのコホートとは異なるB型慢性肝炎患者1,300人と健常者2,100人からなる新たな日本人およびタイ人コホート（バリデーションコホート）を用いて，当該領域の2種類のSNPを調べ，異なるコホートでもこれらSNPがB型慢性肝炎疾患と有意に相関していることを確認した．全症例でのP値とオッズ比はそれぞれ$P=6.34\times10^{-39}$，オッズ比は0.57および$P=2.31\times10^{-38}$，オッズ比は0.56であった．また，ハプロタイプ解析を行い，リスク型（発症しやすい）ハプロタイプ（HLA-DPA1*0202-DPB1*0501およびHLA-DPA1*0202-DPB1*0301．それぞれオッズ比1.45および2.31）と，保護型（発症しにくい）ハプロタイプ（HLA-DPA1*0103-DPB1*0402およびHLA-DPA1*0103-DPB1*0401．それぞれオッズ比0.52および0.57）を同定した．これらのことから，HLA-DP遺伝子に存在する遺伝的多型が，HBVの持続感染に強く関連していることが示された．

2. HCV

HCVに慢性的に感染している人（キャリア）は世界中で約1億7千万人おり，わが国でもHCV感染者は約200万人にもおよび，また，毎年3万人以上が命を失う肝細胞癌患者の約80％がHCVによるものであり，C型肝炎は，わが国における健康上の大いなる脅威である．

HCVの場合，ほとんどの感染（70～80％）は持続性であり，持続感染している患者は将来的に高率に肝硬変や肝細胞癌になる．宿主側因子が，HCV感染からの回復か持続感染するかの違いに関与しており，HCVに対する強い宿主免疫応答はウイルス排除に有利に働くといわれている．最近のゲノムワイド関連解析で，IL28B遺伝子の3,000塩基上流の1塩基多型（rs12979860）が，ペグインターフェロン，リバビリン併用療法に対する治療効果と強く関連することが示された．そこで，Thomasらは自然経過でrs12979860変異がHCV感染の転帰にどのような影響を与えるかを調べた[2]．すなわち，HCVを自然に排除した患者388例および持続感染患者620例からなるコホートで，この変異の遺伝子型検査を行った．すると，ペグインターフェロン，リバビリン併用療法が効果的であるC/C遺伝子型では，HCV感染からの回復，すなわちHCVの自然排除を促進していることがわかった．HCV感染の回復に，IL28Bが重要な役割を担っていることが判明した．

3 慢性ウイルス肝炎の病態進展と遺伝子多型

本来，ウイルス感染により，宿主の自然免疫系が活性化され，インターフェロンを始めとする抗ウイルスシステムが作動し，その結果，ウイルス増殖は抑制され，最終的にウイルスは排除される．インターフェロンにより誘導される分子群（インターフェロン誘導遺伝子，Interferon stimulated genes: ISGs）は，直接的あるいは間接的にウイルス増殖を抑制し，ウイルスを排除する働きを有している．

今までの検討から，HCVは確かに宿主の自然免疫系により認識され，主たる抗ウイルスシステムであるインターフェロン誘導系を活性化することが明らかになっている[3]．HCV感染においても，これらインターフェロン誘導遺伝子は，HCV増殖と密接に関連し，C型肝炎の病態形成に大きく寄与しているものと思われる．そこで，これら分子の質的あるいは量的な個人差は，C型肝炎の病態に関連している可能性がある．

1．2-5AS

2'-5'-oligoadenylate synthetase（2-5AS）は二重鎖RNAにより活性化され，活性化した2-5ASはRNaseLを活性化し，RNAを分解する．このことにより，ウイルスRNAが分解され，ウイルス増殖は抑制される．実際に，2-5ASをHCVサブゲノムレプリコンの増殖している肝癌細胞に強制発現したところ，その増殖を著しく抑制することがわかった．すなわち，2-5ASにはHCV増殖を抑制する働きがある．

そこで，409例のC型肝炎患者について，2-5ASの6カ所のSNPにつき検討した[4]．6カ所のSNPはプロモーター部分などに存在し，2-5ASの量的な個人差が予測されるもの，あるいは2-5ASのアミノ酸を変化させ，2-5ASの質的な個人差が予測されるものを選んだ．そうしたところ，2-5ASのSNP A4119Gは，AST/ALT値と関連があり，また，肝硬変・肝線維化と関連していることが明らかとなった．この2-5ASのSNP A4229Gは，2-5ASのエクソン3に存在し，二重鎖RNA結合モチーフをコードしており，Ser162Glyアミノ酸置換を引き起こす．そこで，このアミノ酸置換は抗HCV活性に影響を及ぼす可能性があると考え，それぞれのアミノ酸を持つ2-5ASをHCVサブゲノムレプリコンが増殖している肝癌細胞に導入し，その抗HCV効果を検定した．すると，Serタイプの2-5ASはHCV増殖抑制効果がGlyタイプ2-5ASより高いことが判明した．すなわち，抗HCV効果の弱い2-5ASを有する患者では，炎症が惹起されやすく，線維化が促進されることが明らかとなった．

2．TLR3

Toll-like receptor 3（TLR3）はウイルス由来の二重鎖RNAを認識，インターフェロンの産生を誘導し，抗ウイルス効果を惹起する自然免疫系分子の一つである．

そこで，437例のC型肝炎患者について，TLR3の5カ所のSNPにつき検討した．5カ所のSNPはプロモーター部分などに存在し，TLR3の量的な個人差が予測されるもの，あるいはTLR3のアミノ酸を変化させ，TLR3の質的な個人差が予測されるものを選んだ．そうしたところ，TLR3のSNP C6300Tは，ALT値，血小板と関連があった．このTLR3のSNP C6300Tは，TLR3のエクソン4に存在し，細胞内領域をコードしており，Leu412Pheアミノ酸置換を引き起こす．そこで，このアミノ酸置換は抗HCV活性に影響を及ぼす可能性があると考え，それぞれのアミノ酸を持つTLR3をHCVサブゲノムレプリコンが増殖している肝癌細胞に導入し，そのインターフェロン誘導能を検定した．すると，LeuタイプのTLR3はインターフェロン誘導能がPheタイプTLR3より高いことが判明した．すなわち，インターフェロン誘導能の弱いTLR3を有する患者では，炎症が惹起されやすく，線維化が促進されることが明らかとなった．

2-5ASとTLR3のSNPの解析から，HCV増殖抑制効果が弱い，あるいは，インターフェロン誘導能が弱い遺伝子型を有する患者で

は，肝病変が進展しやすいことが明らかとなった．

4 肝炎ウイルスによる発癌と遺伝子多型

HCV感染から肝硬変，肝細胞癌への進展には著しい個人差がある．このような肝病態進展の個人差にはSNPに代表される宿主因子が寄与していると考えられる．今回，肝病態の進展，特に肝癌に関わるSNPにつき候補遺伝子解析を試みてきた．

1. IL-1 β

われわれは274例のHCV感染患者について，炎症性サイトカインであるIL-1ファミリーのIL-1 β，IL-1raおよびTNF αの遺伝子多型と肝細胞癌の関連につき調べてみた[5]．すると，IL-1 βの-31塩基(プロモーター領域)のT/T遺伝子型を有する肝癌患者は55％(C/C遺伝子型に対するオッズ比2.63，P=0.009)であり，T/C遺伝子型(44％，C/C遺伝子型に対するオッズ比1.51，P=0.149)やC/C遺伝子型(35％)に比し，有意に頻度が高かった．IL-1 βの-31のCがTに変わることにより，IL-1 βプロモーター領域に新たなTATAボックスが出現，転写因子の結合に変化を生じ，IL-1 βの転写を修飾，その結果，肝発癌に寄与するのではないかと考えている．

2. UGT1A7

さまざまな変異原の解毒に働くUDP-glucuronosyltransferase1A7（UGT1A7）の遺伝子多型についても，高酵素活性型(H)ハプロタイプと低酵素活性型ハプロタイプ(L)に分類し，280例のHCV感染患者について肝細胞癌との関連の有無につき検討を行った[6]．肝細胞癌患者においてH/HとH/Lの比率はそれぞれ25％と45％と，肝細胞癌なし患者における比率15％と39％に比し有意に高率であり，H/Hに対するオッズ比はそれぞれ2.73と1.80であった．おそらく，低酵素活性型では癌原性物質の代謝(無毒化)能力が低く，肝発癌につながるのではないかと考えている．

3. MDM2

癌抑制遺伝子であるp53の不活化に重要な役割を有する癌遺伝子MDM2のプロモーターSNP309の遺伝子型と肝細胞癌の関連を，435例のC型肝炎患者にて調べてみた[7]．肝細胞癌患者においてG/G遺伝子型は33％を占め，肝細胞癌なしの患者での23％に比し，オッズ比2.82で高率であった．MDM2のプロモーターSNPのG/G遺伝子型は高レベルのMDM2を発現しp53を不活化する．それにより，肝発癌につながると考えられる．

4. SCYB14，CRHR2，GFRA1

肝病態の進展，特に肝発癌に関わるSNPにつき網羅的候補遺伝子解析を試みた[8]．肝発癌に関与する可能性のある遺伝子として，細胞の生と死，炎症に関わる遺伝子171遺伝子，393SNPsを選択した．トレーニングケースとして，C型肝炎患者188例(慢性肝炎＋肝硬変111例，肝細胞癌77例)において，上記393 SNPsをTaqman PCR法により解析し肝細胞癌との関連につき検討した．有意に肝細胞癌と関連している29遺伝子，31SNPsを抽出した．次に，トレーニングにより絞り込まれたSNPにつき，新たに選んだC型肝炎患者188例(慢性肝炎＋肝硬変95例，肝細胞癌93例)において，同様に検討した．テスティングにより，29遺伝子，31SNPsから，有意に肝細胞癌と関連している3遺伝子(SCYB14，GFRA1，CRHR2)，3SNPsを同定した．これらは肝発癌メカニズムの解明に重要のみならず，肝発癌の超高危険群，超低危

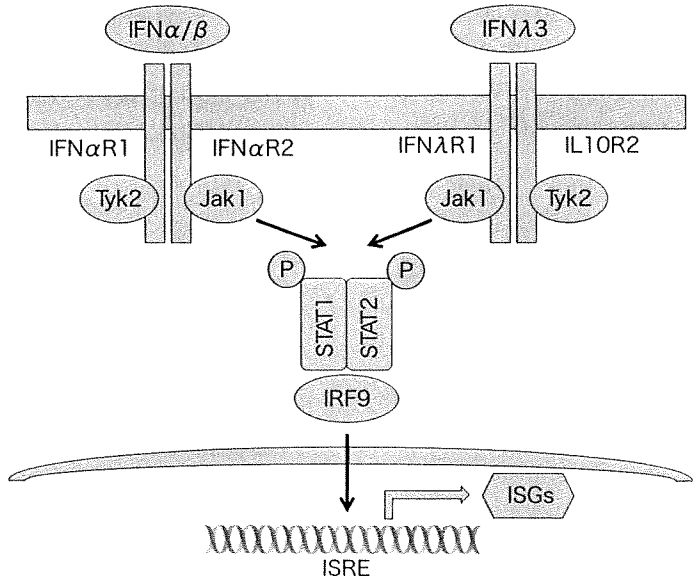

図1　インターフェロンα/βシグナルとIL-28B（インターフェロンλ3）シグナル（文献9を参照）

険群の設定を可能にすることが期待される．

5 インターフェロン治療効果と遺伝子多型

1. IL-28B

ゲノムワイド関連解析により，わが国を含む3つのグループからIL-28Bの遺伝子多型がペグインターフェロン，リバビリン併用療法の治療効果を規定することが報告され，ホットな話題になっている．IL-28B遺伝子はインターフェロン-λ3をコードしており，また，インターフェロン-λ3は細胞のリセプターこそインターフェロンα/βと異なるものの，JAK/STAT系を活性化し，ISRE（interferon-stimulated response element）を介してインターフェロン誘導遺伝子（ISGs）の発現を誘導する（図1）[9]．

ペグインターフェロンとリバビリンとの48週間の併用療法は，C型慢性肝炎に対する標準治療であり，また，現時点で最強の治療である．しかしながら，本治療をもってしてもウイルス駆除に至らない患者が多いこと，またヨーロッパ系患者はアフリカ系患者よりも治りやすいことが知られていた．Geらは IL-28B遺伝子近傍の遺伝的多型が，治療反応性と関連することを報告した（オッズ比は約2倍）[10]．この関連は，ヨーロッパ系患者（$P=1.06\times10^{-25}$）とアフリカ系アメリカ人患者（$P=2.06\times10^{-3}$）の両方で認められたが，治療効果の高い遺伝子型はアフリカ系患者よりもヨーロッパ系患者の方に高い頻度で認められ，これがアフリカ系患者とヨーロッパ系患者の間での治療反応性の違いの一つの理由であることを見出した．

わが国では，Tanakaらがペグインターフェロン，リバビリン併用療法を行ったgenotype 1型HCV日本人患者で，ウイルス減少を示さない（null virological response: NVR）ことが，IL28B遺伝子近傍の2つのSNPと強く関連することを見出した（rs12980275,

P=1.93 × 10^{-13} および rs8099917, P=3.11 × 10^{-15})[11]. これらのSNPはウイルス学的著効(sustained virological response: SVR)とも関連を示した(rs12980275, P=3.99 × 10^{-24}, および rs8099917, P=1.11 × 10^{-27}). 末梢血単核細胞でのリアルタイム定量PCRにより, マイナー対立遺伝子をもつ人はIL-28Bの発現レベルが低いことが判明し(P=0.015), 実際にIL-28BがC型肝炎のペグインターフェロン, リバビリン併用療法で重要な役目を担っているだろうことを示した.

もう一つの報告はオーストラリア人でのゲノムワイド関連研究であるが, やはりIL-28Bの遺伝子多型がSVRと関連したことを明らかにしている[12].

2. MAPKAPK3

Tsukadaらは, MAPKAPK3 (mitogen-activated protein kinase-activated protein kinase 3)の2カ所のSNPsがC型慢性肝炎のインターフェロン治療効果と関連することを報告している[13]. また, MPAKAPK3はインターフェロンαにより誘導される抗ウイルス遺伝子群の発現を抑制することを突き止めている.

6 おわりに

多数症例のゲノムワイド関連解析により, 極めて低いP値を持つ, 非常に信頼性の高いウイルス肝炎関連遺伝子が見つかるようになってきた. これら遺伝子の多型は, 例えば発癌の高危険群の絞り込みやペグインターフェロン, リバビリン併用療法の治療効果予測に有用のみならず, 病態に密接に関連していることから, 直接的に治療への応用も考えられる. これら生体の肝炎ウイルスの感染・複製・増殖に関わるキープレイヤーとその役割を明らかにし, ゲノム解析を行うことで, 肝病態進展の個人差の解明, オーダーメイド治療を含む新たな治療法の開発, また, 新たなHCV感染培養系の確立にもつながっていくものと期待される.

文 献

1) Kamatani Y, Wattanapokayakit S, Ochi H et al : A genome-wide association study identifies variants in the HLA-DP locus associated with chronic hepatitis B in Asians. Nat Genet 41 : 591–595, 2009
2) Thomas DL, Thio CL, Martin MP et al : Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 461 : 798–801, 2009
3) Moriyama M, Kato N, Otsuka M et al : Intrerferon-beta is activated by hepatitis C virus NS5B and inhibited by NS4A, NS4B, and NS5A. Hepatol Int 1 : 302–310, 2007
4) Li CZ, Kato N, Chang JH et al : Polymorphism of OAS-1 determines liver fibrosis progression ih hepatitis C by reduced ability to inhibit viral replication. Liver Int 29 : 1413–1421, 2009
5) Wang Y, Kato N, Hoshida Y et al : Interleukin-1beta gene polymorphisms associated with hepatocellular carcinoma in hepatitis C virus infection. Hepatology 37 : 65–71, 2003
6) Wang Y, Kato N, Hoshida Y et al : UDP-glucuronosyltransferase 1A7 genetic polymorphisms are associated with hepatocellular carcinoma in Japanese patients with hepatitis C virus infection. Clin Cancer Res 10 : 2441–2446, 2004
7) Dharel N, Kato N, Muroyama R et al : MDM2 promoter SNP309 is associated with the risk of hepatocellular carcinoma in patients with chronic hepatitis C. Clin Cancer Res 12 : 4867–4871, 2006
8) Kato N, Ji G, Wang Y et al : Large-scale search of single nucleotide polymorphisms for hepatocellular carcinoma susceptibility genes in patients with hepatitis C. Hepatology 42 : 846–853, 2005
9) O'Brien TR : Interferon-alfa, interferon-lamda and hepatitis C. Nat Genet 41 : 1048–1050, 2009
10) Ge D, Fellay J, Thompson AJ et al : Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461 : 399–401, 2009
11) Tanaka Y, Nishida N, Sugiyama M et al : Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41 : 1105–1109, 2009

12) Suppiah V, Moldovan M, Ahlenstiel G et al : IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41 : 1100–1104, 2009

13) Tsukada H, Ochi H, Maekawa T et al : a polymorphism in MAPKAPK3 affects response to interferon therapy for chronic hepatitis C. Gastroenterology 136 : 1796–1805, 2009

*　　*　　*

〔お知らせ〕

第7回　日本免疫治療学研究会（JRAI）学術集会

会　期：2010年2月20日（土）9:30 ～ 18:00
会　場：東京ガーデンパレス（〒133–0034 東京都文京区湯島 1–7–5）
会　長：木下　秀樹（千葉県がんセンター副センター長）
テーマ：免疫療法の標準化と今後の可能性
参加費：【会員】7,000円【一般】9,000円【コメディカル】4,000円【学生】2,000円
共　催：日本免疫治療学研究会／株式会社メディネット
プログラム：
　シンポジウム　『免疫療法の効果判定』
　教育講演1
　　『がん免疫療法の標準化を目指して』
　　中面　哲也先生（国立がんセンター東病院臨床開発センターがん治療開発部機能再生室）
　教育講演2
　　『肝癌／肝炎に対する免疫細胞療法の可能性』
　　大段　秀樹先生（広島大学大学院先進医療開発科学講座外科学）
　教育講演3
　　『新時代の抗体療法』
　　石田　高司先生（名古屋市立大学大学院医学研究科腫瘍・免疫内科学）
　ワークショップ　『免疫細胞療法の可能性』
　特別講演
　　『自然免疫による病原体認識』
　　審良　静男先生（大阪大学 WPI 免疫学フロンティア研究センター）

第7回日本免疫治療学研究会学術集会 HP：http://www.jrai.gr.jp/7thjrai
株式会社メディネット HP：http://www.medinet-Inc.co.jp

事務局：〒223–0033 横浜市港北区新横浜 2–5–14 臼井ビル 9F
　　TEL : 045–478–0222　FAX : 045–478–0083
　　E-mail：7thjrai@jrai.gr.jp

IRF7多型とC型肝硬変進展リスク

室山良介* 加藤直也* 小俣政男**

索引用語：IRF7，SNP，C型肝硬変，インターフェロン

1 はじめに

C型肝炎ウイルス(HCV)は慢性肝炎・肝硬変・肝細胞癌の主要な病原因子であるが，その肝病変の進展程度には個人差が認められる．その進展程度に影響する要因としては，年齢，性別，アルコール飲酒などがあげられるが，それに加えて遺伝子多型などの宿主ゲノムの要因も報告されている[1~3]．

HCVが宿主体内に侵入すると，宿主側ではHCVを排除しようとする防御システムが働く．この防御システムの主要なものの1つとして「内因性インターフェロン(IFN)産生経路」があげられ，産生されたIFNは強力な抗HCV効果を発揮する．したがって，IFN誘導遺伝子の遺伝子多型が，HCV感染における肝病変の進展程度の個人差を生み出す要因となっている可能性がある．

本稿では，IFN誘導遺伝子の1つであるIFN regulatory factor 7 (IRF7)の1塩基多型(SNP)とC型肝硬変進展リスクとの関連性について概説する[4]

2 IRF7

IRF7はIFN誘導遺伝子の1つであり，内因性IFN産生経路によって産生されたIFN-βにより発現が亢進する．産生されたIRF7はIFN-αプロモーターに結合してIFN-α産生を誘導するが，これによりIRF7発現がさらに促進される[5]．このようなIRF7とIFN-α間の「positive-feedback loop」により，IFNが体内で飛躍的に産生され，抗ウイルス効果を発揮する．

IRF7は，Type I IFN産生において中心的役割を果たすことが今までに数多く報告されており，そのためIRF7は「Type I IFNのmaster regulator」と呼ばれている[6]．

3 IRF7のSNPとC型肝硬変との関連

1．対象と方法

対象は日本人のHCV感染患者406人で，内訳は男：女＝227：179人，肝硬変：非肝

Ryosuke MUROYAMA et al: Association of interferon regulatory factor-7 gene polymorphism with liver cirrhosis in chronic hepatitis C patients
*東京大学医科学研究所 疾患制御ゲノム医学ユニット［〒108–8639 東京都港区白金台 4–6–1］
**山梨県特別顧問

表1　患者背景

	総数 (n=406)	肝硬変なし (n=228)	肝硬変あり (n=178)	P値
男性	227 (55.91%)	127 (50.70%)	100 (56.18%)	0.92
肝細胞癌あり	177 (43.60%)	51 (22.37%)	126 (70.79%)	< 0.0001
年齢(歳)	62 (22-84)	60 (22-83)	65 (37-84)	< 0.0001
血小板数(× 10^4/ μl)	12.7 (1.7-38.67)	16 (4.1-38.67)	9.2 (1.7-27.1)	< 0.0001
アルブミン値(g/dl)	3.9 (2.3-5)	4.1 (2.8-5)	3.6 (2.3-4.8)	< 0.0001
ALT値(U/L)	65 (9-429)	63.5 (9-341)	66 (13-429)	0.09
総ビリルビン値(mg/dl)	0.8 (0.2-6.7)	0.7 (0.2-6.7)	0.9 (0.3-3.5)	< 0.0001
AFP値(μg/L)	12 (1-21700)	6 (1-3222)	25 (2-21700)	< 0.0001
PT活性値(%)	77.8 (7.3-100)	85 (12.6-100)	71.3 (7.3-100)	< 0.0001
HCV RNA量(KIU/ml)	429 (0.5-1910)	453 (7.1-1910)	409.5 (0.5-1407)	0.3
アルコール摂取量 >50 g/日	50 (12.92%)	24 (11.32%)	26 (14.86%)	0.36
HCV genotype 1	259 (75.73%)	143 (75.26%)	116 (76.32%)	0.8

表2　genotypingを行ったIRF7遺伝子のSNP

SNP	位置	アレル	機能	アミノ酸変異	方法
rs10902179	-667	C/T	Promoter	-	Sequencing
rs2277270	-486	A/G	Intron	-	Sequencing
rs3832720	-424-425	-/GCCTCC	Intron	-	Sequencing
rs11544076	-206	C/G	Intron	-	Sequencing
rs1061502	1047	A/G	Nonsynonymous	Lys/Glu	Realtime PCR
rs1061505	2068	C/A	Synonymous	-	Sequencing
rs3178010	2157	A/G	Nonsynonymous	Gln/Arg	Sequencing
rs12422022	2173	A/G	Intron	-	Sequencing
rs1051390	2200	C/G	Intron	-	Sequencing

硬変＝178：228人であった(表1)．これら患者の白血球よりGenomic DNAを抽出し，SNPのgenotypingに用いた．

調べたIRF7遺伝子のSNPは計9カ所で，そのうちの2カ所はIRF7のアミノ酸変異を生じるものであった．またSNPのジェノタイピングは，Direct sequencing法あるいはTaqman realtime PCR法を用いて行った(表2)．

2．IRF7のSNP genotyping結果

IRF7のSNPジェノタイピング結果を表3に示す．SNP1047，SNP2068，SNP2157，SNP2173，SNP2200の5 SNPsは完全に連鎖していたため(complete linkage disequilibrium)，SNP1047を代表例として後の解析を行った．

HCV感染患者を「肝硬変あり，なし」で2つのグループに分け，SNP1047のジェノタイプ頻度を比較したものを表4に示す．SNP1047のジェノタイプAGの頻度が，ジェノタイプAAに比し，肝硬変患者中で有意に高いことが分かる(5.62 vs. 1.75%；P=0.034)．SNP1047のアレル頻度の比較でも，Gアレルが肝硬変患者中で有意に高かった(1 vs. 4%，P=0.036)．また，その他のSNPsについても同様の解析を行ったが，SNP1047以外に肝

表3　HCV感染患者におけるIRF7遺伝子のSNP頻度

SNP	位置	機能	Homozygote	Heterozygote	Homozygote
rs10902179	-667C/T	Promoter	355 (87.44%) CC	50 (12.31%) CT	1 (0.25%) TT
rs2277270	-486A/G	Intron	224 (55%) (4 Repeat)	153 (37.6%)	30 (7.4%) (3 Repeat)
rs3832720	-424-425 -/GCCTCC	Intron	371 (91.38%) AA	35 (8.62%) AG	0 (0%) GG
rs11544076	-206C/G	Intron	406 (100%) CC	0 (0%) CG	0 (0%) GG
rs1061502	1047A/G	Nonsynonymous (Lys/Glu)	392 (96.55%) AA	14 (3.45%) AG	0 (0%) GG
rs1061505	2068A/C	Synonymous	392 (96.55%) AA	14 (3.45%) AC	0 (0%) CC
rs3178010	2157A/G	Nonsynonymous (Gln/Arg)	392 (96.55%) AA	14 (3.45%) AG	0 (0%) GG
rs12422022	2173A/G	Intron	392 (96.55%) AA	14 (3.45%) AG	0 (0%) GG
rs1051390	2200C/G	Intron	392 (96.55%) CC	14 (3.45%) CG	0 (0%) GG

表4　SNP1047とC型肝硬変との関連

SNP1047	肝硬変なし (n=228)	肝硬変あり (n=178)	オッズ比 (95% CI)	P値
ジェノタイプ				
AA	224 (98.25%)	168 (94.4%)	1	0.034
AG	4 (1.75%)	10 (5.6%)	3.34	
GG	0 (0%)	0 (0%)	(1.03-10.8)	
アレル				
A	456 (99%)	350 (96%)	1	0.036
G	4 (1%)	14 (4%)	3.27 (1.02-10.5)	

硬変の有無と関連するものは存在しなかった．

「肝硬変あり，なし」の2グループの患者背景を単変量解析で比較すると，「年齢，アルブミン値，AFP値，血小板数，総ビリルビン値」で有意差を認めたため，ロジスティック回帰分析による多変量解析にてSNP1047と肝硬変との関連を検討した．その結果，

表5　肝硬変の有無と関連する因子の多変量解析結果

因子		単変量解析			多変量解析		
		OR	95% CI	P値	OR	95% CI	P値
年齢	> 60歳	2.6	1.7-3.9	< 0.0001	1.5	1.2-1.9	0.003
アルブミン値	< 3.3 g/dl	5.9	2.8-12.7	< 0.0001	-	-	-
AFP値	> 20 μg/L	4.8	3.1-7.5	< 0.0001	1.7	1.3-2.2	<0.0001
血小板数	< 12.5 × 10^4/ μl	10.7	6.7-17.1	< 0.0001	2.7	2.1-3.5	<0.0001
総ビリルビン値	> 0.7 mg/dl	4.8	3.0-7.8	< 0.0001	2	1.5-2.6	<0.0001
SNP1047	A/A	1			1		0.02
	A/G	3.34	1.03-10.8	0.03	2.5	1.2-5.6	

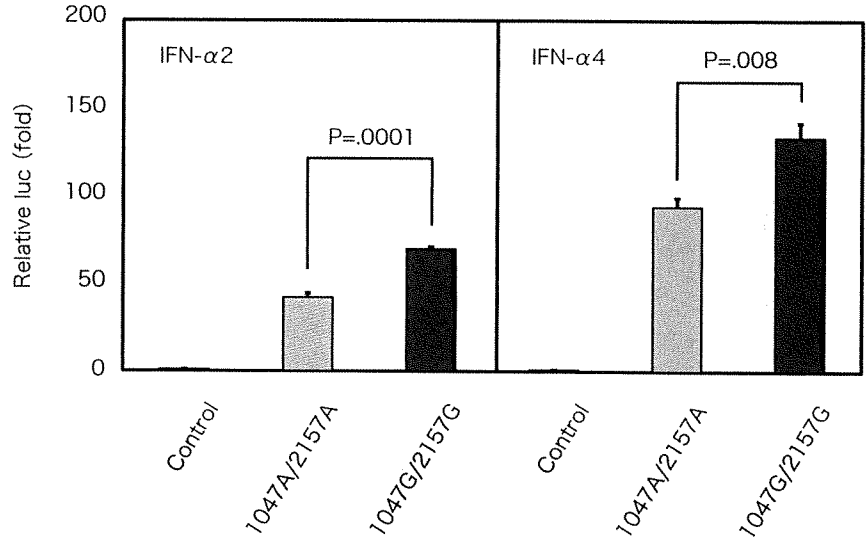

図1　IRF7のSNPsによるアミノ酸変異とIFN-α誘導能

SNP1047は肝硬変に寄与する独立した因子として選択され，SNP1047は肝硬変の有無と関連するSNPであることが示唆された（OR=2.5, 95% CI=1.2-5.6, P=0.02）（表5）．

3. SNP1047とIFN-α誘導能との関連性

今回の検討で肝硬変との関連性が見いだされたIRF7のSNP1047は，SNP2068, SNP2157, SNP2173, SNP2200の4 SNPsと完全に連鎖しており，その中のSNP1047, SNP2157はアミノ酸変異を伴うものであった．そこで，このアミノ酸変異がIFN-α誘導能に及ぼす影響につき，SNP1047A/SNP2157AもしくはSNP1047G/SNP2157Gを有するIRF7の発現プラスミドを構築し，IFN-α2もしくはIFN-α4のレポータープラスミドを用いたルシフェラーゼアッセイにて検討した．

その結果，SNP1047G/SNP2157Gを有するIRF7は，IFN-α2，IFN-α4のルシフェラーゼ活性を上昇させることが判明した（図1）．すなわち，SNP1047G/SNP2157Gを有するIRF7は，IFN-αプロモーターの転写活性を亢進させ，IFN-αをより強く誘導するもの

と考えられた.

4 考 察

今回の検討では9カ所のIRF7遺伝子のSNPジェノタイピングを行ったが，5 SNPsは完全に連鎖しており，その中でアミノ酸変異を伴うものはSNP1047，SNP2157の2つであった．そしてSNP1047AG，SNP2157AGジェノタイプがC型肝硬変患者に多く認められ，SNP1047G，SNP2157Gを有するIRF7は，IFN-αプロモーターの転写活性を亢進させることが明らかとなった．

IRF7中にはいくつかのdomainが存在するが，SNP1047は「constitutive activation domain」，SNP2157は「inhibitory domain」に中に存在する[7~10]．とりわけ「Constitutive activation domain」は「acidic region」を含んでおり，SNP1047によるアミノ酸変異（Lys/Glu）は，IRF7の転写活性を変化させる可能性がある．

また，IRF7はIFN-α産生を誘導するだけでなく，RANTES（regulated on activation normal T cell expressed and secreted; CCL5），TRAIL（TNF-related apoptosis-inducing ligand），IL-12，IL-15といった種々のケモカインやサイトカインを活性化することも報告されている[11~18]．したがって，SNP1047G，SNP2157Gを有するIRF7では，これらの経路を介して肝臓での炎症がより惹起され，C型肝硬変への進展を助長させる可能性もある．

5 むすび

今回の検討にて，SNP1047，SN2157のGアレルがC型肝硬変のリスクファクターであることが示された．したがって，これらのSNPsはHCV感染患者の中から，C型肝硬変へ進展するハイリスク患者を選別するマーカーとなりうる可能性がある．ただし，その詳細なメカニズムはまだ十分に解明されているとはいえず，今後の検討課題である．

また，「内因性インターフェロン（IFN）産生経路」を構成する遺伝子は他にも多数存在するため，IRF7以外の遺伝子のSNPsがC型肝硬変への進展と関連している可能性も十分に存在する．そのため今後は，これら多数の遺伝子を含めた網羅的なSNP解析が望まれる．

文 献

1) Dharel N, Kato N, Muroyama R et al : MDM2 promoter SNP309 is associated with the risk of hepatocellular carcinoma in patients with chronic hepatitis C. Clin Cancer Res 12 : 4867–4871, 2006
2) Kato N, Ji G, Wang Y et al : Large-scale search of single nucleotide polymorphisms for hepatocellular carcinoma susceptibility genes in patients with hepatitis C. Hepatology 42 : 846–853, 2005
3) McIlroy D, Theodorou I, Ratziu V et al : FAS promoter polymorphisms correlate with activity grade in hepatitis C patients. Eur J Gastroenterol Hepatol 17 : 1081–1088, 2005
4) Sermasathanasawadi R, Kato N, Muroyama R et al : Association of interferon regulatory factor-7 gene polymorphism with liver cirrhosis in chronic hepatitis C patients. Liver Int 28 : 798–806, 2008
5) Seth RB, Sun L, Chen ZJ : Antiviral innate immunity pathways. Cell Res 16 : 141–147, 2006
6) Honda K, Yanai H, Negishi H et al : IRF-7 is the master regulator of type-I interferon-dependent immune responses. Nature 434 : 772–777, 2005
7) Au WC, Moore PA, LaFleur DW et al : Characterization of the interferon regulatory factor-7 and its potential role in the transcription activation of interferon A genes. J Biol Chem 273 : 29210–29217, 1998
8) Lin R, Genin P, Mamane Y et al : Selective DNA binding and association with the CREB binding protein coactivator contribute to differential activation of alpha/beta interferon genes by interferon regulatory factors 3 and 7. Mol Cell Biol 20

: 6342–6353, 2000
9) Lin R, Mamane Y, Hiscott J : Multiple regulatory domains control IRF-7 activity in response to virus infection. J Biol Chem 275 : 34320–34327, 2000
10) Marie I, Smith E, Prakash A et al : Phosphorylation-induced dimerization of interferon regulatory factor 7 unmasks DNA binding and a bipartite transactivation domain. Mol Cell Biol 20 : 8803–8814, 2000
11) Apolinario A, Majano PL, Alvarez-Perez E et al : Increased expression of T cell chemokines and their receptors in chronic hepatitis C: relationship with the histological activity of liver disease. Am J Gastroenterol 97 : 2861–2870, 2002
12) Barnes BJ, Richards J, Mancl M et al : Global and distinct targets of IRF-5 and IRF-7 during innate response to viral infection. J Biol Chem 279 : 45194–45207, 2004
13) Bone-Larson CL, Simpson KJ, Colletti LM et al : The role of chemokines in the immunopathology of the liver. Immunol Rev 177 : 8–20, 2000
14) Genin P, Algarte M, Roof P et al : Regulation of RANTES chemokine gene expression requires cooperativity between NF-kappa B and IFN-regulatory factor transcription factors. J Immunol 164 : 5352–5361, 2000
15) Hellier S, Frodsham AJ, Hennig BJ et al : Association of genetic variants of the chemokine receptor CCR5 and its ligands, RANTES and MCP-2, with outcome of HCV infection. Hepatology 38 : 1468–1476, 2003
16) Leroy V, Vigan I, Mosnier JF et al : Phenotypic and functional characterization of intrahepatic T lymphocytes during chronic hepatitis C. Hepatology 38 : 829–841, 2003
17) Nischalke HD, Nattermann J, Fischer HP et al : Semiquantitative analysis of intrahepatic CC-chemokine mRNas in chronic hepatitis C. Mediators Inflamm 13 : 357–359, 2004
18) Romieu-Mourez R, Solis M, Nardin A et al : Distinct roles for IFN regulatory factor (IRF) -3 and IRF-7 in the activation of antitumor properties of human macrophages. Cancer Res 66 : 10576–10585, 2006

* * *

Virus Research 146 (2009) 41–50

Contents lists available at ScienceDirect

Virus Research

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

Efficient replication systems for hepatitis C virus using a new human hepatoma cell line

Nobuyuki Kato[a,*], Kyoko Mori[a], Ken-ichi Abe[a], Hiromichi Dansako[a], Misao Kuroki[a], Yasuo Ariumi[a], Takaji Wakita[b], Masanori Ikeda[a]

[a] Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan
[b] Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

ARTICLE INFO

Article history:
Received 29 May 2009
Received in revised form 12 August 2009
Accepted 21 August 2009
Available online 29 August 2009

Keywords:
Hepatitis C virus (HCV)
HCV RNA replication
Li23 cells
Reporter assay for anti-HCV reagents
Infectious HCV production

ABSTRACT

Persistent hepatitis C virus (HCV) infection causes chronic liver diseases and is a serious global health problem. Cell culture-based persistent HCV RNA replication systems and infectious HCV production systems are widely used in HCV research. However, persistent HCV production systems have been developed only for HuH-7 hepatoma cells. Here we found a new human hepatoma cell line, Li23, that enables persistent HCV production and anti-HCV reagent assay. Li23's cDNA expression profile differed from HuH-7's, although the two cells had similar liver-specific expression profiles. We used HCV RNA with a specific combination of adaptive mutations to develop an HCV replicon system and genome-length HCV RNA replicating systems including a reporter assay system. Finally, Li23-derived cells persistently produced infectious virus of an HCV strain. Li23-derived cells are potentially useful for understanding the HCV life cycle and for finding antiviral targets.

1. Introduction

Hepatitis C virus (HCV) infection frequently causes active liver diseases such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Choo et al., 1989; Saito et al., 1990; Thomas, 2000). Although the combination of pegylated-interferon (PEG-IFN) and ribavirin is the standard therapy worldwide, only half of the patients receiving this treatment exhibit a sustained virological response (Chevaliez and Pawlotsky, 2007; Hadziyannis et al., 2004). Since more than 170 million people are infected with HCV worldwide, the virus remains a serious global health problem (Thomas, 2000). HCV is an enveloped positive single-stranded RNA virus of the *Flaviviridae* family. The HCV RNA genome encodes a large polyprotein precursor of approximately 3000 amino acids, which is cleaved into 10 proteins in the following order: core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Hijikata et al., 1991, 1993; Kato et al., 1990). Although many issues have been addressed since HCV was first identified, the lack of a virus culture system has long been a serious handicap in the fight against HCV infection (Kato and Shimotohno, 2000). The development of an HCV replicon system enabling HCV subgenomic RNA replication in HuH-7 human hepatoma cells has allowed the study of the mechanisms underlying HCV replication (Lohmann et al., 1999). After the first replicon of genotype 1b was developed, HCV replicons derived from several HCV strains appeared, and tissue, genotype, and host ranges were expanded (Ali et al., 2004; Date et al., 2004; Ikeda et al., 2002; Kato et al., 2003a,b; Kishine et al., 2002; Zhu et al., 2003). However, most of RNA replication systems using the culture cells other than HuH-7 cells have been fairly low-level. Furthermore, genome-length HCV RNA replication systems and drug assay systems have been developed (Blight et al., 2002; Ikeda et al., 2002, 2005; Mori et al., 2008; Pietschmann et al., 2002). To date, however, robust genome-length HCV RNA replication and anti-HCV reagent assays have been developed for only one human cell line, HuH-7 (Bartenschlager and Sparacio, 2007; Lindenbach and Rice, 2005). In 2005, an efficient virus production system using the JFH1 genotype 2a strain was developed using HuH-7-derived cell lines (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). In this system also, HuH-7 is still the only cell line that enables persistent HCV production without additional host factors such as CD81 (Gottwein and Bukh, 2008), although transient virus production in human hepatoma cell line LH86 was recently reported (Zhu et al., 2007). Furthermore, it is uncertain whether or not the recent advances obtained from the HuH-7 cell system reflect the general features of the HCV life cycle. Here, we found a new human hepatoma cell line, Li23, that enables robust genome-length

Abbreviations: HCV, hepatitis C virus; PEG-IFN, pegylated-interferon; E1, envelope 1; NS2, non-structural 2; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline; p.i., post-infection; dsRNA, double-stranded RNA; EC_{50}, 50% effective concentration.

* Corresponding author. Tel.: +81 86 235 7385; fax: +81 86 235 7392.
E-mail address: nkato@md.okayama-u.ac.jp (N. Kato).

0168-1702/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2009.08.006

HCV RNA replication. Using Li23-derived cell lines, we developed a novel drug assay system and a persistent HCV production system.

2. Materials and methods

2.1. Cell line

The Li23 cell line, established in 1987, consists of human hepatoma cells from a Japanese male (age 56) and was kindly provided by Drs. Y. Ishikawa and S. Hirohashi (National Cancer Center Research Institute, Tokyo). The Li23 cell line is free of both the hepatitis B virus antigen and HCV (Kato et al., 1995).

2.2. Cell culture

The six HuH-7-derived cell lines: sO cells, harboring the subgenomic replicon RNA of HCV-O (genotype 1b) (Kato et al., 2003a); O cells, harboring a replicative genome-length HCV-O RNA (Ikeda et al., 2005); Oc cured cells, which were created by eliminating HCV RNA from O cells by IFN treatment (Ikeda et al., 2005); OAc cured cells, which were created by eliminating HCV RNA from genome-length HCV-O RNA replicating OA cells (Abe et al., 2007); OR6 cells, harboring the genome-length HCV-O RNA with luciferase as a reporter (Ikeda et al., 2005); RSc cured cells that cell culture-generated HCV-JFH1 (HCVcc) (JFH1 strain of genotype 2a) (Wakita et al., 2005) could infect and efficiently replicate (Ariumi et al., 2007, 2008; Kuroki et al., 2009), or their parental HuH-7 cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The sO, O, and OR6 cells were maintained in the presence of G418 (0.3 mg/ml, Geneticin; Invitrogen). Li23 cells were maintained in modified culture medium for the PH5CH8 human immortalized hepatocyte cell line (Ikeda et al., 1998). The medium for Li23 cells consists of F12 medium and DMEM (1:1 in volume) supplemented with 1% FBS, epidermal growth factor (50 ng/ml), insulin (10 μg/ml), hydrocortison (0.36 μg/ml), transferrin (5 μg/ml), linoleic acid (5 μg/ml), selenium (20 ng/ml), prolactin (10 ng/ml), gentamycin (10 μg/ml), kanamycin monosulfate (0.2 mg/ml), and fungizone (0.5 μg/ml). Li23-derived cells harboring the HCV replicon or genome-length HCV RNA were cultured in the above medium supplemented with G418 (0.3 mg/ml). The cured Li23-derived cells were maintained in the above medium without G418. The HeLa and HEK293 cells were cultured in DMEM supplemented with 10% FBS.

2.3. Plasmid construction

To introduce the mutations into plasmid pON/3-5B, pON/C-5B, or pORN/C-5B (Ikeda et al., 2005) (GenBank accession no. AB191333; Supplemental Fig. S1), a PCR-based site-directed mutagenesis method was used as previously described (Abe et al., 2007; Mori et al., 2008).

2.4. RNA synthesis

Plasmid DNAs were linearized with XbaI and used for RNA synthesis with the T7 MEGAscript kit (Ambion) as previously described (Kato et al., 2003a). Synthesized RNA was purified by lithium chloride precipitation and dissolved in nuclease-free water.

2.5. RNA transfection and selection of G418-resistant cells

RNA was transfected to Li23 or Li23-derived cells as previously described (Lohmann et al., 1999). Cells were selected in complete medium with G418 (0.3 mg/ml) and sodium bicarbonate solution (0.15%) for 3 weeks as previously described (Kato et al., 2003a). For the staining of G418-resistant colonies, Coomassie brilliant blue (0.06% in 50% methanol–10% acetic acid) was used as previously described (Ikeda et al., 2005).

2.6. Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting analysis with a PVDV membrane were performed as previously described (Kato et al., 2003a). The antibodies used for the O strain in this study were those against core (CP11; Institute of Immunology), E1 and E2 (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), NS3 (Novocastra Laboratories), NS4A and NS5A (a generous gift from Dr. A. Takamizawa, Research Foundation for Microbial Diseases, Osaka University), and NS5B (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science). The antibodies used for the JFH1 strain were those against core (CP11; Institute of Immunology) and NS5B (Murakami et al., 2008). β-Actin antibody (Sigma) was used as the control for the amount of protein loaded per lane. Immunocomplexes were detected by the Renaissance enhanced chemiluminescence assay (PerkinElmer Life Sciences).

2.7. Northern blot analysis

Total RNA from the cultured cells was prepared using an RNeasy extraction kit (Qiagen). Three micrograms of total RNA was used for the analysis. HCV-specific RNA and β-actin mRNA were detected according to a method described previously (Ikeda et al., 2005; Kato et al., 2003a). The synthetic RNA transcribed from pON/3-5B, pON/C-5B, or pORN/C-5B (10^7 and 10^8 genome equivalents spiked into cellular total RNA) was used to compare HCV RNA levels.

2.8. Quantification of HCV RNA

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis for HCV RNA was performed using a real-time LightCycler PCR as described previously (Ikeda et al., 2005). We used the following forward and reverse primer sets for the real-time LightCycler PCR: HCV-O, 5′-AGAGCCATAGTGGTCTGCGG-3′ (forward), 5′-CTTTCGCGACCCAACACTAC-3′ (reverse); and HCV-JFH1, 5′-AGAGCCATAGTGGTCTGCGG-3′ (forward), 5′-CTTTCGCAACCCAACGCTAC-3′ (reverse). Experiments were done in triplicate.

2.9. Preparation of cured cells

To prepare cured cells, the cells harboring the HCV replicon or genome-length HCV RNA were treated with IFN-γ as described previously (Abe et al., 2007). Briefly, the cells were treated with IFN-γ (1000 IU/ml) in the absence of G418. The treatment was continued for 3 weeks with the addition of IFN-γ at 4-day intervals. The cured cells obtained from O, OA, sOL, OL8, OL11, OL14, ORL8, and ORL11 cells were named Oc, OAc, sOLc, OL8c, OL11c, OL14c, ORL8c, and ORL11c, respectively. RT-PCR confirmed the absence of HCV RNA in these cured cells.

2.10. Immunofluorescence and confocal microscopic analyses

Four days after the cells were seeded on the collagen-coated coverslip, they were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) and then permeabilized in 0.1% Triton X-100 in PBS at room temperature. After blocking with 1% bovine serum albumin, the cells were incubated with the primary antibodies and then with the secondary antibody. The primary antibodies used to detect the core and dsRNA were anti-core (CP11; Institute of Immunology) and anti-double-stranded (ds) RNA (K1; English and

Scientific Consulting), respectively. The primary antibodies used to detect NS5B of O strain and JFH1 strain were anti-NS5B (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science) and anti-NS5B (Murakami et al., 2008), respectively. The secondary antibody was Cy2-conjugated anti-mouse secondary antibody or FITC-conjugated anti-rabbit secondary antibody (for NS5B of JFH1) (Jackson ImmunoResearch). The nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma). The coverslips were mounted on glass slides by PermaFluor Aqueous Mountant (ThermoFisher) and then the cells were photographed under a confocal laser scanning microscope (LSM510; Carl Zeiss).

2.11. cDNA microarray analysis

HuH-7, Oc, OAc, Li23, OL8, OL11, OL8c, and OL11c cells (1×10^6 each) were plated onto 10-cm diameter dishes and cultured for 2 days in the absence of G418. Total RNAs from these cells were prepared using the RNeasy extraction kit (Qiagen). cDNA microarray analysis was performed by Dragon Genomics Center of Takara Bio. (Otsu, Japan) through an authorized Affymetrix service provider. cDNA was synthesized by the GeneChip T7-Oligo(dT) Promoter Primer Kit (Affymetrix) and TaKaRa cDNA Synthesis Kit (Takara Bio) from 3 μg total RNA. Biotinylated complementary RNA (cRNA) was synthesized by the IVT Labeling Kit (Affymetrix). Following fragmentation, 10 μg of cRNA was hybridized for 16 h at 45 °C on the GeneChip Human Genome U133 Plus 2.0 Array. GeneChips were washed and stained in the Affymetrix Fluidics Station 450, and then were scanned using GeneChip Scanner 3000 7G. Single array analysis was calculated by Microarray Suite version 5.0 (MAS5.0) with the Affymetrix setting. Differentially expressed genes were selected by comparing HuH-7-derived cells and Li23-derived cells.

2.12. RT-PCR

RT-PCR was performed to detect cellular mRNA as described previously (Dansako et al., 2003). Briefly, total RNA (2 μg) was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen) using an oligo dT primer (Invitrogen) according to the manufacturer's protocol. One-tenth of synthesized cDNA was used for PCR. The primers and PCR cycles used in this study are listed in Supplemental Table 1.

2.13. Quantification of HCV core protein

The levels of the core protein in the culture supernatants were determined by enzyme-linked immunosorbent assay (Mitsubishi Kagaku Bio-Clinical Laboratories).

2.14. Renilla luciferase assay for anti-HCV reagents

To monitor the effects of anti-HCV reagents, the cells were plated onto 24-well plates (2×10^4 cells per well) and cultured with the medium for Li23-derived cells in the absence of fungizone and G418 for 24 h. The cells were then treated with anti-HCV reagent at several concentrations for 72 h (sometimes 24 or 48 h), or the cells were treated with a combination of IFN-α and another anti-HCV reagent at several concentrations for 72 h. After treatment, the cells were subjected to luciferase assay using the renilla luciferase assay system according to the manufacturer's protocol (Promega). A manual Lumat LB 9501/16 luminometer (EG&G Berthold) was used to detect luciferase activity. The experiments were performed in at least triplicate.

2.15. Cell viability

To examine the cytotoxic effects of anti-HCV reagents on the cells, the cells were plated onto 24-well plates (2×10^4 cells per well) and cultured for 24 h. They were then treated with or without anti-HCV reagents for 72 h in the absence of G418. The viable cells were then counted in an improved Neubauer-type hemotocytometer after Trypan blue dye (Invitrogen) treatment. The experiments were performed in triplicate.

2.16. Infection of cells with secreted HCV

The inoculum for HCV infection was the culture medium of RSc cells (Ariumi et al., 2007, 2008; Kuroki et al., 2009) at 145 days after transfection with JFH1 RNA *in vitro* synthesized from pJFH1 (Wakita et al., 2005). This inoculum was passed through a 0.2-μm filter after low-speed centrifugation before use for infection. We seeded cells 24 h before infection at a density of 2×10^4 cells per well in a 24-well plate. We infected cells with 100 μl (equivalent to $10^{4.3}$ $TCID_{50}$) of inoculum for 2 h, washed them, added complete medium and cultured them for a maximum of 30 days with adequate passage of the cells. In some cases, at 7 or 8 days p.i., supernatant was used as an inoculum for the next HCV infection. The cells at 7 or 14 days p.i. were used to detect HCV proteins by Western blot analysis, to quantify HCV RNA by quantitative RT-PCR or to analyze the immunofluorescence of HCV proteins or dsRNA.

3. Results

3.1. Efficient replication system with HCV replicon or genome-length HCV RNA using human hepatoma Li23 cells

We previously established several genome-length HCV RNA (O strain of genotype 1b) replicating cell lines and found that a specific combination of adaptive mutations – either Q1112R, P1115L, and S2200R (QR,PL,SR) or Q1112R, K1609E, and S2200R (QR,KE,SR) – drastically enhanced the level of genome-length HCV RNA replication (Abe et al., 2007; Ikeda et al., 2005). This finding led us to hypothesize that such combinations of adaptive mutations may overcome the barrier that has made HuH-7 the only cell line thus far to allow the robust replication of genome-length HCV RNA. To test this hypothesis, HCV replicon RNA (ON/3-5B) possessing QR,PL,SR or QR,KE,SR (Supplemental Fig. 1) was transfected into various kinds of human cell lines (HuH-6, Li21, Li23, Li24, PH5CH, OUMS29, IHH10.3, IHH12 etc.), and the G418 selection was performed as described previously (Kato et al., 2003a). Although we failed to obtain the G418-resistant colonies in the most cell lines, fortunately, we found that the Li23 human hepatoma cell line gave only G418-resistant colonies (Fig. 1A). Approximately 200 and 700 colonies obtained from ON/3-5B/QR,PL,SR and ON/3-5B/QR,KE,SR-transfected cells, respectively, were pooled. Western blot analysis revealed that the expression levels of HCV proteins NS5A and NS5B were much higher in ON/3-5B/QR,KE,SR-derived colonies than in ON/3-5B/QR,PL,SR-derived colonies (Fig. 1B). We used the former for further analysis and referred to them as sOL cells. We demonstrated that the replicon in sOL cells showed a high level of sensitivity to anti-HCV reagents, similar to the level shown by the replicon (ON/3-5B/SR) in sO cells (Kato et al., 2003a) (Fig. 1C).

To obtain a source of cells with which to develop a genome-length HCV RNA replication system, we prepared cured cells (sOLc) from sOL cells by IFN-γ treatment, because cured cells are known to extremely enhance HCV RNA replication levels (Ikeda et al., 2005; Kato et al., 2003a). A genome-length HCV RNA (ON/C-5B/QR,KE,SR; Supplemental Fig. 1) was transfected into sOLc cells. Following G418 selection, many colonies were obtained (Fig. 2A). Fourteen

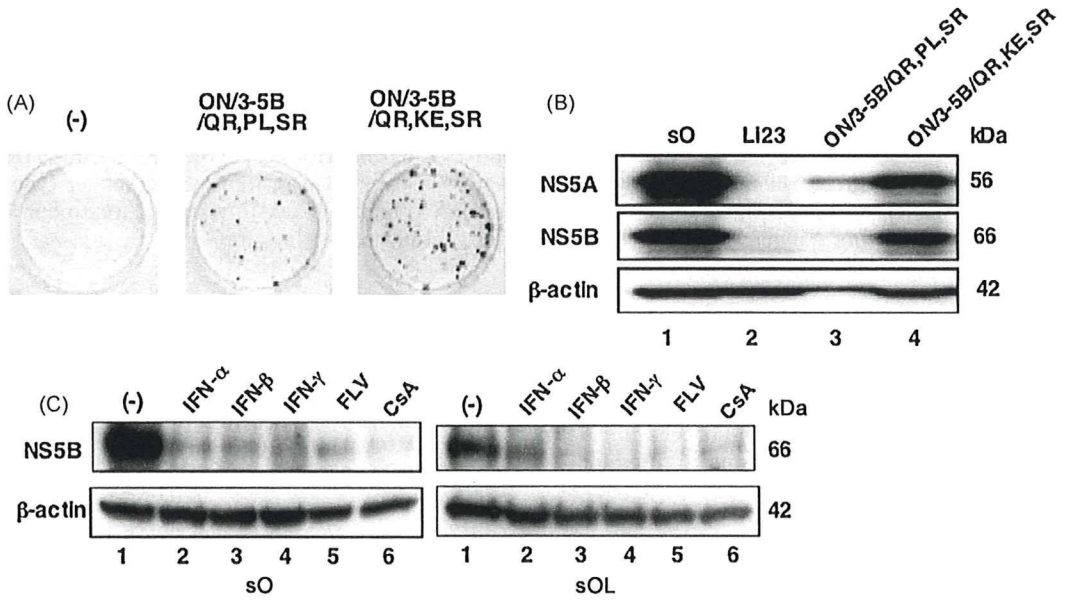

Fig. 1. Li23-derived cells harboring HCV replicon. (A) G418-resistant colonies from Li23 cells transfected with replicon RNA. ON/3-5B RNA with three additional mutations (ON/3-5B/QR,PL,SR or ON/3-5B/QR,KE,SR) was transfected into Li23 cells. The panels show G418-resistant colonies (57 colonies/μg RNA for ON/3-5B/QR,PL,SR and 132 colonies/μg RNA for ON/3-5B/QR,KE,SR) that were stained with Coomassie brilliant blue at 3 weeks after RNA transfection. (B) Western blot analysis of Li23-derived G418-resistant cells for HCV proteins NS5A and NS5B. Lane 1, sO (HuH-7-derived cell line harboring HCV replicon, ON/3-5B/SR); lane 2, Li23 as a negative control; lane 3, polyclonal G418-resistant cells obtained by transfection with ON/3-5B/QR,PL,SR RNA; lane 4, polyclonal G418-resistant cells (sOL) by transfection with ON/3-5B/QR,KE,SR RNA. (C) Sensitivity of sOL replicon to anti-HCV reagents. sOL cells were treated with IFN-α (lane 2, 20 IU/ml), IFN-β (lane 3, 20 IU/ml), IFN-γ (lane 4, 20 IU/ml), fluvastatin (FLV) (lane 5, 5 μM), or cyclosporine A (CsA) (lane 6, 0.5 μg/ml) for 5 days. Lane 1 shows no treatment. For comparison, sO cells were treated as well as sOL cells. NS5B was detected by Western blot analysis.

colonies (referred to as OL1–OL14) and a mixture of approximately 200 other colonies (referred to as OL) were successfully proliferated as cell lines. Using quantitative RT-PCR, we selected OL8, OL11, and OL14 because of their high levels (>9 × 10^6 copies/μg total RNA) of HCV RNA, although the titer of HCV RNA from genome-length HCV RNA replicating HuH-7-derived O cells (Ikeda et al., 2005) was approximately 4.5 × 10^7 copies/μg total RNA (Supplemental Fig. 2). We also demonstrated that the HCV sequence was not

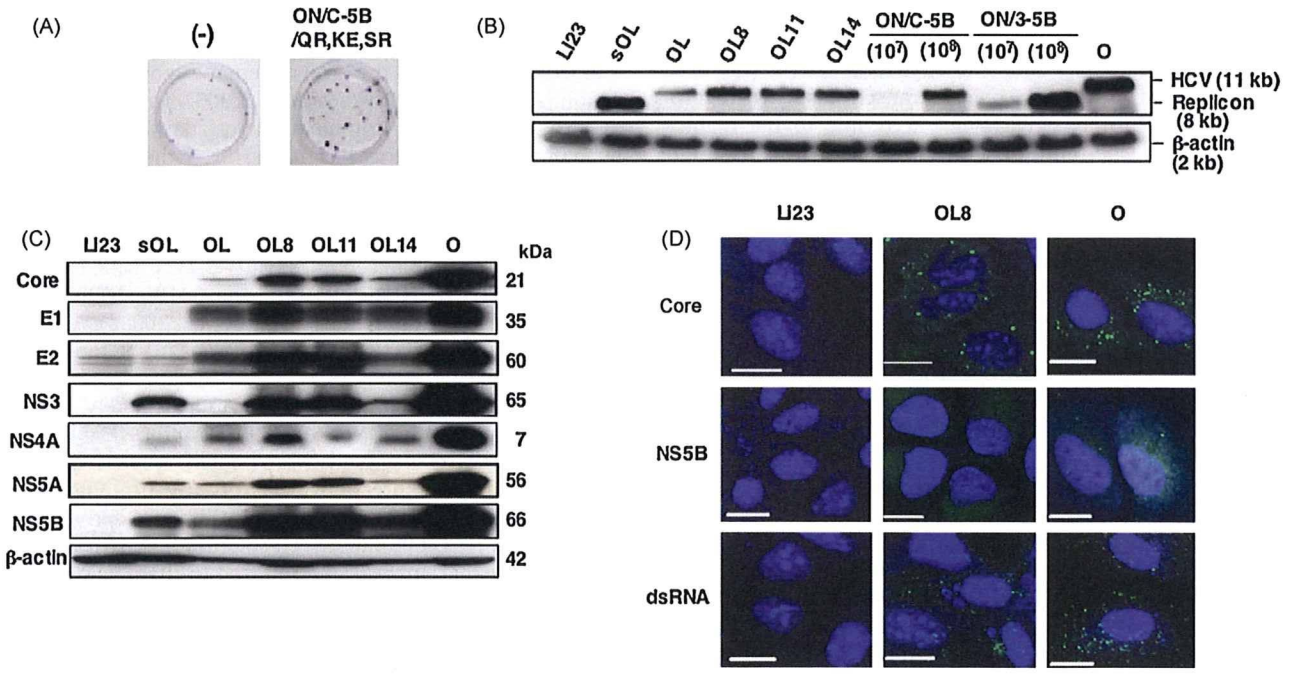

Fig. 2. Establishment of Li23-derived cell lines harboring replicative genome-length HCV RNA. (A) G418-resistant colonies from sOLc cells transfected with genome-length HCV RNA (ON/C-5B/QR,KE,SR). The panels show G418-resistant colonies (100 colonies/μg RNA) that were stained with Coomassie brilliant blue at 3 weeks after RNA transfection. (B) Northern blot analysis of total RNA prepared from sOL cells and genome-length HCV RNA-replicating cells (OL, OL8, OL11, and OL14). Synthetic RNA, given number of ON/C-5B or ON/3-5B RNA. HuH-7-derived O cells harboring replicative genome-length HCV RNA (ON/C-5B/KE,SR) and Li23 cells served as positive and negative controls, respectively. (C) Western blot analysis of sOL and genome-length HCV RNA-replicating cells (OL, OL8, OL11, and OL14) for HCV proteins, core, E1, E2, NS3, NS4A, NS5A, and NS5B. O cells and Li23 cells served as positive and negative controls, respectively. (D) Immunofluorescence analysis of OL8 cells. The cells were processed and stained with anti-core, anti-NS5B, or anti-dsRNA antibodies and Cy2-conjugated secondary antibody. The O cells and Li23 cells served as positive and negative controls, respectively. Bar, 20 μm.

integrated into the genomic DNA in OL, OL8, OL11, OL14, or sOL cells (data not shown). Northern and Western blot analyses also showed that the levels of HCV RNA and proteins in OL8 and OL11 cells were somewhat lower than those in O cells (Fig. 2B and C). Immunofluorescence analysis of the intracellular localization of HCV proteins and dsRNA, which is an intermediate of RNA replication, showed that the staining levels of HCV proteins and dsRNA located in the cytoplasm of OL8 cells, were also comparable to those in O cells (Fig. 2D). Both OL8 and O cells had two types of core protein staining patterns (detergent-resistant dots or patches and detergent-sensitive ring-like structures), as described previously (Matto et al., 2004) in HuH-7 cells harboring the genome-length HCV RNA (Con1 strain of genotype 1b) (Fig. 2D). These results suggest that robust replication of genome-length HCV RNA occurs in OL8 and OL11 cells. We performed sequence analysis of HCV RNAs derived from OL8, OL11, and OL14 cells, but no additional mutations were detected commonly among the three independent clones sequenced (data not shown). This suggested that no mutations other than Q1112R, K1609E, and S2200R are needed for genome-length HCV RNA replication in Li23-derived cells.

3.2. Genes differentially expressed between Li23- and HuH-7-derived cells

RT-PCR analysis revealed that Li23 and HuH-7 cells had similar liver-specific gene expression profiles (Fig. 3A). However, there is no information regarding the Li23-specific gene expression profile. To address this, we performed cDNA microarray analysis using total RNAs prepared from Li23, OL8, OL11, cured OL8 (OL8c), and OL11c cells in addition to HuH-7, Oc (Ikeda et al., 2005), and OAc (Abe et al., 2007). As the first step in this analysis, we selected 206 and 326 genes whose expression levels were upregulated and downregulated at ratios of more than 2^5 and less than 2^{-5} in Li23 vs. HuH-7, respectively. Then, from among those selected in the first step, we performed an additional selection of genes whose expression levels were commonly upregulated or downregulated among Li23-derived cells when compared with HuH-7-derived cells, and each of several already-known genes were identified (data not shown). Fig. 3B shows the results of RT-PCR regarding the representative genes belonging to such a category in the expression levels between Li23- and HuH-7-derived cells. The most characteristic feature of Li23-derived cells was the high expression levels of cancer antigens (NY-ESO-1, MAGEA, etc.) compared with no expression in HuH-7-derived cells (Fig. 3B). We demonstrated that such drastic differences were not attributable to differences in culture media (Supplemental Fig. 3). These results exclude the possibility that OL8 and OL11 cells are derived from contamination of HuH-7-derived cells. On the other hand, this microarray analysis revealed that HuH-7- and Li23-derived cells showed similar expression levels of CD81, scavenger receptor class B type I (SR-BI), Claudin-1, and Occludin, which have been identified as the host factors for HCV entry (Burlone and Budkowska, 2009). RT-PCR analysis confirmed them (Fig. 3C).

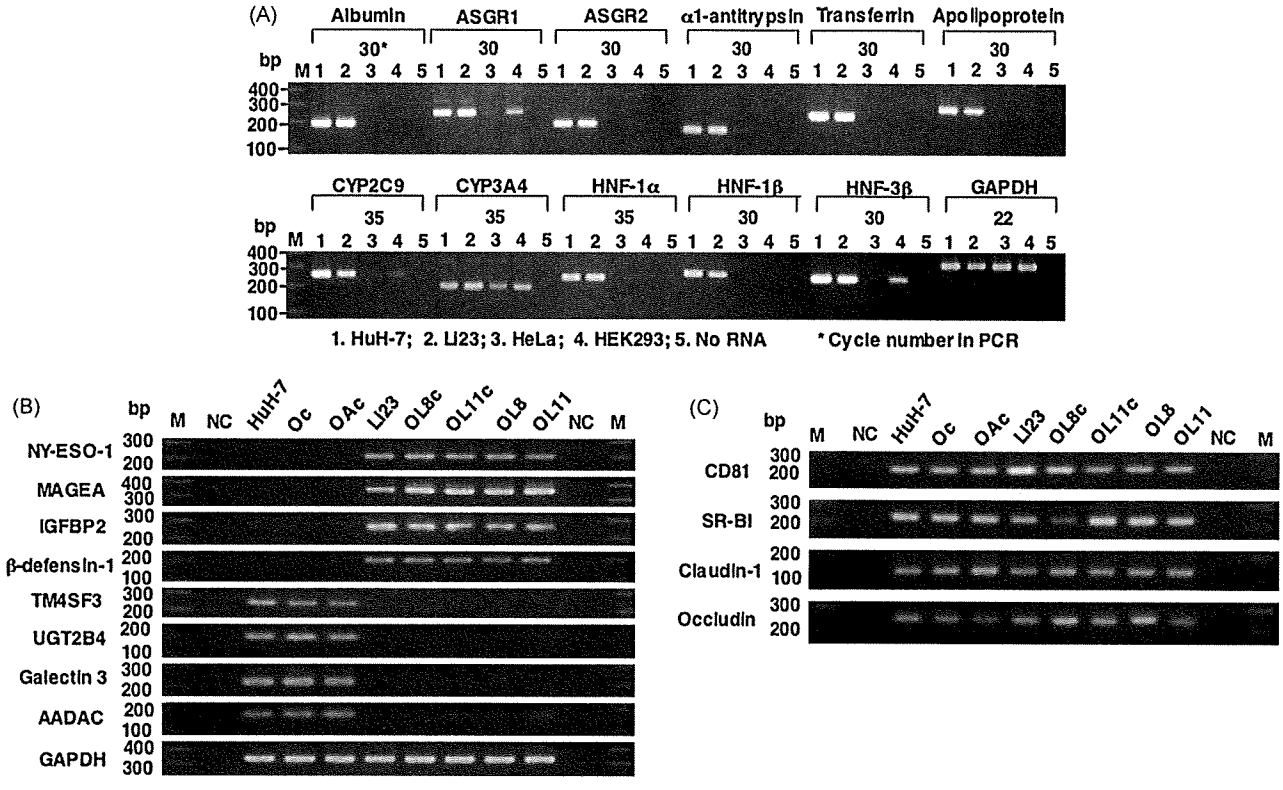

Fig. 3. Representative genes differentially expressed among Li23- and HuH-7-derived cells. (A) Li23 and HuH-7 cells showed similar liver-specific gene expression profiles. Total RNAs prepared from HuH-7, Li23, HeLa, and HEK293 cells were subjected to RT-PCR to detect liver-specific mRNAs using the primer sets listed in Supplementary Table 1. Presented data are the results of the following mRNA species: albumin, asialoglycoprotein receptor 1 (ASGR1), ASGR2, α1-antitrypsin, transferrin, apolipoprotein, cytochrome P450 2C9 (CYP2C9), CYP3A4, hepatocyte nuclear factor 1α (HNF-1α), HNF-1β, and HNF-3β. (B) Representative genes that were differentially expressed between HuH-7-derived cell lines and Li23-derived cell lines. Total RNAs prepared from HuH-7-derived cells (HuH-7, Oc, and OAc) and Li23-derived cells (Li23, OL8c, OL11c, OL8, and OL11) were subjected to RT-PCR using the primer sets listed in Supplementary Table S1. Lane M, 100 bp DNA ladder; NC, no RNA. The data presented are the results of the following mRNA species: cancer testis antigen (NY-ESO-1), melanoma-specific antigen family A (MAGEA), insulin-like growth factor binding protein 2 (IGFBP2), β-defensin-1, transmembrane 4 superfamily member 3 (TM4SF3), UDP glycosyltransferase 2 family polypeptide B4 (UGT2B4), galectin 3, and arylacetamide deacetylase (AADAC). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control. (C) Expression levels of CD81, SR-BI, Claudin-1, and Occludin between HuH-7- and Li23-derived cells. RNA preparation and RT-PCR were performed as described in (B).

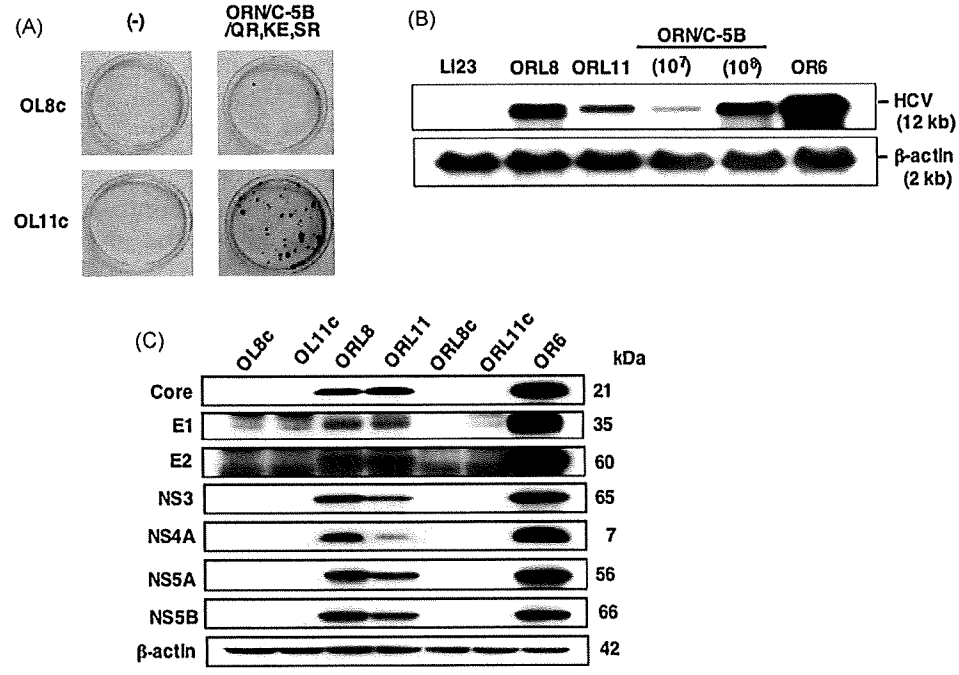

Fig. 4. Establishment of OL8- and OL11-derived cell lines harboring replicative genome-length HCV RNA encoding renilla luciferase. (A) G418-resistant colonies from OL8c or OL11c cells transfected with genome-length HCV RNA (ORN/C-5B/QR,KE,SR) encoding renilla luciferase gene. The panels show G418-resistant colonies that were stained as described in Fig. 1A. (B) Northern blot analysis of total RNA prepared from genome-length HCV RNA replicating ORL8 and ORL11 cells. Synthetic RNA, given number of synthetic ORN/C-5B RNA; Li23, negative control. HuH-7-derived OR6 cells replicating genome-length HCV RNA encoding renilla luciferase gene (ORN/C-5B/KE,SR) served as positive control. (C) Western blot analysis of ORL8 and ORL11 cells for HCV proteins, core, E1, E2, NS3, NS4A, NS5A, and NS5B. OL8c, OL11c, ORL8c, and ORL11c, negative controls; OR6, positive control.

3.3. Development of new luciferase reporter assay systems that facilitate the quantitative monitoring of HCV RNA replication

Since the reporter assay system using HuH-7-derived OR6 cells, which robustly replicates genome-length HCV RNA encoding renilla luciferase, is potentially useful for the quantitative evaluation of anti-HCV activity (Ikeda et al., 2005, 2006; Ikeda and Kato, 2007), we have tried to develop an Li23-derived assay system corresponding to the OR6 assay system. A genome-length HCV RNA encoding renilla luciferase (ORN/C-5B/QR,KE,SR) (Supplemental Fig. 1) was transfected into OL8c or OL11c cells. Following G418 selection, several OL8c colonies and several hundred OL11c colonies were obtained from the cells transfected with ORN/C-5B/QR,KE,SR (Fig. 4A). Regarding ORN/C-5B/QR,KE,SR, 9 OL8c-derived clones and 16 OL11c-derived clones were successfully proliferated as cell lines. Each clone possessing the highest

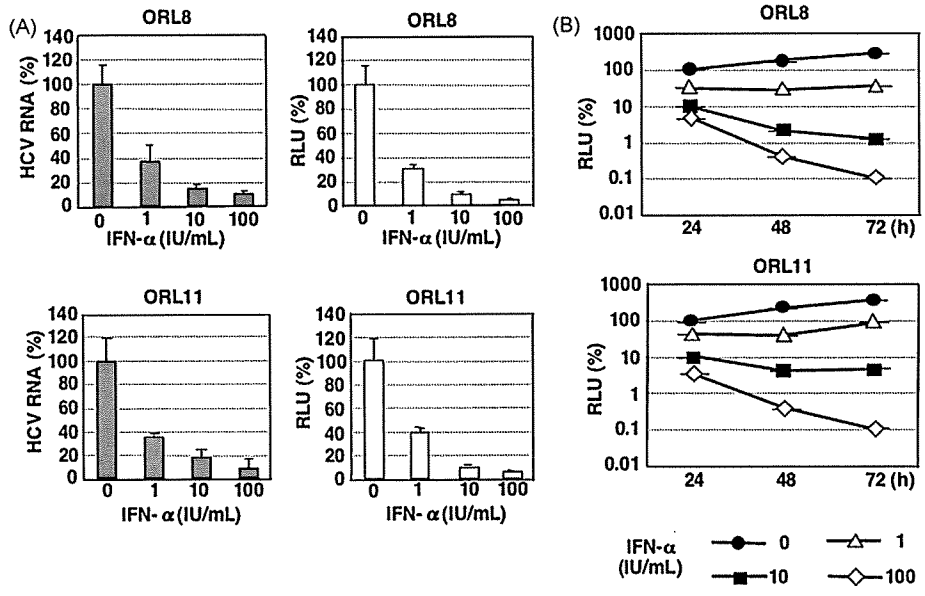

Fig. 5. ORL8 and ORL11 reporter assay system to monitor genome-length HCV RNA replication. (A) Renilla luciferase activity is correlated with HCV RNA level. The ORL8 (upper panels) and ORL11 (lower panels) cells were treated with IFN-α (0, 1, 10, and 100 IU/ml) for 24 h, and then luciferase reporter assay (right panels) and quantitative RT-PCR (left panels) were performed. The relative luciferase activity (RLU) (%) or HCV RNA (%) calculated at each point, when the level of luciferase activity or HCV RNA in non-treated cells was assigned to be 100%, is presented here. (B) IFN-α sensitivity of HCV RNA replication in ORL8 and ORL11 cells. The ORL8 (upper panel) and ORL11 (lower panel) cells were treated with IFN-α (0, 1, 10, and 100 IU/ml); the luciferase assay was performed at 24, 48, and 72 h after the treatment. The RLU (%) calculated at each point, when the luciferase activity of non-treated cells at 24 h was assigned to be 100%, is presented here. The experiments were performed in at least triplicate.

titer of HCV RNA was selected by quantitative RT-PCR and was thereafter referred to as ORL8 and ORL11 (data not shown). We demonstrated that the HCV RNA sequence was not integrated into the genomic DNA in ORL8 or ORL11 cells (data not shown). Northern and Western blot analyses showed that ORL8 and ORL11 cells expressed sufficient levels of HCV RNA and proteins for the quantitative monitoring of HCV RNA replication, although these levels were somewhat lower than those in OR6 cells (Fig. 4B and C). We performed sequence analysis of HCV RNAs derived from ORL8 and ORL11 cells, but no additional mutations were detected commonly among the three independent clones sequenced (data not shown). We demonstrated good correlations between the levels of luciferase activity and HCV RNA in ORL8 and ORL11 cells (Fig. 5A), as we previously demonstrated in OR6 cells treated with IFN-α for 24 h (Ikeda et al., 2005). Time course assays (24, 48, and 72 h) on IFN-α treatment demonstrated that the luciferase activity decreased in a dose- and time-dependent manner, and revealed that the luciferase activity had decreased to less than 0.1% at 72 h after treatment with 100 IU/ml IFN-α (Fig. 5B).

3.4. ORL8 and ORL11 assay systems are frequently more sensitive than the OR6 assay system

Using ORL8 and ORL11 assay systems, we evaluated the anti-HCV activities of representative reagents identified by HuH-7-derived assay systems (Ikeda and Kato, 2007; Moriishi and Matsuura, 2007). For the sake of comparison, we also evaluated these activities using the OR6 assay system along with the same culture medium that we used for the ORL8 and ORL11 assays, since we had already confirmed that HCV RNA in OR6 cells was efficiently replicated using this culture medium (data not shown). First, we measured the 50% effective concentration (EC_{50}) of IFN-α against HCV RNA replication. The EC_{50} values of IFN-α in ORL8, ORL11, and OR6 assays were assigned as 0.13, 0.30, and 0.40 IU/ml, respectively, without suppression of cell growth (Fig. 6A). Regarding IFN-β, IFN-γ, and cyclosporine A also, the ORL8 and ORL11 assays were each more sensitive than the OR6 assay (Fig. 6B). It is noteworthy that the EC_{50} values of fluvastatin and simvastatin in the ORL8 and ORL11 assays were fairly lower than those in the OR6 assay (Fig. 6B). In contrast, we observed that the OR6 assay for geldanamycin was slightly more sensitive than the ORL8 or ORL11 assay (Fig. 6B). When the number of cells without treatment was compared to that of cells with treatment, no significant decrease in cell number was observed following treatment with anti-HCV reagents used in Fig. 6B (data not shown). Co-treatment of IFN-α and fluvastatin also demonstrated that the ORL8 and ORL11 assays were much more sensitive than the OR6 assay (Fig. 6C), indicating that these two systems are powerful biosensors of RNA viral replication.

3.5. Persistent reproduction of HCV life cycle in Li23-derived cells

A most interesting point is whether or not infectious HCV is produced in Li23-derived cell lines and thus enables robust HCV RNA replication. To clarify this point, we used HCV-JFH1 (genotype 2a), the only infectious HCV molecular clone identified in a cell culture to date (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al.,

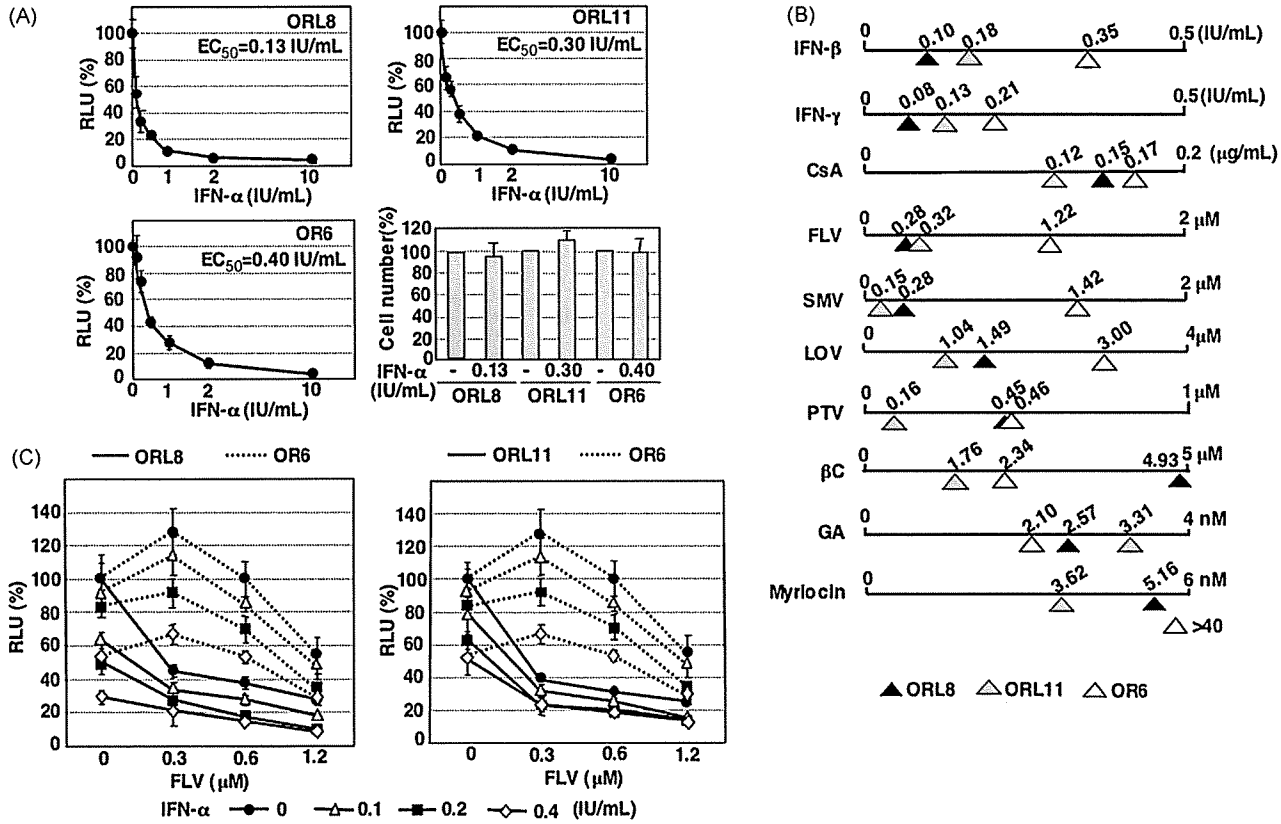

Fig. 6. The diverse effects of anti-HCV reagents in ORL8, ORL11, and OR6 assay systems. (A) IFN-α sensitivities on genome-length HCV RNA replication in ORL8, ORL11, and OR6 assay systems. The ORL8, ORL11, and OR6 cells were treated with IFN-α (0, 0.06, 0.13, 0.25, 0.5, 1, 2, and 10 IU/ml) for 72 h, and then luciferase assay was performed as described in Fig. 5A. ORL8, ORL11, and OR6 cells were cultured in the absence or presence of IFN-α at each 50% effective concentration (EC_{50}) for 72 h, and then the cells were counted as described in Section 2. (B) Diverse EC_{50} values of anti-HCV reagents on genome-length HCV RNA replication in ORL8, ORL11, and OR6 cells. ORL8, ORL11, and OR6 cells were treated with several different concentrations of IFN-β, IFN-γ, CsA, FLV, simvastatin (SMV), lovastatin (LOV), pitavastatin (PTV), β-carotene (βC), geldanamycin (GA), or myriocin for 72 h, after which luciferase assay was performed as described in Fig. 5A. EC_{50} values were calculated from the data of each triplicate assay. (C) ORL8 and ORL11 assay systems are more sensitive than the OR6 assay system in the combination analysis of IFN-α and FLV. ORL8, ORL11, and OR6 cells were treated with a combination of IFN-α (0, 0.1, 0.2, and 0.4 IU/ml) and FLV (0, 0.3, 0.6, and 1.2 μM) for 72 h, after which a luciferase assay was performed as described in Fig. 5A.