III. 新規抗ウイルス薬の開発動向と展望 新規抗肝炎ウイルス薬

新規抗HCV薬

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New antivirals against hepatitis C virus

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Abstract

Recently, first direct acting antiviral (DAA) against hepatitis C virus (HCV) has just approved in Japan. It is a first generation protease inhibitor, telaprevir. Telaprevir inhibits HCV NS3 & 4A serine protease, and combination with pegylated-interferon and ribavirin has now become a standard of care (SOC) for patients with genotype 1 high viral load hepatitis C. Fortunately, more than 50 new antivirals against HCV are under development including antivirals in preclinical trials. New antivirals are classified into several categories; ①NS3 & 4A protease inhibitor, ②NS5B polymerase inhibitor, ③NS5A inhibitor, ④host factor targeting antivirals, ⑤interferon-related antivirals, and others. Combination of different classes of antivirals without interferon is expected to become a future SOC for hepatitis C.

Key words: direct acting antiviral (DAA), protease inhibitor, polymerase inhibitor, NS5A inhibitor, cyclophilin inhibitor

はじめに

世界中では1億7千万人もの人が肝硬変・肝癌を引き起こすC型肝炎ウイルス(hepatitis C virus: HCV)に感染している．治療に対するニーズは高い．また，最近のHCV研究の進歩により，HCVのライフサイクルが明らかになりつつある．増殖に必須のHCV機能タンパクであるNS3/NS4Aプロテアーゼや NS5B RNA依存的RNAポリメラーゼについては，その構造についても明らかになった．このような状況を踏まえ，メガファーマやベンチャーによるC型肝炎に対する新たな薬剤の開発が進んでいる(表1)．

HCVの機能タンパクを標的としたプロテアーゼ阻害薬やポリメラーゼ阻害薬などの薬剤はdirect acting antiviral (DAA)と呼ばれる．NS5AはHCV増殖に重要だが機能の明らかでないウイルスタンパクである．このNS5Aを標的とするNS5A阻害薬も開発されつつある．また，HCV増殖に関連する宿主因子を標的とする宿主細胞標的薬も開発されつつある．本稿では，主に臨床第II相試験あるいは第III相試験が行われている有望な新規抗HCV薬につき概説する．

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表1 新たな抗C型肝炎ウイルス薬

薬剤	臨床試験	製薬会社	備考
プロテアーゼ阻害薬			
boceprevir(SCH503034)	III	MSD	linear
telaprevir(VX-950, MP-424)	III	Vertex/田辺三菱	linear
ABT450	II	ABT	
BI201335	II	Boehringer Ingelheim	macrocyclic
BMS-650032	II	Bristol-Myers Squibb	macrocyclic
danoprevir(RG7227)	II	Roche	macrocyclic
MK-7009	II	MSD	macrocyclic
narlaprevir(SCH900518)	II	MSD	linear
TMC435	II	Tibotec	macrocyclic
ポリメラーゼ阻害薬			
RG7128	II	Roche	NI
ABT-333	II	Abbott	NNI
BI 207127	II	Boehringer Ingelheim	NNI
ABT-072	II	Abbott	NNI
PSI-7977	II	Pharmasset	NI
ANA598	II	Anadys Pharma.	NNI
IDX184	II	Idenix	NI
filibuvir(PF-00868554)	II	Pfizer	NI
その他			
silibinin	III		botanical medicine
taribavirin	III	Valeant	ribavirin prodrug
amantadine	III		
thymosin alfa-1	III		
nitazoxanide	II	Romark Laboratories	thiazolide
IPH1101	II	Innate Pharma	

NI: nucleoside inhibitor, NNI: non-nucleoside inhibitor.

1. プロテアーゼ阻害薬

HCVは2つのプロテアーゼを有している．1つはNS2/NS3金属プロテアーゼであり，NS2とNS3の間を切断する．もう1つはNS3/NS4Aセリンプロテアーゼであり，NS3とNS4Aの間，NS4AとNS4Bの間，NS4BとNS5Aの間，NS5AとNS5Bの間を切断する．NS3がセリンプロテアーゼであり，NS4Aはそのコファクターとして機能している．

現在，開発が進んでいるのは，NS3/NS4Aセリンプロテアーゼ阻害薬である．NS3/NS4Aプロテアーゼ阻害薬は，構造上，基質であるペプチド類似の非環状(linear)の第一世代プロテアーゼ阻害薬と，環状(macrocyclic)の第二世代プロテアーゼ阻害薬の2種に分類される(表2)．多くのプロテアーゼ阻害薬が開発中であるが，今までに世界で承認されたものは，linear型の第一世代プロテアーゼ阻害薬と呼ばれるテラプレビル(telaprevir，商品名テラビック)とboceprevirの2種である．

プロテアーゼ阻害薬の単独治療では，HCVは治療早期に耐性変異を獲得するため，単剤でのHCV駆除は期待できない．したがって，ペグインターフェロン(pegylated-interferon: PEG-IFN)＋リバビリン(ribavirin: RBV)をはじめとした薬剤との併用が必須である．薬剤耐性に関する一番の問題点は，各プロテアーゼ阻害薬間に交差耐性が認められることである．

表2 第一世代プロテアーゼ阻害薬と第二世代プロテアーゼ阻害薬の比較

	第一世代プロテアーゼ阻害薬	第二世代プロテアーゼ阻害薬
構造	非環状	環状
1日の投与回数	3回	1回
1日のcap数	9	1-2
genotype	1a/b	1a/b
副作用	皮膚症状，貧血	皮膚症状，消化器症状 肝障害
SVR(初回)	約70%	80-90%
NVR(non responders)	約30%	約40%

1) BILN2061(ciluprevir，ベーリンガーインゲルハイム)

最初に開発されたプロテアーゼ阻害薬はBILN2061である．プロテアーゼの基質ペプチドの構造アナログとしてデザインされ，臨床第II相試験において，2日間の内服投与によってHCV RNAが1/100-1/1,000に減少するなど劇的な効果を示した．しかしながら，BILN2061を4週間投与したサルに心毒性が出現し，その後の開発が中止された．それでも，本薬剤は，理論に基づいてデザインされたプロテアーゼ阻害薬が実際に極めて有効であることを証明した点で，その後のプロテアーゼ阻害薬開発にとって大きな意義があった．

2) テラプレビル

テラプレビルは我が国で初めて承認されたDAAで，1回750mg，1日3回の投与が必要なlinear型の第一世代プロテアーゼ阻害薬である．詳しくは他稿に譲るが，我が国での臨床第III相試験のgenotype 1，高ウイルス量患者に対する成績を示す．

①PR48(標準治療standard of care: SOC): PEG-IFNα2b+RBV，48週の標準治療でSVR(sustained viral response)は49%(31/63)

②PRT12/PR12: PEG-IFNα2b+RBV+テラプレビルを12週に引き続きPEG-IFNα2b+RBVを12週の治療でSVRは73%(92/126)

標準治療より約20%治療効果が改善し，現時点での新たな標準治療となっている．

3) Boceprevir(SCH503034，MSD)

boceprevirはlinear型のプロテアーゼ阻害薬で，1回800mg，1日3回の投与が必要な第一世代のプロテアーゼ阻害薬である．プロテアーゼ阻害薬としての抗ウイルス効果はテラプレビルに劣るものの，副作用が少ないため，PEG-IFN+RBV療法と長期の併用が可能であり，テラプレビルに匹敵する治療効果が得られている．boceprevirは米国で2011年5月23日に承認，欧州でも2011年7月18日に承認されている．しかしながら，我が国ではPEG-IFNα2b(MSD)はテラプレビルとの併用療法があるためと思われるが，臨床試験の計画はいまだない．

SPRINT-1試験では，genotype 1の未治療C型慢性肝炎患者に対し，

①PR48(SOC): PEG-IFNα2b+RBV，48週の標準治療でSVRは38%(39/104)

②PRB28: PEG-IFNα2b+RBV+boceprevir(2,400mg/日)，28週の治療でSVRは54%(58/107)

③PR4/PRB24: PEG-IFNα2b+RBVを4週に引き続きPEG-IFNα2b+RBV+boceprevirを24週の治療でSVRは56%(58/103)

④PRB48: PEG-IFNα2b+RBV+boceprevirを48週の治療でSVRは67%(69/103)

⑤PR4/PRB44: PEG-IFNα2b+RBVを4週に引き続きPEG-IFNα2b+RBV+boceprevirを44週の治療でSVRは75%(77/103)

⑥Low-dose PRB48: PEG-IFNα2b+少量

RBV+boceprevirを48週の治療でSVRは36％(21/59)

という成績であった[1]．すなわち，boceprevirを含んだプロトコールは，少量リバビリンのプロトコールを除き，すべて標準治療を有意に上回る成績であった．約30％の上乗せ効果が認められた．また，リバビリンが必須であることも明らかになった．本試験では，4週間のPEG-IFN+RBVのlead-inを行うことにより，標準治療の約2倍のSVRが得られており，本プロトコールがboceprevirの標準使用法となった．また，boceprevirによる有害事象は少なく，貧血と味覚異常が多い以外は標準治療と同等であった．

米国での最新の処方データから，プロテアーゼ阻害薬のマーケットシェアは，テラプレビルが80％，boceprevirが20％と伝えられたが，テラプレビルの皮膚障害の副作用から，boceprevirに切り替えるなど，boceprevirの処方が増えつつあるという報告もある．

4) TMC435(Tibotec)

TMC435はmacrocyclic型で1日1回75-150 mgの投与でよい第二世代のプロテアーゼ阻害薬である．臨床第II相試験であるPILLAR試験結果が報告された．副作用は少なく，テラプレビルやboceprevirを上回る治療効果が得られており，非常に期待されている．genotype 1の未治療C型慢性肝炎に対するPILLAR試験の成績を示す．

①PRT(75 mg)12/PR12: SVRは83.6％
②PRT(75 mg)24: SVRは76.1％
③PRT(150 mg)12/PR12: SVRは83.1％
④PRT(150 mg)24: SVRは84.4％

TMC435投与群とプラセボ群に副作用の差は認められていない．また，TMC435群の治療中止率は低く，プラセボ群と同程度であった．本臨床試験の結果にて，PRT(150 mg)24が臨床第III相試験として行われた．我が国でも同様に臨床第III相試験が行われており，2-3年後に市場に登場することが期待されている．

5) Danoprevir(RG7227，ロシュ)

genotype 1，未治療C型慢性肝炎患者に対する臨床第II相のATLAS試験では，900-1,800 mg/日のdanoprevirを服用した．

①PR48(SOC): complete early virological response(cEVR)は43％
②PRD12/PR12-36: cEVRは88-92％

との結果であった．

6) Vaniprevir(MK-7009，MSD)

genotype 1，未治療C型慢性肝炎患者に対する臨床第II相試験では，600-1,200 mg/日のvaniprevirを服用した．

①PR48(SOC): SVRは63％
②PRV4/PR44: SVRは78-84％

との結果が得られている．

7) BI201335(ベーリンガーインゲルハイム)

genotype 1，未治療C型慢性肝炎患者に対する臨床第II相試験(SILEN-C1試験)では，120-240 mg/日のBI201335を服用した．

①PR48(SOC): SVRは56％
②PRB24±PR24: SVRは71-83％

との結果が得られている．

8) ACH-806/GS-9132(Acillion Pharmaceutical)

ACH-806/GS-9132は，NS3プロテアーゼのコファクターであるNS4AのNS3への結合をブロックするというユニークな作用機序を有する薬剤である．腎毒性にて現在，開発が中止されているが，新たなプロテアーゼ阻害薬の形を示した点で意義があった．なお，現在NS4Aを標的とする新薬の開発も行われている．

2. ポリメラーゼ阻害薬

ポリメラーゼ阻害薬には，ポリメラーゼの基質アナログとして働き，RNA鎖伸長反応を阻害してターミネーターとして作用する核酸系ポリメラーゼ阻害薬(nucleoside inhibitor: NI)と，ポリメラーゼの活性中心とは異なるサイトに作用して阻害効果を及ぼす非核酸系ポリメラーゼ阻害薬(non-nucleoside inhibitor: NNI)の2種がある．ポリメラーゼ阻害薬には，幾つかの有望な薬剤が含まれるのみならず，PEG-IFNを使わない経口薬のみによる治療に用いられている．

1) RG7128(mericitabine, ロシュ)

ヌクレオシドアナログであるmericitabineのgenotype 1あるいは4, 未治療のC型慢性肝炎患者に対しての臨床第II相試験(PROPEL study)が行われている. RG7128は500 mgあるいは1,000 mgを1日2回服用した.

①PR48(SOC): cEVRは49%

②PRR12/PR12-36: cEVRは80-88%

との結果が得られている[2].

2) PSI-7977(Pharmasset)

2011年に行われた米国肝臓学会での最大のトピックの一つである. たった40人のgenotype 2および3での試験ながら, PEG-IFNの投与の有無にかかわらず, RBV併用にて, 治療後24週間の経過観察を行い得た症例全例でのウイルス駆除が達成されていた.

genotype 2および3の未治療のC型慢性肝炎患者40例に, 400 mgのPSI-7977と体重に応じたRBVを12週間投与する試験である. また, PEG-IFNは投与しない群, 4週投与群, 8週投与群, 12週投与群の4群であった. ウイルス学的ブレークスルー(耐性ウイルス出現)は認められず, PSI-7977が高いgenetic barrierを有していることが証明された. genotype 1でも同様の試験が行われており, こちらも良好な成績であることが紹介された.

本試験の結果を受け, Pharmasset社は, PEG-IFNなしのPSI-7977+RBVの経口2剤併用療法の第III相試験を開始することを発表した.

3. NS5A阻害薬

NS5Aは, HCV複製やIFN感受性との関連が示唆されているが, その機能はいまだ明らかではない. しかしながら, NS5Aは宿主タンパク質に類似のものがなく, 宿主タンパク質とのアミノ酸配列の相同性も低く, NS5Aの機能を阻害することにより, 特異性の高い薬剤の開発が期待される.

1) AZD2836(A-831, アストラゼネカ)

AZD2836は, アストラゼネカに買収されたArrow Therapeuticsが開発した初のNS5A阻害薬で, IRES依存性の翻訳反応を阻害する. しかしながら, その後の開発は中止されている. 現在, 同社開発のもう一つのNS5A阻害薬, AZD7295(A-689)の臨床第II相試験が行われている.

2) BMS-790052(ブリストルマイヤーズ・スクイブ)

BMS-790052は今までで最も強力といわれるほどのHCV増殖阻害効果を有する. すなわち, ピコモルレベルでの強力な抗HCV活性をもち, かつ, 薬理動態から1日1回投与でよい[3]. genotype 1および4, 未治療のC型慢性肝炎に対する臨床第II相試験では,

①PR48(SOC): cEVRは43%(31/72)

②PRB(20 mg)24/48: cEVRは78%(114/147)

③PRB(60 mg)24/48: cEVRは75%(110/146)

との成績が報告されている. 目立った副作用は報告されていない.

4. DAA併用療法

DAAの併用療法は, PEG-IFNを必要としない経口薬でのC型肝炎治療を可能にするかもしれないと期待されている. 経口2剤を有しているロシュ, ブリストルマイヤーズ・スクイブ, Vertex, Gileadの4社による臨床試験が開始されている.

1) RG7128(ポリメラーゼ阻害薬)+danoprevir(プロテアーゼ阻害薬)(ロシュ)

INFORM-1試験では, genotype 1の未治療のC型慢性肝炎患者73例に対し, RG7128(1,000-2,000 mg/日)+danoprevir(300-1,800 mg/日)の13日間投与で, HCV RNAは3.7-5.2 log10 IU/mL低下が認められている[4]. また, 前治療無効の患者でもHCV RNAは4.9 log10 IU/mL低下が認められている. 特別な有害事象は認められておらず, ウイルス学的リバウンドは1例に認められたのみであった. 本試験では, 引き続きPEG-IFN+RBV治療が行われているため, PEG-IFNなしでのウイルス駆除が可能かどうかは将来的な試験により確かめられなければならない.

2) BMS-790052(NS5A阻害薬)+BMS-650032(プロテアーゼ阻害薬)(ブリストルマイヤーズ・スクイブ)

genotype 1のPEG-IFN+RBV治療無効例に対するNS5A阻害薬BMS-790052とプロテアーゼ阻害薬BMS-650032による臨床第II相試験が行われた. BMS-790052(60 mg/日)+BMS-650032(1,200 mg/日)による2剤併用療法(11例)あるいはPEG-IFN+RBVを加えた4剤併用療法(10例)の24週間の比較試験である.

①BB24: SVRは36%(4/11)

②PRBB24: SVRは90%(9/10)

との成績であった.

興味深いことに, 2剤併用療法でウイルス学的ブレークスルーの認められた症例は全例genotype 1aであり, genotype 1aでのSVRが22%(2/9)であるのに対し, genotype 1bでのSVRは症例数が少ないながらも100%(2/2)であった. また, ウイルス学的ブレークスルーの認められた症例では, 耐性変異について検討したところ, 両剤に対する耐性が出現していた. それでも, PEG-IFN+RBVなしで実際にウイルス駆除が可能であることが示された意義は大きい.

我が国でも同様の試験が行われ, 2011年11月の米国肝臓学会でその成績が報告された. genotype 1bのPEG-IFN+RBV治療無効例10例に対する2剤併用療法の試験で, SVRは90%(9/10)であったが, 脱落例もウイルス駆除を達成しており, 実質100%のウイルス駆除を達成している.

3) テラプレビル(プロテアーゼ阻害薬)+VX-222(ポリメラーゼ阻害薬)(Vertex)

2010年12月, Vertexはテラプレビルとポリメラーゼ阻害薬VX-222併用臨床第II相試験の一部中止を発表した. ウイルス再燃に関する中止ルールに合致したことにより, テラプレビルとVX-222の2剤治療が中止された.

4) GS-9256(プロテアーゼ阻害薬)+tegobuvir(GS-9190, ポリメラーゼ阻害薬)(Gilead)

genotype 1, 未治療のC型慢性肝炎患者に対し, プロテアーゼ阻害薬GS-9256を75 mg, 1日2回とポリメラーゼ阻害薬tegobuvirを40 mg, 1日2回投与のみ, それらにRBVを加えたもの, それにPEG-IFNを加えた3つのアームの試験が行われている.

① GT4/PR44: rapid virological response (RVR)は7%(1/15)

②RGT4/PR44: RVRは38%(5/13)

③PRGT4/PR44: RVRは100%(15/15)

との成績が得られている.

5. 宿主細胞標的薬

宿主因子を標的にした薬剤の開発が行われている. 宿主因子を標的にする利点は, 耐性が生じにくいことと, どのgenotypeでも有効性が期待されることである. 宿主細胞標的薬ではサイクロフィリン阻害薬の開発が最も進んでいる.

1) DEBIO-025(Alisporivir, DEBIO Pharm)

サイクロスポリンに抗HCV活性があることが報告され, その標的はサイクロフィリンであることが明らかになった. サイクロスポリンは強力な免疫抑制剤であるが, 抗HCV効果は免疫抑制効果によらないことが示された. 現在開発中のDEBIO-025はDEBIO Pharm社による免疫抑制作用のないサイクロフィリン阻害薬である[5]. 臨床第II相試験が行われており, genotype 1あるいは4の未治療C型慢性肝炎患者に対し, 600-1,000 mg/日のDEBIO-025とPEG-IFN+RBVを組み合わせ, 4週で4.6-4.8 log10 IU/mLのHCV RNAの低下を認めている.

6. その他

1) Zalbin/joulferon/albuferon(albinterferon α-2b)(ノバルティス)

IFNα2bにアルブミンを結合したalbuferonは, PEG-IFNよりも長期間安定であり, 2-4週間に1度の投与でPEG-IFNに匹敵する治療効果が得られるとのことで, 大変に期待されていたIFN製剤である. しかしながら, 2010年10月, ノバルティスは, Human Genome Sciencesと提携のもとで開発してきたalbuferonの開発

中止を発表した．安全性への懸念(特に間質性肺炎)を指摘されたためである．

2) PEG-IFNλ(ブリストルマイヤーズ・スクイブ)

PEG-IFNλ+RBV併用療法で，PEG-IFNα2aを上回る治療効果が得られている．PEG-IFNλはPEG-IFNα2a同様，interferon stimulated genes(ISGs)を誘導するが，異なるレセプターを利用しており，PEG-IFNλのレセプターを発現している組織が限られているために，PEG-IFNα2aよりも全身的副作用が少ない．

PEG-IFNλの120μg/180μg/240μgと体重換算のRBVを，genotype 1および4に対しては48週間投与，genotype 2および3に対しては24週間投与を行う試験が行われている．genotype 1および4に対する試験結果を示す．

①PR48(SOC)：cEVRは37.9％

②PEG-IFNλ(120μg)+RBV48：cEVRは55.0％

③PEG-IFNλ(180μg)+RBV48：cEVRは55.9％

④PEG-IFNλ(240μg)+RBV48：cEVRは56.3％

との成績であり，有意にPEG-IFNα2aより高率にcEVRが得られていた．

現在までの試験結果をまとめると，

・genotype 1，2，3，4におけるPEG-IFNλ 180/240μgのRVRはPEG-IFNα2aより優れていた．

・genotype 1，4におけるPEG-IFNλ全用量のcEVRはPEG-IFNα2aより優れていた．

・genotype 2，3におけるPEG-IFNλ全用量のcEVRはPEG-IFNα2aと同様であった．

・IL28Bにかかわらず抗ウイルス効果はPEG-IFNλでPEG-IFNα2aより増加した．

・PEG-IFNλ全用量でIFNおよびRBVの減量率はPEG-IFNα2aより減少した．

・PEG-IFNλでは血球系の異常は少なかった．

・PEG-IFNλ240μgでのみAST/ALTの上昇が認められた．

・PEG-IFNλでの高ビリルビン血症4例は中止により全例回復した．

となる．

前述のように，発熱，関節痛，筋肉痛，悪寒，発疹などの副作用は，PEG-IFNα2aに比しPEG-IFNλでは非常に少ない．また，PEG-IFNλそのものの減量も少ないが，RBVの減量も少なく，cEVRの増加に貢献していると思われる．

おわりに

我が国のC型肝炎患者は高齢化してきており，IFN治療の適応とならないことも多い．そのため，将来的に作用機序の異なる複数の経口薬の併用療法にてウイルス駆除が可能となることが強く期待される．

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Development of a drug assay system with hepatitis C virus genome derived from a patient with acute hepatitis C

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Abstract We developed a new cell culture drug assay system (AH1R), in which genome-length hepatitis C virus (HCV) RNA (AH1 strain of genotype 1b derived from a patient with acute hepatitis C) efficiently replicates. By comparing the AH1R system with the OR6 assay system that we developed previously (O strain of genotype 1b derived from an HCV-positive blood donor), we demonstrated that the anti-HCV profiles of reagents including interferon-γ and cyclosporine A significantly differed between these assay systems. Furthermore, we found unexpectedly that rolipram, an anti-inflammatory drug, showed anti-HCV activity in the AH1R assay but not in the OR6 assay, suggesting that the anti-HCV activity of rolipram differs depending on the HCV strain. Taken together, these results suggest that the AH1R assay system is useful for the objective evaluation of anti-HCV reagents and for the discovery of different classes of anti-HCV reagents.

Keywords HCV · Acute hepatitis C · Anti-HCV drug assay system · Anti-HCV activity of rolipram

Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. HCV is an enveloped virus with a positive single-stranded 9.6 kb RNA genome, which encodes a large polyprotein precursor of approximately 3,000 amino acid (aa) residues [1, 2]. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [1].

Human hepatoma HuH-7 cell culture-based HCV replicon systems derived from a number of HCV strains have been widely used for various studies on HCV RNA replication [3, 4] since the first replicon system (based on the Con1 strain of genotype 1b) was developed in 1999 [5]. Genome-length HCV RNA replication systems (see Fig. 2 for details) derived from a limited number of HCV strains (H77, N, Con1, O, and JFH-1) are also sometimes used for such studies, as they are more useful than the replicon systems lacking the structural region of HCV, although the production of infectious HCV from the genome-length HCV RNA has not been demonstrated to date [3, 4]. Furthermore, these RNA replication systems have been improved enough to be suitable for the screening of anti-HCV reagents by the introduction of reporter genes such as luciferase [3, 4, 6]. We also developed an HuH-7-derived cell culture assay system (OR6) in which genome-length HCV RNA (O strain of genotype 1b derived from an HCV-positive blood donor) encoding renilla luciferase (RL) efficiently replicates [7]. Such reporter assay systems could save time and facilitate the mass screening of anti-HCV reagents, since the values of luciferase correlated well with the level of HCV RNA after treatment with anti-HCV reagents. Furthermore, OR6 assay system became more useful as a drug assay system than the HCV subgenomic replicon-based reporter assay systems developed to date [3, 4], because the older systems lack the Core-NS2 regions containing structural proteins likely to be involved in the events that take place in the HCV-infected human liver.

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Indeed, by the screening of preexisting drugs using the OR6 assay system, we have identified mizoribine [8], statins [9], hydroxyurea [10], and teprenone [11] as new anti-HCV drug candidates, indicating that the OR6 assay system is useful for the discovery of anti-HCV reagents.

On the other hand, we previously established for the first time an HuH-7-derived cell line (AH1) that harbors genome-length HCV RNA (AH1 strain of genotype 1b) derived from a patient with acute hepatitis C [12]. In that study, we noticed different anti-HCV profiles of interferon (IFN)-γ or cyclosporine A (CsA) between AH1 and O cells supporting genome-length HCV RNA (O strain) replication [7]. From these results, we supposed that the diverse effects of IFN-γ or CsA were attributable to the difference in HCV strains [12].

To test this assumption in detail, we first developed an AH1 strain-derived assay system (AH1R) corresponding to the OR6 assay system, and then performed a comparative analysis using AH1R and OR6 assay systems. In this article, we report that the difference in HCV strains causes the diverse effects of anti-HCV reagents, and we found unexpectedly by AH1R assay that rolipram, an anti-inflammatory drug, is an anti-HCV drug candidate.

Materials and methods

Reagents

IFN-α, IFN-γ, and CsA were purchased from Sigma-Aldrich (St. Louis, MO). Rolipram was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Plasmid construction

The plasmid pAH1RN/C-5B/PL,LS,TA,$(VA)_3$ was constructed from pAH1 N/C-5B/PL,LS,TA,$(VA)_3$ encoding genome-length HCV RNA clone 2 (See Fig. 2) obtained from AH1 cells [12], by introducing a fragment of the RL gene from pORN/C-5B into the *Asc*I site before the neomycin phosphotransferase (Neo^R) gene as previously described [7].

RNA synthesis

The plasmid pAH1RN/C-5B/PL,LS,TA,$(VA)_3$ DNA was linearized by *Xba*I, and used for RNA synthesis with T7 MEGAscript (Ambion, Austin TX) as previously described [7].

Cell cultures

AH1R and OR6 cells supporting genome-length HCV RNAs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.3 mg/mL of G418 (Geneticin; Invitrogen, Carlsbad, CA). AH1c-cured cells, which were created by eliminating HCV RNA from AH1 cells [12] by IFN-γ treatment, were also cultured in DMEM supplemented with 10% FBS.

RNA transfection and selection of G418-resistant cells

Genome-length HCV (AH1RN/C-5B/PL,LS,TA,$(VA)_3$) RNA synthesized in vitro was transfected into AH1c cells by electroporation, and the cells were selected in the presence of G418 (0.3 mg/mL) for 3 weeks as described previously [13].

RL assay for anti-HCV reagents

To monitor the effects of anti-HCV reagents, RL assay was performed as described previously [14]. Briefly, the cells were plated onto 24-well plates (2×10^4 cells per well) in triplicate and cultured with the medium in the absence of G418 for 24 h. The cells were then treated with each reagent at several concentrations for 72 h. After treatment, the cells were subjected to a luciferase assay using the RL assay system (Promega, Madison, WI). From the assay results, the 50% effective concentration (EC_{50}) of each reagent was determined.

Quantification of HCV RNA

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis for HCV RNA was performed using a real-time LightCycler PCR (Roche Applied Science, Indianapolis, IN, USA) as described previously [7]. The experiments were done in triplicate.

IFN-α treatment to evaluate the assay systems

To monitor the anti-HCV effect of IFN-α on AH1R cells, 2×10^4 cells and 5×10^5 cells were plated onto 24-well plates (for luciferase assay) and 10 cm plates (for quantitative RT-PCR assay) in triplicate, respectively, and cultured for 24 h. The cells were then treated with IFN-α at final concentrations of 0, 1, 10, and 100 IU/mL for 24 h, and subjected to luciferase and quantitative RT-PCR assays as described above.

Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting analysis with a PVDF membrane were performed as described previously [13]. The antibodies used in this study were those against HCV Core (CP11 monoclonal antibody;

Institute of Immunology, Tokyo), NS5B, and E2 (generous gifts from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science, Japan). Anti-β-actin antibody (AC-15; Sigma, St. Louis, MO, USA) was used as a control for the amount of protein loaded per lane. Immunocomplexes were detected with the Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA).

WST-1 cell proliferation assay

The cells were plated onto 96-well plates (1×10^3 cells per well) in triplicate and then treated with rolipram at several concentrations for 72 h. After treatment, the cells were subjected to the WST-1 cell proliferation assay (Takara Bio, Otsu, Japan) according to the manufacturer's protocol. From the assay results, the 50% cytotoxic concentration (CC_{50}) of rolipram was estimated. The selective index (SI) value of rolipram was also estimated by dividing the CC_{50} value by the EC_{50} value.

RT-PCR and sequencing

To amplify the genome-length HCV RNA, RT-PCR was performed separately in two fragments as described previously [7, 15]. Briefly, one fragment covered from 5′-untranslated region to NS3, with a final product of approximately 6.2 kb, and the other fragment covered from NS2 to NS5B, with a final product of approximately 6.1 kb. These fragments overlapped at the NS2 and NS3 regions and were used for sequence analysis of the HCV open reading frame (ORF) after cloning into pBR322MC. PrimScript (Takara Bio) and KOD-plus DNA polymerase (Toyobo, Osaka, Japan) were used for RT and PCR, respectively. The nucleotide sequences of each of the three independent clones obtained were determined using the Big Dye terminator cycle sequencing kit on an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Differences between AH1R and OR6 cell lines were tested using Student's t test. P values <0.05 were considered statistically significant.

Results

Development of a luciferase reporter assay system that facilitates the quantitative monitoring of genome-length HCV-AH1 RNA replication

To develop an HCV AH1 strain-derived assay system corresponding to the OR6 assay system [7], a genome-length HCV RNA encoding RL (AH1RN/C-5B/PL,LS,TA,$(VA)_3$) was transfected into AH1c cells. Following 3 weeks of culturing in the presence of G418, more than 10 colonies were obtained, and then 8 colonies (#2, #3, #4, #5, #6, #8, #13, and #14) were successfully proliferated. We initially selected colonies #2, #3, and #14 because they had high levels of RL activity ($>4 \times 10^6$ U/1.6×10^5 cells) (Fig. 1a). However, RT-PCR and the sequencing analyses revealed that the genome-length HCV-AH1 RNAs obtained from these colonies each had an approximately 1 kb deletion in the E2 region (data not shown). In this regard, we previously observed similar phenomenon and described the difficulty of the development of a luciferase reporter assay system using the genome-length HCV RNA of more than 12 kb [7], suggesting that the NS5B polymerase possesses the limited elongation ability (probably up to a total length of 12 kb). Indeed, in that study, we could overcome this obstacle by the selection of the colony harboring a complete genome-length HCV RNA among the obtained G418-resistant colonies [7]. Therefore, we next carried out the selection among the other colonies. Fortunately, we found that colony #4, showing a rather high level of RL activity (2×10^6 U/1.6×10^5 cells), possessed a complete genome-length HCV-AH1 RNA without any deleted forms, although most of the other colonies possessed some amounts of a deleted form in addition to a complete genome-length HCV-AH1 RNA (data not shown). We demonstrated that the HCV RNA sequence was not integrated into the genomic DNA in colony #4 (data not shown). From these results, we finally selected colony #4, and it was thereafter referred to as AH1R and used for the following studies.

We first demonstrated that AH1R cells expressed sufficient levels of HCV proteins (Core, E2, and NS5B) by Western blot analysis for the evaluation of anti-HCV reagents, and the expression levels were almost equivalent to those in OR6 cells (Fig. 1b). In this analysis, we confirmed that the size of the E2 protein in AH1R cells was 7 kDa larger than that in OR6 cells (Fig. 1b), as observed previously [12]. This result indicates that AH1R cells express AH1 strain-derived E2 protein possessing two extra N-glycosylation sites [12]. We next demonstrated good correlations between the levels of RL activity and HCV RNA in AH1R cells (Fig. 1c), as we previously demonstrated in OR6 cells treated with IFN-α for 24 h [7]. These correlations indicate that AH1R cells were as useful as OR6 cells as a luciferase assay system.

Aa substitutions detected in genome-length HCV RNA in AH1R cells

To examine whether or not genome-length HCV RNA in AH1R cells possesses additional conserved mutations such as adaptive mutations, we performed a sequence analysis of HCV RNA in AH1R cells. The results (Fig. 2) revealed that

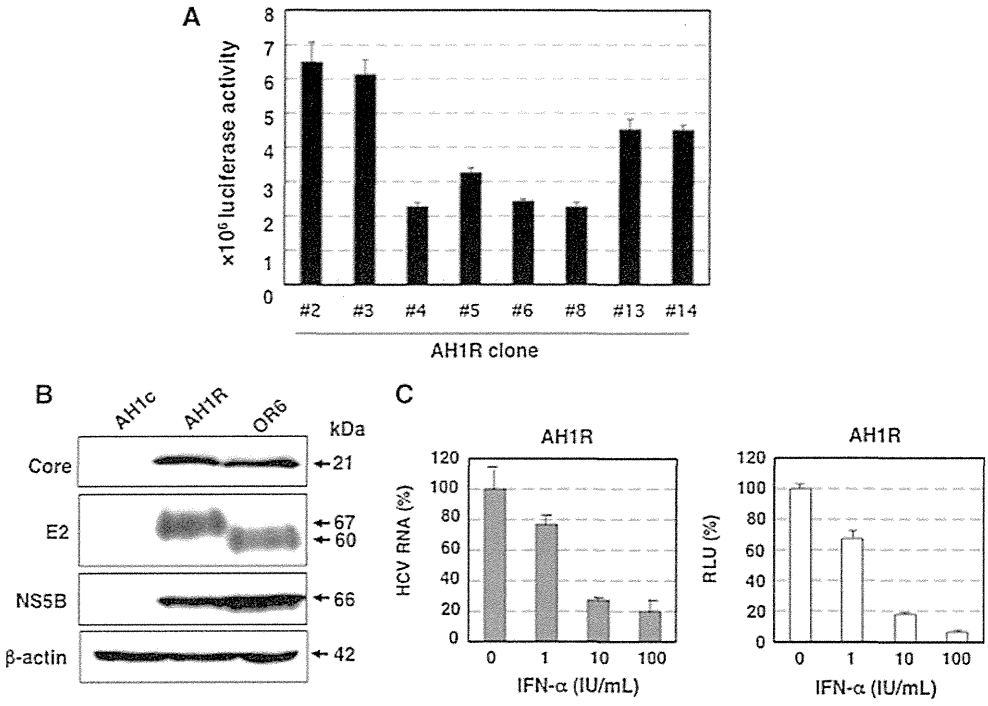

Fig. 1 Characterization of AH1R cells harboring genome-length HCV RNA. **a** Selection of G418-resistant cell clones. The levels of HCV RNA in G418-resistant cells were monitored by RL assay. **b** Western blot analysis. AH1c, AH1R, and OR6 cells were used for the comparison. *Core, E2,* and *NS5B* were detected by Western blot analysis. β-actin was used as a control for the amount of protein loaded per lane. **c** RL activity is correlated with HCV RNA level. The AH1R cells were treated with IFN-α (0, 1, 10, and 100 IU/mL) for 24 h, and then a luciferase reporter assay (*right panel*) and quantitative RT-PCR (*left panel*) 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

two additional mutations accompanying aa substitutions (W860R (NS2) and A1218E (NS3)) were detected commonly among the three independent clones sequenced, suggesting that these additional mutations are required for the efficient replication or stability of genome-length HCV RNA. The P1115L (NS3), L1262S (NS3), V1897A (NS4B), and V2360A (NS5A) mutations derived from the sAH1 replicon [12] were conserved in AH1R cell-derived clones. However, AH1-clone-2-specific mutations (T1338A and V1880A) were almost reverted to the consensus sequences of AH1 RNA [12] except for V1880A in AH1R clone 2 (Fig. 2). In addition, the Q63R (Core) mutation was observed in two of three clones (Fig. 2).

Comparison between the AH1R and OR6 assay systems regarding the sensitivities to IFN-α, IFN-γ, and CsA

Using quantitative RT-PCR analysis, we previously examined the anti-HCV activities of IFN-α, IFN-γ, and CsA in AH1 and O cells, and noticed different anti-HCV profiles of IFN-γ and CsA between AH1 and O cells [12]. In that study, AH1 cells seemed to be more sensitive than the O cells to CsA (significant difference was observed when 0.063, 0.12, or 0.25 μg/mL of CsA was used). Conversely, AH1 cells seemed to be less sensitive than the O cells to IFN-γ (significant difference was observed when 1 or 10 IU/mL of IFN-γ was used). However, we were not able to determine precisely the EC_{50} values of these reagents, because of the unevenness of the data obtained by RT-PCR.

After developing the AH1R assay system in this study, we determined the EC_{50} values of IFN-α, IFN-γ, and CsA using the AH1R assay and compared the values with those obtained by the OR6 assay. The results revealed that AH1R assay was more sensitive than OR6 assay to IFN-α (EC_{50}; 0.31 IU/mL for AH1R, 0.45 IU/mL for OR6) (Fig. 3a) and CsA (EC_{50}; 0.11 μg/mL for AH1R, 0.42 μg/mL for OR6) (Fig. 3b), and that the OR6 assay was more sensitive than the AH1R assay to IFN-γ (EC_{50}; 0.69 IU/mL for AH1R, 0.28 IU/mL for OR6) (Fig. 3c). Regarding these anti-HCV reagents, the anti-HCV activities observed between the AH1R and OR6 assays differed significantly in all of the concentrations examined (Fig. 3). In addition, regarding these anti-HCV reagents, cell growth was not suppressed within the concentrations used. Regarding IFN-γ and CsA, the present results clearly support those of our previous

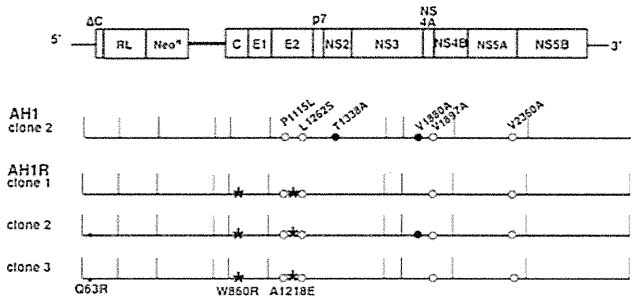

Fig. 2 Aa substitutions detected in intracellular AH1R genome-length HCV RNA. The *upper portion* shows schematic gene organization of genome-length HCV RNA encoding the *RL* gene developed in this study. Genome-length HCV RNA consists of 2 cistrons. In the first cistron, RL is translated as a fusion protein with Neo^R by HCV-IRES, and in the second cistron, all of HCV proteins (C-NS5B) are translated by encephalomyocarditis virus (EMCV)-IRES introduced in the region upstream of C-NS5B regions. Genome-length HCV RNA-replicating cells possess the G418-resistant phenotype because Neo^R is produced by the efficient replication of genome-length HCV RNA. Therefore, when genome-length HCV RNA is excluded from the cells or when its level is decreased, the cells are killed in the presence of G418. In this system, anti-HCV activity is able to evaluate the value of the reporter (RL activity) instead of the quantification of HCV RNA or HCV proteins. In addition, it has been known that the infectious HCV is not produced from this RNA replication system [3, 4, 6]. Core to NS5B regions of three independent clones (*AH1R clones 1–3*) sequenced are presented. W860R and A1218E conserved substitutions are indicated by *asterisks*. Q63R substitutions detected in two of three clones are each indicated by a *small dot*. Core to NS5B regions of *AH1 clone 2*, used to establish the AH1R cell line, are also presented. AH1-specific conserved substitutions and *AH1-clone-2*-specific substitutions are indicated by *open circles* and *black circles*, respectively

study [12]. Therefore, we suggest that the diverse effects of these anti-HCV reagents are due to the difference in HCV strains, although we are not able to completely exclude the possibility that AH1R cells are compromised cells causing the different responses against anti-HCV reagents. In summary, the previous and present findings suggest that the AH1R assay system is also useful for the evaluation of anti-HCV reagents as an independent assay system.

Anti-HCV activity of rolipram was clearly observed in the AH1R assay, but not in the OR6 assay

From the above findings, we supposed that the anti-HCV reagents reported to date might show diverse effects between the drug assay systems derived from the different HCV strains. To test this assumption, we used the AH1R and OR6 assay systems to evaluate the anti-HCV activity of more than 10 pre-existing drugs (6-Azauridine, bisindoly maleimide 1, carvedilol, cehalotaxine, clemizole, 2′-deoxy-5-fluorouridine, esomeprazole, guanazole, hemin, homoharringtonine, methotrexate, nitazoxanide, resveratrol, rolipram, silibinin A, Y27632, etc.), which other groups had evaluated using an assay system derived from the Con1 strain (genotype 1b) or JFH-1 strain (genotype 2a). The results revealed that most of these reagents in the AH1R assay showed similar levels of anti-HCV activities compared with those in the OR6 assay or those of the previous studies (data not shown). However, we found that only rolipram, a selective phosphodiesterase 4 (PDE4) inhibitor [16] that is used as an anti-inflammatory drug, showed moderate anti-HCV activity (EC_{50} 31 μM; $CC_{50} > 200$ μM; $SI > 6$) in the AH1R assay, but no such activity in the OR6 assay (upper panel in Fig. 4a). This remarkable difference was confirmed by Western blot analysis (lower panel in Fig. 4a). It is unlikely that rolipram's anti-HCV activity is due to the inhibition of exogenous RL, Neo^R or encephalomyocarditis virus internal ribosomal entry site (EMCV-IRES), all of which are encoded in the genome-length HCV RNA, because the AH1R and OR6 assay systems possess the same structure of genome-length HCV RNA except for HCV ORF. To demonstrate that rolipram's anti-HCV activity is not due to the clonal specificity of the cells or the specificity of genome-length HCV RNA, we examined the anti-HCV activity of rolipram using the monoclonal HCV replicon RNA-replicating cells (sAH1 cells for AH1 strain [12], and sO cells for O strain [13]). The results

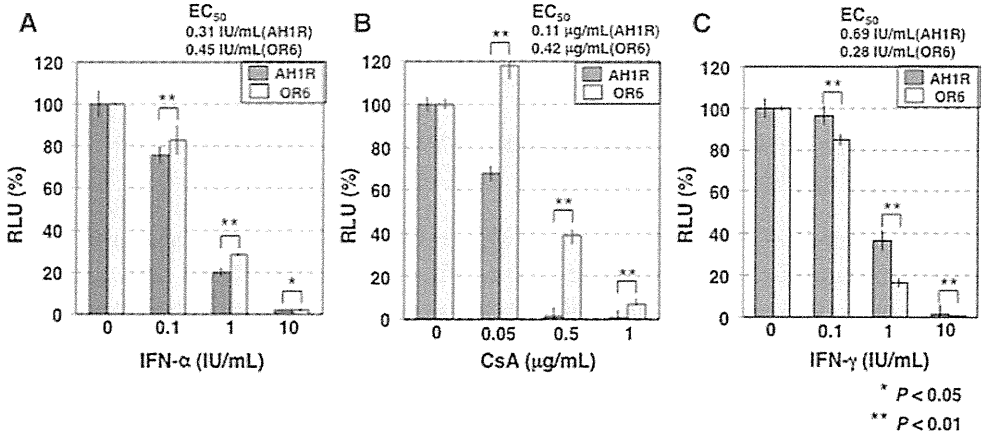

Fig. 3 The diverse effects of anti-HCV reagents on AH1R and OR6 assay systems. AH1R and OR6 cells were treated with anti-HCV reagents for 72 h, and then the RL assay was performed as described in Fig. 1c. **a** Effect of IFN-α. **b** Effect of CsA. **c** Effect of IFN-γ

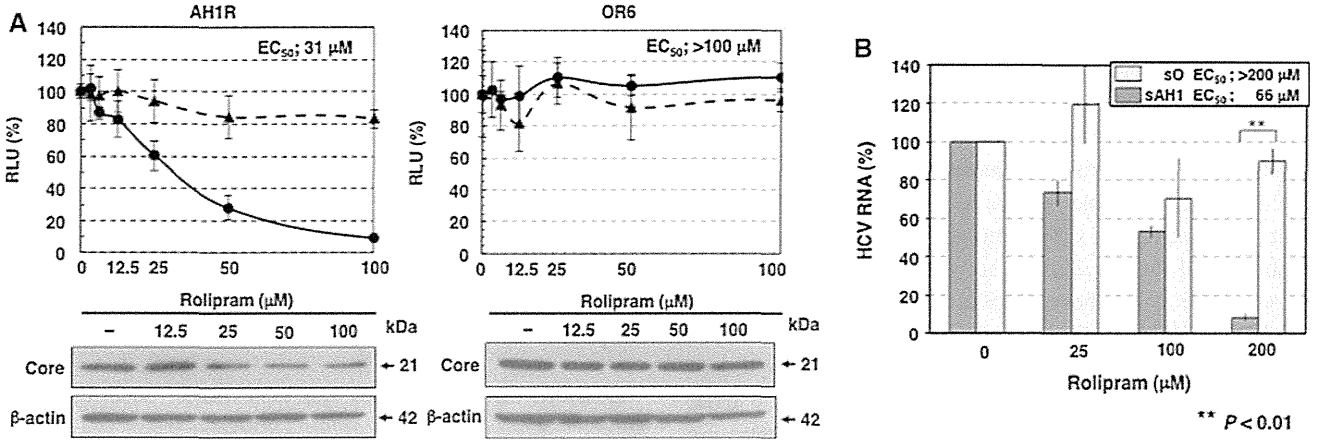

Fig. 4 Anti-HCV activity of rolipram. **a** Rolipram sensitivities on genome-length HCV RNA replication in AH1R and OR6 assay systems. AH1R and OR6 cells were treated with rolipram for 72 h, followed by RL assay (*black circle* with *linear line* in the *upper panels*) and WST-1 assay (*black triangle* with *broken line* in the *upper panels*). The relative value (%) calculated at each point, when the level in non-treated cells was assigned to 100%, is presented here. Western blot analysis of the treated cells for the HCV Core was also performed (*lower panels*). **b** Rolipram sensitivities on HCV replicon RNA replication in sAH1 and sO cells. sAH1 and sO cells were treated with rolipram for 72 h, and extracted total RNAs were subjected to quantitative RT-PCR for HCV 5′ untranslated region as described previously [7]. The HCV RNA (%) calculated at each point, when the level of HCV RNA in non-treated cells was assigned to be 100%, is presented here

revealed by quantitative RT-PCR that rolipram showed moderate anti-HCV activity (EC_{50} 66 μM) in sAH1 cells, but no such activity in sO cells (Fig. 4b). Anti-HCV activity of rolipram in sAH1 cells was a little weaker than that in AH1R cells (Fig. 4b). The similar phenomenon that the anti-HCV activity in genome-length HCV RNA-based reporter assay is stronger than that in HCV subgenomic replicon-based reporter assay was observed regarding other anti-HCV reagents in our previous studies [14, 17, 18]. This result suggests that the anti-HCV activity of rolipram is not either a clone-specific or genome-length HCV RNA-specific phenomenon. In our previous studies also [14, 18], we demonstrated that anti-HCV activities of several reagents including ribavirin and statins were not due to the clonal specificity of the cells. On the other hand, it was recently reported that rolipram did not show anti-HCV activity in the JFH-1 strain-derived assay [19]. Taken together, the previous and present results suggest that rolipram's anti-HCV activity differs depending on the HCV strain. In summary, rolipram was identified as a new anti-HCV candidate using the AH1R assay system.

Discussion

In the present study, we developed for the first time a drug assay system (AH1R), derived from the HCV-AH1 strain (from a patient with acute hepatitis C), in which HCV-AH1 RNA is efficiently replicated. Using this system, we found that rolipram, an anti-inflammatory drug, had potential anti-HCV activity. This potential had not been detected by preexisting assay systems such as OR6, in which HCV-O RNA was derived from an HCV-positive blood donor. Since an HCV replicon harboring the sAH1 cell line, the parent of the AH1R cell line, was obtained from OR6-cured cells [12], the divergence in rolipram's effects between AH1R and OR6 cells is probably attributable to the difference in HCV strains rather than to the difference in cell clones. Indeed, rolipram's anti-HCV activity was not observed in another ORL8 assay system (O strain), which was recently developed using a new hepatoma Li23 cell line (data not shown) [15]. Therefore, we propose that multiple assay systems derived from different HCV strains are required for the discovery of anti-HCV reagents such as rolipram or for the objective evaluation of anti-HCV activity.

Comparative evaluation analysis of anti-HCV activities of IFN-α, IFN-γ, and CsA using AH1-strain-derived AH1R and O-strain-derived OR6 assay systems demonstrated that each of these anti-HCV reagents showed significantly diverse antiviral effects between the two systems. Regarding IFN-γ and CsA, the present results obtained using a luciferase reporter assay fully supported our previous findings [12] using quantitative RT-PCR analysis. However, in the present analysis, we noticed that IFN-α also showed significantly diverse effects (especially at less than 1 IU/mL) between the AH1R and OR6 assays.

The differences in IFN-α sensitivity may be attributable to the difference in aa sequences in the IFN sensitivity-determining region (ISDR; aa 2209-2248 in the HCV-1b genotype), in which aa substitutions correlate well with IFN sensitivity in patients with chronic hepatitis C [20], because the AH1 strain possesses three aa substitutions (T2217A, H2218R, and A2224 V) in ISDR, whereas the O strain possesses no aa substitutions. However, no report has demonstrated the correlation between IFN sensitivity and the substitution numbers in ISDR using the cell culture-based HCV RNA replication system.

Alternatively, Akuta et al. [21] reported that aa substitutions at position 70 and/or position 91 in the HCV Core region of patients infected with the HCV-1b genotype are pretreatment predictors of null virological response (NVR) to pegylated IFN/ribavirin combination therapy. In particular, substitutions of arginine (R) by glutamine (Q) at position 70, and/or leucine (L) by methionine (M) at position 91, were common in NVR. The patients with position-70 substitutions often showed little or no decrease in HCV RNA levels during the early phase of IFN-α treatment [21]. Regarding this point, it is interesting that position 70 in the AH1 strain is R (wild type) and that in the O strain is Q (mutant type), whereas position 91 is L (wild type) in both strains. Therefore, wild-type R in position 70 of the AH1 strain may contribute to the high sensitivity to IFN-α in the AH1R assay. Regarding positions 70 and 91 of the HCV Core, it is noteworthy that, among all of the HCV strains used thus far to develop HCV replicon systems, only the AH1 strain possesses double wild-type aa (data not shown). Therefore, the AH1R assay system may be useful for further study of sensitivity to IFN/ribavirin treatment.

The anti-HCV activity of rolipram, which is currently used as an anti-inflammatory drug, is interesting, although its anti-HCV mechanism is unclear. As a selective PDE4 inhibitor [16], rolipram may attenuate fibroblast activities that can lead to fibrosis and may be particularly effective in the presence of transforming growth factor (TGF)-β1-induced fibroblast stimulation [22]. On the other hand, HCV enhances hepatic fibrosis progression through the generation of reactive oxygen species and the induction of TGF-β1 [23]. Taken together, the previous and present results suggest that rolipram may inhibit both HCV RNA replication and HCV-enhanced hepatic fibrosis. However, it is unclear that rolipram shows anti-HCV activity against the majority of HCV strains, because rolipram has been effective for AH1 strain, but not for O strain. Although rolipram's anti-HCV activity would be HCV-strain-specific, it is not clear which HCV strain is the major type regarding the sensitivity to rolipram. Since developed assay systems using genome-length HCV RNA-replicating cells are limited to several HCV strains including O and AH1 strains to date, further analysis using the assay systems of other HCV strains will be needed to clarify this point.

In this study, we demonstrated that the AH1R assay system, which was for the first time developed using an HCV strain derived from a patient with acute hepatitis C, showed different sensitivities against anti-HCV reagents in comparison with assay systems in current use, such as OR6 assay. Therefore, AH1R assay system would be useful for various HCV studies including the evaluation of anti-HCV reagents and the identification of antiviral targets.

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Identification of host genes showing differential expression profiles with cell-based long-term replication of hepatitis C virus RNA

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ABSTRACT

Persistent hepatitis C virus (HCV) infection frequently causes hepatocellular carcinoma. However, the mechanisms of HCV-associated hepatocarcinogenesis and disease progression are unclear. Although the human hepatoma cell line, HuH-7, has been widely used as the only cell culture system for robust HCV replication, we recently developed new human hepatoma Li23 cell line-derived OL, OL8, OL11, and OL14 cells, in which genome-length HCV RNA (O strain of genotype 1b) efficiently replicates. OL, OL8, OL11, and OL14 cells were cultured for more than 2 years. We prepared cured cells from OL8 and OL11 cells by interferon-γ treatment. The cured cells were also cultured for more than 2 years. cDNA microarray and RT-PCR analyses were performed using total RNAs prepared from these cells. We first selected several hundred highly or moderately expressed probes, the expression levels of which were upregulated or downregulated at ratios of more than 2 or less than 0.5 in each set of compared cells (e.g., parent OL8 cells versus OL8 cells cultured for 2 years). From among these probes, we next selected those whose expression levels commonly changed during a 2-year culture of genome-length HCV RNA-replicating cells, but which did not change during a 2-year culture period in cured cells. We further examined the expression levels of the selected candidate genes by RT-PCR analysis using additional specimens from the cells cultured for 3.5 years. Reproducibility of the RT-PCR analysis using specimens from recultured cells was also confirmed. Finally, we identified 5 upregulated genes and 4 downregulated genes, the expression levels of which were irreversibly altered during 3.5-year replication of HCV RNA. These genes may play roles in the optimization of the environment in HCV RNA replication, or may play key roles in the progression of HCV-associated hepatic diseases.

1. Introduction

Hepatitis C virus (HCV) is a causative agent of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma (HCC) (Choo et al., 1989; Saito et al., 1990; Thomas, 2000). However, the mechanisms of HCV-associated hepatocarcinogenesis and disease progression are still unclear. HCV is an enveloped virus with a positive single-stranded 9.6 kb RNA genome, which encodes a large polyprotein precursor of approximately 3000 amino acid residues. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Hijikata et al., 1991, 1993; Kato et al., 1990).

The initial development of a cell culture-based replicon system (Lohmann et al., 1999) and a genome-length HCV RNA-replication system (Ikeda et al., 2002) using genotype 1b strains enabled the rapid progression of investigations into the mechanisms underlying HCV replication (Bartenschlager, 2005; Lindenbach and Rice, 2005). Furthermore, these RNA replication systems have been improved such that they have become suitable for the screening of anti-HCV reagents by the introduction of reporter genes such as luciferase (Ikeda et al., 2005; Krieger et al., 2001). Moreover, in 2005, an efficient virus production system using the JFH1 genotype 2a strain was developed using human hepatoma cell line HuH-7-derived cells (Wakita et al., 2005). However, to date, HuH-7-derived cells are used as the only cell culture

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; E1, envelope 1; EGF, epidermal growth factor; RT-PCR, reverse transcription-polymerase chain reaction; IFN, interferon; ACSM3, acyl-CoA synthetase medium-chain family member 3; ANGPT1, angiopoietin 1; CDKN2C, cyclin-dependent kinase inhibitor 2C; PLA1A, phospholipase A1 member A; SEL1L3, Sel-1 suppressor of lin-12-like 3; SLC39A4, solute carrier family 39 member 4; TBC1D4, TBC1 domain family member 4; WISP3, WNT1 inducible signaling pathway protein 3; ANXA1, annexin A1; AREG, amphiregulin; BASP1, brain abundant, membrane attached signal protein 1; CIDEC, cell death activator CIDE-3; CPB2, carboxypeptidase B2; HSPA6, heat-shock 70 kDa protein B'; PI3, peptidase inhibitor 3; SLC1A3, solute carrier family 1 member 3; THSD4, thrombospondin type-1 domain-containing protein 4; ICAM-1, intercellular adhesion molecule-1; ALXR, ANXA1 receptor.

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system for robust HCV replication (Bartenschlager and Sparacio, 2007; Lindenbach and Rice, 2005). Most studies of HCV replication or anti-HCV reagents are currently carried out using a HuH-7-derived cell culture system. Therefore, it remains unclear whether or not recent advances obtained from the HuH-7-derived cell culture system reflect the general features of HCV replication or anti-HCV targets. To resolve this issue, we aimed to find a cell line other than HuH-7 that enables robust HCV replication. We recently found a new human hepatoma cell line, Li23, that enables efficient HCV RNA replication and persistent HCV production (Kato et al., 2009b). In that study, we established genome-length HCV RNA replicating cell lines, OL (polyclonal; a mixture of approximately 200 clones), OL8 (monoclonal), OL11 (monoclonal), and OL14 (monoclonal), and characterized them (Kato et al., 2009b). We further developed Li23-derived drug assay systems (ORL8 and ORL11) (Kato et al., 2009b), which are relevant to the HuH-7-derived OR6 assay system (Ikeda et al., 2005). Since we demonstrated that the gene expression profile of Li23 cells was distinct from that of HuH-7 cells (Mori et al., 2010), we expected to find that the host factors required for HCV replication or anti-HCV targets in Li23-derived cells would also be distinct from those in HuH-7-derived cells. Indeed, we found that treatment of the cells with approximately 10 μM (a clinically achievable concentration) of ribavirin, an anti-HCV drug, efficiently inhibited HCV RNA replication in both the Li23-derived ORL8 and ORL11 assay systems, but not in the HuH-7-derived OR6 assay system (Mori et al., 2011). We further demonstrated that more than half of the 26 anti-HCV reagents that have been reported by other groups as anti-HCV candidates using HuH-7-derived assay systems other than OR6 assay system exhibited different anti-HCV activities from those of the previous studies (Ueda et al., 2011). In addition, we observed that the anti-HCV activities evaluated by the OR6 and ORL8 assay systems were also frequently different (Ueda et al., 2011). Furthermore, Li23-derived cells showed epidermal growth factor (EGF)-dependent growth (Kato et al., 2009b)-like immortalized or primary hepatocyte cells (e.g., PH5CH8 (Ikeda et al., 1998)), whereas HuH-7-derived cells can grow in an EGF-independent manner. Our findings, when taken together, suggested that a study using Li23-derived cells might yield unexpected results, since only HuH-7-derived cells are commonly used in a wide range of HCV studies.

Moreover, our findings to date suggested that the long-term replication of HCV RNA may cause irreversible changes in the gene expression profiles of host cells, yielding an environment for facilitative viral replication or progression of a malignant phenotype. To investigate this possibility, we carried out cDNA microarray and/or reverse transcription-polymerase chain reaction (RT-PCR) analyses using Li23-derived cells (OL, OL8, OL11, and OL14) in order to identify host genes for which expression levels were irreversibly altered by the long-term replication of HCV RNA. Here we report the identification of such host genes.

2. Materials and methods

2.1. Cell culture

The Li23 cell line consists of human hepatoma cells from a Japanese male (age 56) was established and characterized in 2009 (Kato et al., 2009b). Li23 cells were maintained in modified culture medium for the PH5CH8 human immortalized hepatocyte cell line (Ikeda et al., 1998), as described previously (Kato et al., 2009b). Genome-length HCV RNA-replicating cells (Li23-derived OL, OL8, OL11, and OL14 cells) were also maintained in the medium for the Li23 cells in the presence of 0.3 mg/mL of G418 (Geneticin, Invitrogen, Carlsbad, CA). Cured cells (OL8c and OL11c cells), from which the HCV RNA had been eliminated by interferon (IFN)-γ treatment (Abe et al., 2007), were cultured in the medium for the Li23 cells. These cells were passaged every 7 days for 3.5 years. In this study, OL, OL8, OL11, OL14, OL8c, and OL11c cells were renamed as OL(0Y), OL8(0Y), OL11(0Y), OL14(0Y), OL8c(0Y), and OL11c(0Y) cells, respectively, to specify the time at which the cells were established. These "0Y" cells of passage number 3 were used in this study. Two-year cultures of OL(0Y), OL8(0Y), OL11(0Y), OL14(0Y), OL8c(0Y), and OL11c(0Y) cells were designated as OL(2Y), OL8(2Y), OL11(2Y), OL14(2Y), OL8c(2Y), and OL11c(2Y) cells, respectively. The 3.5-year cultures of OL8(0Y), OL11(0Y), OL8c(0Y), and OL11c(0Y) cells were designated as OL8(3.5Y), OL11(3.5Y), OL8c(3.5Y), and OL11c(3.5Y) cells, respectively. The cured cells obtained from OL8(2Y) and OL11(2Y) cells by IFN-γ treatment (Abe et al., 2007) were designated as OL8(2Y)c and OL11(2Y)c cells, respectively, and were maintained in the medium for the Li23 cells.

2.2. cDNA microarray analysis

OL(0Y), OL(2Y), OL8(0Y), OL8(2Y), OL11(0Y), OL11(2Y), OL8c(0Y), OL8c(2Y), OL11c(0Y), and OL11c(2Y) cells were cultured in the medium without G418 during a few passages, and then these cells (1×10^6 each) were plated onto 10-cm diameter dishes and cultured for 2 or 3 days. Total RNAs from these cells (approximately 70–80% confluency) were prepared using the RNeasy extraction kit (QIAGEN, Hilden, Germany). As previously described (Kato et al., 2009b; Mori et al., 2010), cDNA microarray analysis was performed by Dragon Genomics Center of Takara Bio. (Otsu, Japan) through an authorized Affymetrix service provider using the GeneChip Human Genome U133 Plus 2.0 Array. Differentially expressed genes were selected by comparing the arrays from the genome-length HCV RNA-replicating cells, and the selected genes were further compared with the arrays from the cured cells (see Fig. 2 for details).

2.3. RT-PCR

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

2.4. Quantitative RT-PCR analysis

The quantitative RT-PCR analysis for HCV RNA was performed using a real-time LightCycler PCR (Roche Diagnostics, Basel, Switzerland) as described previously (Ikeda et al., 2005; Kato et al., 2009b). Quantitative RT-PCR analysis for the mRNAs of the selected genes was also performed using a real-time LightCycler PCR. The primer sets used in this study are listed in Table 1.

2.5. Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting analysis with a PVDF membrane were performed as previously described (Kato et al., 2003). The antibodies used for the O strain in this study were those against Core (CP9, CP11, and CP14 monoclonal antibodies [Institute of Immunology, Tokyo, Japan]; a polyclonal antibody [a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science]), E1 and NS5B (a generous gift from Dr. M. Kohara), and NS3 (Novocastra Laboratories, Newcastle upon Tyne, UK). β-Actin antibody (Sigma, St. Louis, MO)

Table 1
Primers used for RT-PCR analysis.

Gene (accession no.)	Direction	Nucleotide sequence (5′–3′)	Products (bp)	Gene (accession no.)	Direction	Nucleotide sequence (5′–3′)	Products (bp)
Acyl-CoA synthetase medium-chain family member 3 (ACSM3; NM_005622)	Forward	GCATTCAAGTTCTACCCAACCGAC	258	Brain abundant, membrane attached signal protein 1 (BASP1; NM_006317)	Forward	GGATGAATGCCAGCTTTCAGACAG	247
	Reverse	GGCTGCTGACAACAGCTGACTC			Reverse	ACTGGAACTGCAATGAACGCAGAC	
Angiopoietin 1 (ANGPT1; NM_001146)	Forward	ATACAACATCGTGAAGATGGAAGTC	287	Cell death activator CIDE-3 (CIDEC; NM_022094)	Forward	GATCTGTACAAGCTGAACCCACAG	265
	Reverse	CCGTGTAAGATCAGGCTGCTCTG			Reverse	GACAGGTCGGGATAAGGGATGAG	
Cyclin-dependent kinase inhibitor 2C (CDKN2C; NM_001262)	Forward	AAGACCGAACTGGTTTCGCTGTC	246	Carboxypeptidase B2 (CPB2; NM_001872)	Forward	GGAACTGTCTCTAGTAGCCAGTG	242
	Reverse	CATAGAGCCTGGCCAAATCACAG			Reverse	CAGCGGCAAAAGCTTCTCTACAG	
Phospholipase A1 member A (PLA1A; NM_015900)	Forward	GGAGTTTCACTTGAAGGAACTGAG	292	Heat shock 70 kDa protein B' (HSPA6; NM_002155)	Forward	TGAAGCCGAGCAGTACAAGGCTG	235
	Reverse	GTTCACTGGTTCAGGTAAGCAGAC			Reverse	CTCCCTCTTCTGATGCTCATACTC	
Sel-1 suppressor of lin-12-like 3 (SEL1L3; NM_015187)	Forward	ACCTGCACTTGCGGCTTCTCTG	212	Peptidase inhibitor 3 (PI3; NM_002638)	Forward	GGTTCTAGAGGCAGCTGTCACG	276[a]
	Reverse	AGAGGCATCTGCAGCTGGAGTC			Reverse	CCGCAAGAGCCTTCACAGCAC	
Solute carrier family 39 member 4 (SLC39A4; NM_017767)	Forward	GCCTGTTCCTCTACGTAGCACTC	158	Peptidase inhibitor 3 (PI3; NM_002638)	Forward	GGTTCTAGAGGCAGCTGTCACG	241[b]
	Reverse	GAAGGTGATGTCATCCTCGTACAG			Reverse	GCAGTCAGTATCTTTCAAGCAGC	
TBC1 domain family, member 4 (TBC1D4; NM_014832)	Forward	GGAGAGGGCCAATAGCCAACTG	198	Solute carrier family member 3 (SLC1A3; NM_004172)	Forward	CAATGGCGTGGACAAGCGCGTC	240
	Reverse	AGCTTCCGGAGTTGCTCCACTG			Reverse	CCGACAGATGTCAGCACAATGAC	
WNT1 inducible signaling pathway protein 3 (WISP3; NM_003880)	Forward	AGAGATGCTGTATCCCTAATAAGTC	129	Thrombospondin type-1 domain-containing protein 4 (THSD4; NM_024817)	Forward	TGGAGTCAGTGTTCCATCGAGTG	275
	Reverse	CAGGTTCTCTGCAGTTTCTCTGAC			Reverse	GGGTCACAGAGGTTACTTAGAGTC	
Annexin A1 (ANXA1; NM_000700)	Forward	GACTTGGCTGATTCAGATGCCAG	192	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; NM_002046)	Forward	GACTCATGACCACAGTCCATGC	334
	Reverse	AATGTCACCTTTCAACTCCAGGTC			Reverse	GAGGAGACCACCTGGTGCTCAG	
Amphiregulin (AREG; NM_001657)	Forward	CGGGAGCCGACTATGACTACTC	391				
	Reverse	AAGGCAGCTATGGCTGCTAATGC					

[a] This primer set was used for RT-PCR analysis.
[b] This primer set was used for quantitative RT-PCR analysis.

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, Boston, MA).

2.6. Statistical analysis

Statistical comparison of the mRNA levels between the various time points was performed using Student's *t*-test. *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Efficient replication of genome-length HCV RNA is maintained in long-term cell culture

To prepare specimens for the cDNA microarray analysis, genome-length HCV RNA-replicating OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y) cells were cultured for 2 years, and were designated as OL(2Y), OL8(2Y), OL11(2Y), and OL14(2Y) cells, respectively. OL8c(0Y) and OL11c(0Y) cells were also cultured for 2 years, and were designated as OL8c(2Y) and OL11c(2Y) cells, respectively. We observed that the growth rates of all cell lines increased in a time-dependent manner, while the appreciable changes of cell shapes were not observed. The doubling time of genome-length HCV RNA-replicating cells (OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y)) and cured cells (OL8c(0Y) and OL11c(0Y)) was approximately 41 h and 32 h, respectively. After 2-year culture, these values reduced to approximately 28 h and 23 h.

Using the total RNA specimens obtained from genome-length HCV RNA-replicating cells, the levels of genome-length HCV RNAs were examined by quantitative RT-PCR analysis. The results revealed that the levels of the genome-length HCV RNAs had increased in all cases after a 2-year period of HCV RNA replication (Fig. 1). The levels of HCV proteins (Core, E1, NS3, and NS5B) were also examined by Western blot analysis. The E1, NS3, and NS5B were detected in all specimens, except for the Li23 cells, although a little larger size of E1 was additionally detected in the specimens from 2-year culture (Fig. 1). This phenomenon may indicate the appearance of additional N-glycosylation sites by mutations caused during the 2-year replication of the HCV RNA, as observed in a previous report (Mori et al., 2008). However, genetic analysis of HCV RNAs from 2-year culture of OL8, OL11, and OL14 cell series has detected no additional N-glycosylation sites by mutations (Kato et al., unpublished results). Therefore, the mobility change of E1 may be due to the other modifications such as O-glycosylation. In addition, Core was not detected in the cultures of OL11(2Y) cells, even when polyclonal anti-Core antibody was used (Fig. 1). A similar phenomenon was observed in a previous study using HuH-7-derived genome-length HCV RNA-replicating cells (Kato et al., 2009a). In that study, we showed that the Core region was not deleted, but mutated at several positions within the epitopes of the anti-Core antibody (Kato et al., 2009a). The results of genetic analysis using Li23-derived cells as described above (Kato et al., unpublished results) were also similar with those in the previous study using HuH-7-derived cells (Kato et al., 2009a).

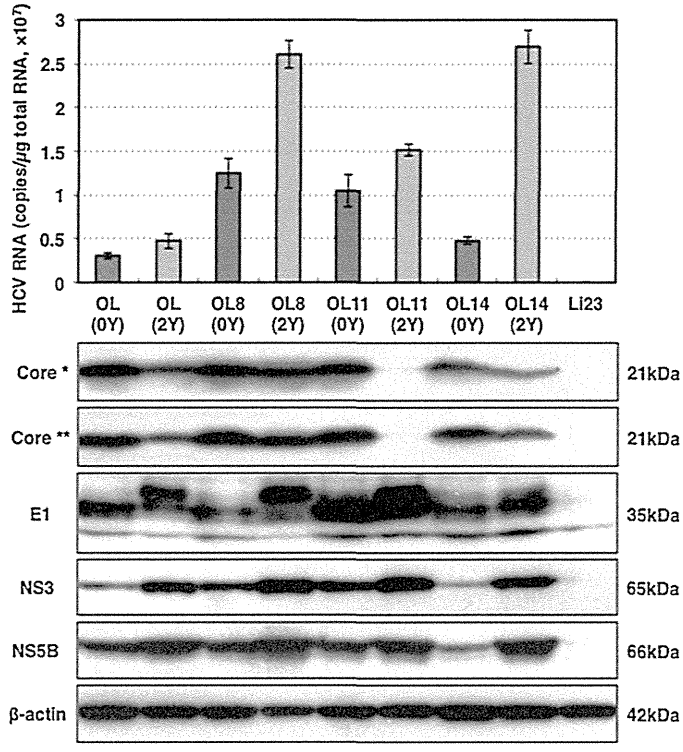

Fig. 1. Characterization of genome-length HCV RNA-replicating cells in long-term cell culture. The upper panel shows the results of a quantitative RT-PCR analysis of intracellular genome-length HCV RNA. Total RNAs from OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y) cells after 2 years [OL(2Y), OL8(2Y), OL11(2Y), and OL14(2Y)] in culture, as well as total RNAs from the parental OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y) cells were used for the analysis. Total RNA from Li23 cells was used as a negative control. The lower panel shows the results of the Western blot analysis. Cellular lysates from cells used for quantitative RT-PCR were also used for comparison. HCV Core, E1, NS3, and NS5B were detected by Western blot analysis. β-Actin was used as a control for the amount of protein loaded per lane. A single asterisk indicates that the anti-Core polyclonal antibody was used for detection. A double asterisk indicates that a mixture of three kinds (CP9, CP11, and CP14) of anti-Core monoclonal antibodies was used for detection.

3.2. Selection of genes showing irreversible changes with long-term HCV RNA replication

To identify those genes whose expression levels were irreversibly altered by the long-term replication of HCV RNA, we performed a combination of cDNA microarray and RT-PCR analyses using several Li23-derived cell lines. An outline of the selection process performed in this study is provided in Fig. 2. The first microarray analysis I was carried out by the comparison of OL(0Y) cells versus OL(2Y) cells, OL8(0Y) cells versus OL8(2Y) cells, and OL11(0Y) cells versus OL11(2Y) cells. In this step, we selected those genes whose expression levels commonly showed changes in at least two of three comparative analyses to avoid the bias caused by the difference of cell clonality, since OL(0Y) was a polyclonal cell line, while OL8(0Y) and OL11(0Y) were monoclonal cell lines (Kato et al., 2009b). As regards the selected genes, a microarray analysis II was performed in which OL8c(0Y) cells were compared to OL8c(2Y) cells, and OL11c(0Y) cells were compared to OL11c(2Y) cells. In this step, the genes were excluded from those selected by the microarray analysis I if their expression levels had changed during the 2-year culture of cured cells. As regards the selected genes, we next performed a RT-PCR analysis I to examine the reproducibility of changes in gene expression levels. In this step, we added the results of a new comparative series, OL14(0Y) versus OL14(2Y), to arrive at the judgment to advance to the next step of analysis. We selected genes for which expression levels had changed in more than five of six comparative series (Fig. 2). At the last step, we confirmed by RT-PCR analysis II whether or not the expression levels of the selected genes in OL8(2Y) or OL11(2Y) cells had changed by HCV RNA replication. When the gene expression levels had not changed in two comparative series (OL8(2Y) versus OL8(2Y)c and OL11(2Y) versus OL11(2Y)c), the genes were selected as the candidates exhibiting irreversible changes after 2-year HCV RNA replication.

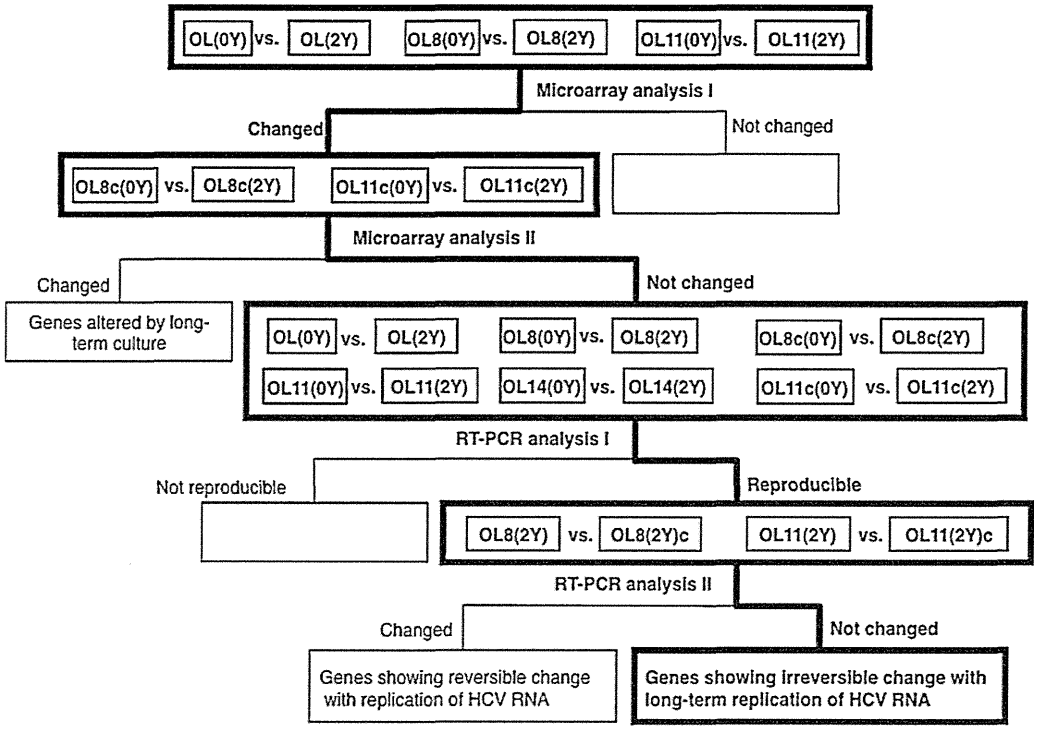

Fig. 2. Outline of selection process performed in this study. To obtain the objective genes, cDNA microarray analyses I and II were performed, and then RT-PCR analyses I and II were also performed.

3.3. Selection and expression profiles of genes showing upregulated expression during long-term HCV RNA replication

The process outlined in Fig. 2 was used to identify those genes that exhibited irreversibly upregulated expression during the 2-year replication of HCV RNA. Microarray analysis I revealed 1912, 1148, and 1633 probes, the expression levels of which were upregulated at a ratio of more than 2 in the case of OL(0Y) cells versus OL(2Y) cells, OL8(0Y) cells versus OL8(2Y) cells, and OL11(0Y) cells versus OL11(2Y) cells, respectively. To avoid the possibility that the genes showing low expression level are selected, the ratios and expression values were used in combination for the selection. As the minimum expression level, more than 100 (actual value of measurement), which was detectable within 30 cycles in RT-PCR analysis, was adopted. From among these probes, we selected those showing ratios of more than 4 with an expression level of more than 100, or those showing ratios of more than 3 with an expression level of more than 200, or those showing an expression level of 1000. By this selection process, 559, 237, and 368 genes (redundant probes excluded) were assigned in the case of OL(0Y) cells versus OL(2Y) cells, OL8(0Y) cells versus OL8(2Y) cells, and OL11(0Y) cells versus OL11(2Y) cells, respectively (Fig. 3A). At this step, we obtained 51 genes as candidates exhibiting upregulation in more than two of three comparisons. Based on the results of the subsequent microarray analysis II, we further selected 14 genes from a total of 51 genes, because the expression levels of the remaining 37 genes increased during the 2-year culture of cured cells (Fig. 3B). The list of these genes was shown in Supplemental Table 1. As regards the 14 selected genes, we performed an RT-PCR analysis I to confirm the results obtained by the cDNA microarray analysis and to examine the status of gene expression in an additional comparison of OL14(0Y) cells versus OL14(2Y) cells. This analysis revealed that the mRNA levels of 6 of 14 genes showed no enhancement in two of four comparative series (data not shown). Therefore, in this step, these 6 genes were excluded from the candidate genes. However, the mRNA levels of the remaining 8 genes (acyl-CoA synthetase medium-chain family member 3 [*ACSM3*], angiopoietin 1 [*ANGPT1*], cyclin-dependent kinase inhibitor 2C [*CDKN2C*], phospholipase A1 member A [*PLA1A*], Sel-1 suppressor of lin-12-like 3 [*SEL1L3*], solute carrier family 39 member 4 [*SLC39A4*], TBC1 domain family member 4 [*TBC1D4*], and WNT1 inducible signaling pathway protein 3 [*WISP3*]) were enhanced in more than three of four comparative series (Fig. 3C). Furthermore, we demonstrated by RT-PCR analysis II that the expression levels of these 8 genes did not return to initial levels, even after elimination of HCV RNA from OL8(2Y) or OL11(2Y) cells (Fig. 3C). It was noteworthy that the mRNA levels of the *ANGPT1* and *PLA1A* genes were enhanced in all comparative series (Fig. 3C).

3.4. Selection and expression profiles of genes showing downregulated expression during long-term HCV RNA replication

To obtain genes showing irreversibly downregulated expression during the 2-year HCV RNA replication period, we performed a selection of genes according to the methods described for the selection of upregulated genes. The first microarray analysis I in this series revealed 1901, 2128, and 1579 probes whose expression levels were downregulated at a ratio of less than 0.5 in the case of OL(0Y) cells versus OL(2Y) cells, OL8(0Y) cells versus OL8(2Y) cells, and OL11(0Y) cells versus OL11(2Y) cells, respectively. As described in Section 3.3, the ratios and expression values were used in combination for the selection. From among these probes, we selected those showing ratios of less than 0.25 with an initial expression level of more than 1000 (actual value of measurement), or those showing ratios of less than 0.33 with an initial expression level of more than 200, or those showing an initial expression level of 100. By this selection process, 828, 622, and 466 genes (redundant probes excluded) were assigned in the case of OL(0Y) cells versus OL(2Y) cells, OL8(0Y) cells versus OL8(2Y) cells, and OL11(0Y) cells versus OL11(2Y) cells, respectively (Fig. 4A). At this step, we obtained 236 genes as candidates showing downregulation in more than two of three comparisons. Based on the results