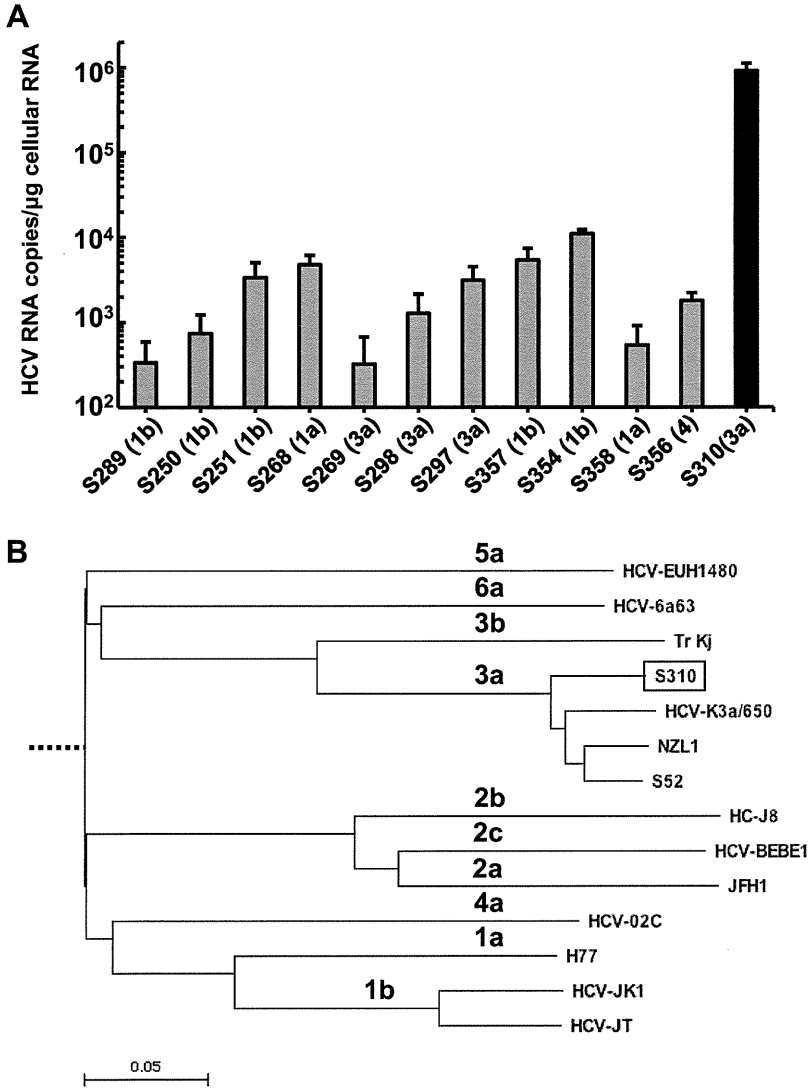
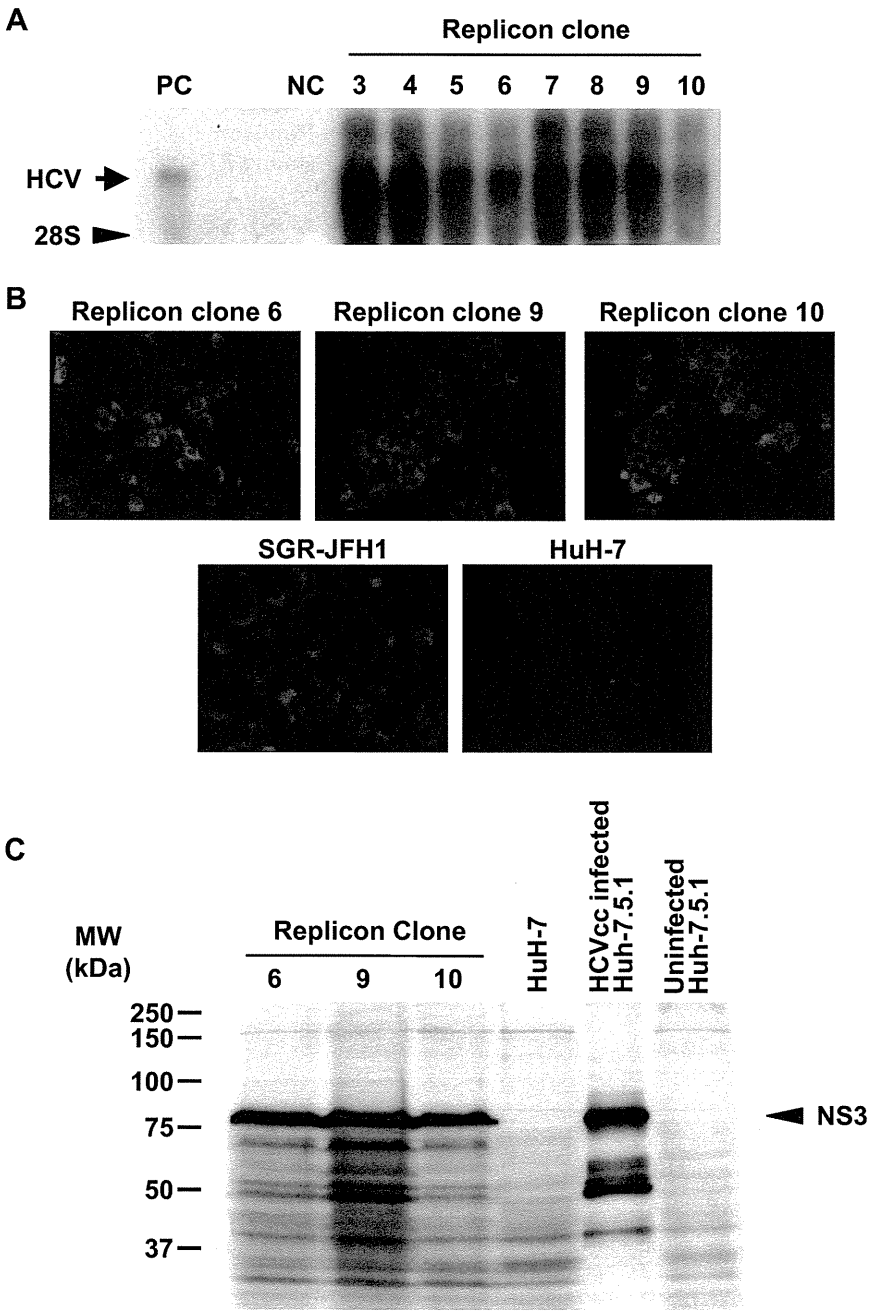
Supplementary Table 1. Primers for Amplification of the S310 HCV Strain

Fragment			Primer sequence (5′→3′)
1 (5′ RACE)[a]	Outer	Antisense	CTTGACGTCCTGTGGGCGA
	Inner	Antisense	TTTTTCTTTGGGGTTTAGG
2	Outer	Sense	GTCTTCACGCGGAAAGCGC
		Antisense	CACCCAAACCACCGACCAC
	Inner	Sense	CCGGGAGAGCCATAGTGGTC
		Antisense	TCCTGAAAGATGGCCTGGGTA
3	Outer	Sense	CTTGGCCCCTCTATGGTAA
		Antisense	GATGTTTCCTGAAGCAGTCG
	Inner	Sense	AGTCATGTGGACCTATTAGT
		Antisense	CACCCAAACCACCGACCAC
4	Outer	Sense	ATGGCTCGTGGCACATCAA
		Antisense	TAGTCATCAGCAGGTCCCAA
	Inner	Sense	GCTCAGCAGCTGCAAGCCCAT
		Antisense	CGCAAAGAATATCTCCGCAAG
5	Outer	Sense	ATTTTTGACATCACTAAGCTAC
		Antisense	AGTGTTGGCTTAAGCCGCA
	Inner	Sense	AATACTTCCAGATGATCATACT
		Antisense	GTGACAGAAAGTGGGCAT
6	Outer	Sense	GTTTCCCGCAGCCAACGT
		Antisense	GTCTCTCAACATCGAGGT
	Inner	Sense	CGGTGAAAGACCGTCTGGA
		Antisense	CAGGGGAGTTGAGATCCT
7	Outer	Sense	GGCCGCGTACATGTGCTAAC
		Antisense	CCGCAGACAAGAAAGTCCGGGT
	Inner	Sense	CTATGGCGCGTGGCTGCCA
		Antisense	ACCCCCAGGTCAGGGTACAC
8	Outer	Sense	CATAACCTAGTCTATTCAACG
		Antisense	TGGTCTTGGTGCGTACCG
	Inner	Sense	GCTCCGTCTGGGAGGACTTGC
		Antisense	CTCGTGCCCGATGTCTCCAA
9	Outer	Sense	TGCTCCTCCAACGTCTCCGT
		Antisense	GCGGCTCACGGACCTTTCAC
	Inner	Sense	GTCGCGGGGACACTCAGGAA
		Antisense	ACTAGGGCTAAGATGGAGCC

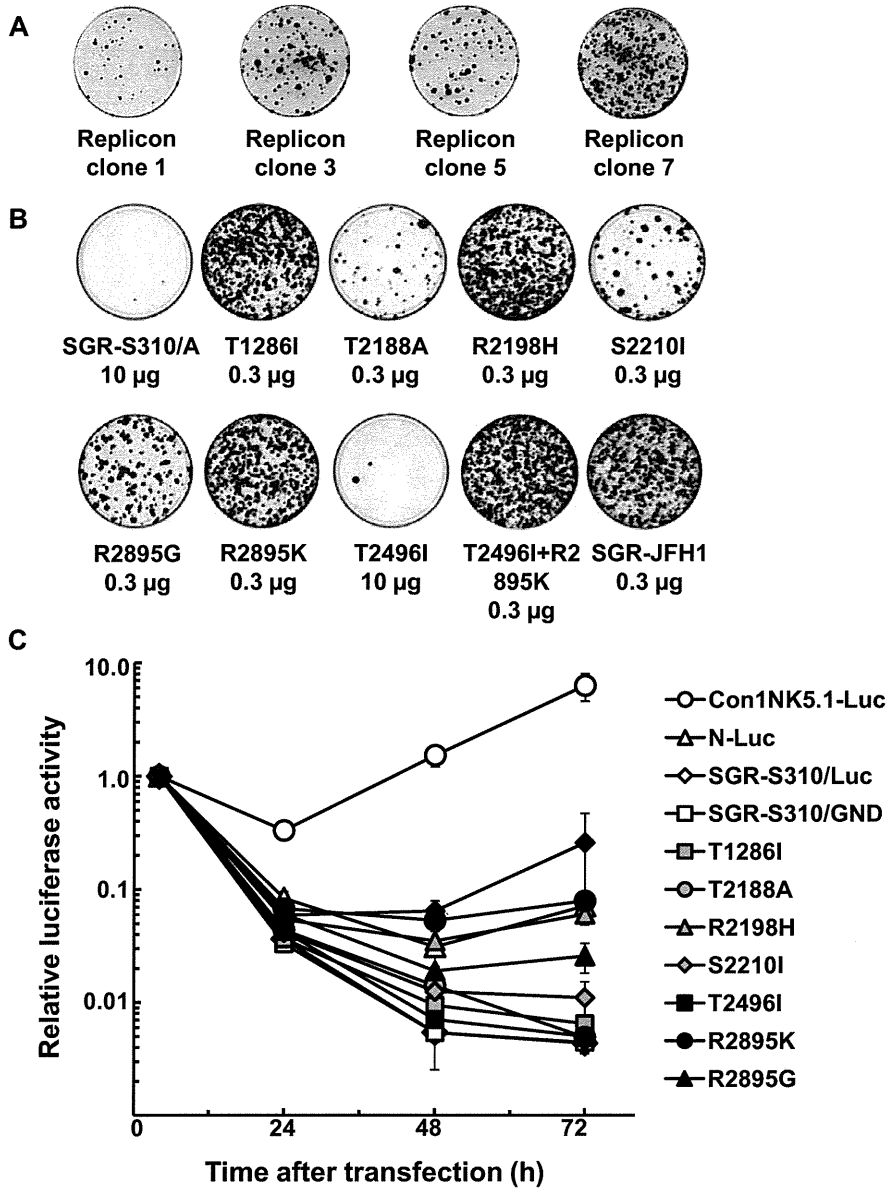
RACE, rapid amplification of complementary DNA ends.
[a]Forward primers used were those in the 5′RACE kit (Abridged Universal Amplification Primer (AUAP) for the first round of PCR and Universal Amplification Primer (UAP) for the second round of PCR).



Supplementary Figure 1. Infection of PHH with HCV patient sera and phylogenetic tree analysis of the S310 strain. (*A*) PHH were exposed to sera of patients infected with genotype 1b (S289, S250, S251, S357, S354), 1a (S268, S358), 3a (S269, S298, S297, S310), and 4 (S356) for 16 h (25 μL/well, except for S310, 10 μL). Intracellular HCV RNA was quantified 72 h post inoculation. Experiments were done in triplicate and data are presented as means ± standard deviation. (*B*) The phylogenetic tree was constructed using the polyprotein region of S310 and HCV strains of different genotypes. The HCV strains analyzed and their corresponding GenBank accession numbers are: K3a/650; D28917, NZL1; NC_009824, S52; GU814263, EUH1480, HCV-6a63; DQ480514, Tr Kj; D49374, HC-J8; D10988, BEBE1; D50409, JFH-1; AB047639, HCV-02C; DQ418784, H77; AF009606, HCV-JK1; X61596 and HCV-JT; D11168. The root of the tree was tentatively taken as the midpoint of the longest path. The length of the horizontal bar indicates the number of nucleotide substitutions per site.



Supplementary Figure 2. Detection and quantification of HCV RNA and proteins in replicon cells. (*A*) Total RNA (3 μg) from replicon cells was analyzed by Northern blot; 5.0×10^7 copies of in vitro–transcribed RNA were loaded in parallel as a positive control (PC), while total RNA from untransfected HuH-7 cells served as the negative control (NC). Replicon RNA was detected using a [α-^{32}P]dCTP-labeled DNA probe. Arrow and arrowhead indicate the positions of the replicon RNA and 28S ribosomal RNA, respectively. (*B*) Subcellular localization of viral proteins determined by immunofluorescence. S310 replicon cell clones, JFH-1 replicon cells, and untransfected HuH-7 cells were grown on glass slides for 24 h. After fixation, cells were incubated with patient serum. (*C*) Western blot analysis. Cell lysates were prepared from replicon clones 6, 9, and 10, untransfected HuH-7, and HCVcc (J6/JFH1)-infected Huh-7.5.1 cells and uninfected Huh-7.5.1 cells. Protein (10 μg) was resolved by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and viral nonstructural protein NS3-specific bands were detected using an anti-NS3 mouse monoclonal antibody (clone 8G2). Arrow indicates the position of NS3.



Supplementary Figure 3. Analysis of the effect of mutations on the colony-forming efficiency and transient replication of the subgenomic replicon S310. (*A*) Total RNA was isolated from the indicated replicon cell clones and 10 μg RNA was introduced into 3 million naïve HuH-7 cells by electroporation. After 3 weeks of G418 selection (500 μg/mL), colonies were stained. (*B*) Three million HuH-7 cells were electroporated with the indicated amounts of transcribed RNA and colonies were selected by a 3-week G418 selection. The JFH-1 subgenomic RNA was included as a positive control. (*C*) Huh-7.5.1 cells were transfected with the transcribed RNA from pSGR-S310/Luc and pSGR-S310/Luc constructs with mutations (GND mutation in NS5B, T1286I, T2188A, R2198H, S2210I, T2496I, R2895K, R2895G, and T2496I+R2895K) and Con1-NK5.1/Luc and N/Luc replicon. Transfected cells were harvested at the indicated time points and at 4 h post transfection. Relative luciferase activity (arbitrary units) was measured in the cell lysate and was normalized to the activity at 4 h post transfection. Assays were performed in triplicate, and data are presented as means ± standard deviation.

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Original Article

Factors responsible for the discrepancy between *IL28B* polymorphism prediction and the viral response to peginterferon plus ribavirin therapy in Japanese chronic hepatitis C patients

Hiroaki Saito,[1,2] Kiyoaki Ito,[1] Masaya Sugiyama,[1] Teppei Matsui,[1] Yoshihiko Aoki,[1] Masatoshi Imamura,[1] Kazumoto Murata,[1] Naohiko Masaki,[1] Hideyuki Nomura,[3] Hiroshi Adachi,[4] Shuhei Hige,[5] Nobuyuki Enomoto,[6] Naoya Sakamoto,[7] Masayuki Kurosaki,[8] Masashi Mizokami[1] and Sumio Watanabe[2]

[1]*The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa,* [2]*Department of Gastroenterology, Juntendo University School of Medicine, Bunkyoku,* [3]*The Center for Liver Diseases, Shin-Kokura Hospital, Kitakyushu, Fukuoka,* [4]*Department of Virology and Liver Unit, Tonami General Hospital, Tonami,* [5]*Department of Internal Medicine, Hokkaido University Graduate School of Medicine, Sapporo,* [6]*Department of Internal Medicine, University of Yamanashi, Kofu,* [7]*Department for Hepatitis Control, Tokyo Medical and Dental University, Tokyo, and* [8]*Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Musashino, Japan*

Aim: *IL28B* polymorphisms serve to predict response to pegylated interferon plus ribavirin therapy (PEG IFN/RBV) in Japanese patients with chronic hepatitis C (CHC) very reliably. However, the prediction by the *IL28B* polymorphism contradicted the virological response to PEG IFN/RBV in some patients. Here, we aimed to investigate the factors responsible for the discrepancy between the *IL28B* polymorphism prediction and virological responses.

Methods: CHC patients with genotype 1b and high viral load were enrolled in this study. In a case–control study, clinical and virological factors were analyzed for 130 patients with rs8099917 TT genotype and 96 patients with rs8099917 TG or GG genotype who were matched according to sex, age, hemoglobin level and platelet count.

Results: Higher low-density lipoprotein (LDL) cholesterol, lower γ-glutamyltransferase and the percentage of wild-type phenotype at amino acids 70 and 91 were significantly associated with the rs8099917 TT genotype. Multivariate analysis showed that rs8099917 TG or GG genotype, older age and lower LDL cholesterol were independently associated with the non-virological responder (NVR) phenotype. In patients with rs8099917 TT genotype (predicted as virological responder [VR]), multivariate analysis showed that older age was independently associated with NVR. In patients with rs8099917 TG or GG genotype (predicted as NVR), multivariate analysis showed that younger age was independently associated with VR.

Conclusion: Patient age gave rise to the discrepancy between the prediction by *IL28B* polymorphism and the virological responses, suggesting that patients should be treated at a younger age.

Key words: aging, genotype, IL28B, low-density lipoprotein cholesterol, single nucleotide polymorphism

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection is a global health problem with worldwide estimates of 120–130 million carriers.[1] Chronic HCV infection, the leading cause of liver transplantation, can lead to progressive liver disease, resulting in cirrhosis and complications, including decompensated liver disease and hepatocellular carcinoma.[2] The current standard-of-care treatment for suitable patients with chronic HCV infection consists of pegylated interferon-α-2a or -2b (PEG IFN) given by injection in combination with oral ribavirin (RBV) for 24 or 48 weeks, depending on HCV genotype. Large-scale treatment in the USA and Europe showed that 42–52% of patients with HCV genotype 1

Correspondence: Dr Masashi Mizokami, The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, 1-7-1 Kohnodai, Ichikawa, Chiba 272-8516, Japan. Email: mmizokami@hospk.ncgm.go.jp

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achieved a sustained virological response (SVR),[3–5] and studies conducted in Japan produced similar results. This treatment is associated with well-known side-effects (e.g. influenza-like syndrome, hematological abnormalities and neuropsychiatric events) resulting in reduced compliance and fewer patients completing treatment.[6] It is important to predict an individual's response before treatment with PEG IFN/RBV to avoid side-effects, as well as to reduce the treatment cost. The HCV genotype, in particular, is used to predict the response: patients with the HCV genotype 2/3 have a relatively high rate of SVR (70–80%) with 24 weeks of treatment, whereas those infected with genotype 1 have a much lower rate of SVR, despite 48 weeks of treatment.[5]

Our recent genome-wide association studies (GWAS) revealed that several highly correlated common single nucleotide polymorphisms (SNP) in the region of the interleukin-28B (*IL28B*) gene on chromosome 19, coding for interferon (IFN)-λ3, are implicated in the non-virological responder (NVR) to PEG IFN/RBV phenotype among patients infected by HCV genotype 1.[7] The association between response to PEG IFN/RBV and SNP associated with *IL28B* was concurrently reported by two other groups who also employed GWAS.[8,9] The *IL28B* polymorphism was highly predictive of the response to PEG IFN/RBV therapy in Japanese chronic hepatitis C (CHC) patients.[10–12] However, this was not always the case. Therefore, we attempted to determine why the *IL28B* polymorphism did not predict the response of all patients. The nature of the functional link between the *IL28B* polymorphism and HCV clearance is unknown, and this must be defined to understand how the *IL28B* polymorphism correlates with HCV clearance. Therefore, we also investigated the association between the *IL28B* polymorphism and clinical characteristics of CHC patients.

METHODS

Patients

A TOTAL OF 696 CHC patients with genotype 1b and high viral load were recruited from the National Center for Global Health and Medicine, Hokkaido University Hospital, Tokyo Medical and Dental University Hospital, Yamanashi University Hospital, Tonami General Hospital, and Shin-Kokura Hospital in Japan. In a case–control study, sex, age, hemoglobin level and platelet count were matched between patients with the rs8099917 TT genotype ($n = 130$) and patients with rs8099917 TG or GG genotypes ($n = 96$) to eliminate background biases.

Each patient was treated with PEG IFN-α-2b (1.5 μg/kg s.c. weekly) or PEG IFN-α-2a (180 μg/body s.c. weekly) plus RBV (600–1000 mg daily, depending on bodyweight). Because a reduction in the dose of PEG IFN/RBV can contribute to a lower SVR rate,[13] only patients with an adherence of more than 80% dose for both drugs during the first 12 weeks were included in this study. Those positive for hepatitis B surface antigen and/or anti-HIV were excluded from this study.

Non-virological response was defined as less than a 2 log-unit decline in the serum level of HCV RNA from the pretreatment baseline value within the first 12 weeks and detectable viremia 24 weeks after treatment. Virological response (VR) was defined as attaining SVR or transient virological response (TVR) in this study; SVR was defined as undetectable HCV RNA in serum 6 months after treatment, whereas TVR was defined as a reappearance of HCV RNA in serum after the treatment was discontinued for a patient who had undetectable HCV RNA during the therapy or on completion of the therapy. At the time of enrollment, written informed consent was obtained for the collection and storage of serum and peripheral blood. This study was conducted in accordance with provisions of the Declaration of Helsinki.

Clinical and laboratory data

The sex, age, hemoglobin (Hb) and platelet counts were matched between study groups. Other parameters determined were as follows: alkaline phosphatase (ALP), alanine transaminase (ALT), total cholesterol, fasting blood sugar (FBS), low-density lipoprotein (LDL) cholesterol, γ-glutamyl transpeptidase (γ-GTP), α-fetoprotein (AFP), HCV RNA level and the rs8099917 polymorphism near *IL28B*.

DNA extraction

Genomic DNA was extracted from the buffy coat fraction of patients' whole blood using a GENOMIX kit (Talent SRL; Trieste, Italy).

IL28B genotyping

We have reported that the rs8099917 polymorphism is the best predictor for the response of Japanese CHC patients to PEG IFN/RBV therapy than other SNP near *IL28B*.[14] Therefore, the rs8099917 polymorphism was genotyped using the InvaderPlus assay (Third Wave Japan, Tokyo, Japan), which combines polymerase

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chain reaction (PCR) and the invader reaction.[15,16] The InvaderPlus assay was performed using the LightCycler LC480 (Roche Applied Science, Mannheim, Germany).

Detection of amino acid substitutions in core and NS5A regions of HCV-1b

In the present study, substitutions of amino acid residues 70 (s-aa 70) and 91 (s-aa 91), and the presence of the IFN sensitivity-determining region (ISDR) were determined by direct nucleotide sequencing. HCV RNA was extracted from serum samples at the start of patients' therapy and reverse transcribed with a random primer and SuperScript III reverse transcriptase (Life Technologies, Carlsbad, CA, USA). Nucleic acids were amplified by PCR as described.[17]

Statistical analysis

Quantitative variables were expressed as the mean ± standard error (SE) unless otherwise specified. Categorical variables were compared using a χ^2-test or Fisher's exact test, as appropriate, and continuous variables were compared using the Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant. Multivariate analysis was performed using a stepwise logistic regression model. We performed statistical analyses using STATA ver. 11.0 (StataCorp, College Station, TX, USA).

RESULTS

Patient characteristics and *IL28B* genotype in a matched case–control study

TABLE 1 SHOWS PATIENT characteristics according to *IL28B* genotype. In a matched case–control study, sex, age, Hb levels and platelet counts were matched between 130 patients with rs8099917 TT genotype and 96 patients with rs8099917 TG or GG genotype. Lower γ-GTP ($P = 0.013$) and higher LDL cholesterol levels ($P < 0.001$) were significantly associated with the TT genotype of rs8099917. The percentages of wild type of s-aa 70 and s-aa 91 of patients with the rs8099917 TT genotype were significantly higher than those of patients with rs8099917 TG or GG genotype (s-aa 70: TT vs TG + GG, 68% vs 37% [$P < 0.001$]; s-aa 91: TT vs TG + GG, 68% vs 51% [$P = 0.017$]).

Factors associated with NVR in total patients

Table 2 shows the factors associated with NVR by univariate and multivariate analyses. Univariate analysis showed that older age ($P = 0.002$), lower platelet counts ($P = 0.01$), higher γ-GTP ($P = 0.013$), lower total cholesterol ($P = 0.017$), lower LDL cholesterol ($P < 0.001$) levels and higher AFP levels ($P = 0.019$) were significantly associated with NVR. The percentage of TG or GG genotype of rs8099917 of patients with NVR was

Table 1 Univariate analysis of *IL28B* TT and TG + GG genotypes

Variable	TT genotype ($n = 130$)	TG + GG genotype ($n = 96$)	*P*-value
Sex (% male)	61 (47)	46 (48)	Matched
Age (years), mean (SE)	57.2 (0.8)	57.5 (0.9)	Matched
Hemoglobin (g/dL), mean (SE)	14.3 (0.3)	13.9 (0.2)	Matched
Platelet count (/μL), mean (SE)	16.2 (0.5)	16.0 (0.5)	Matched
ALT (IU/L), mean (SE)	79.4 (5.4)	80.5 (7.8)	0.281
ALP (IU/L), mean (SE)	273.8 (11.7)	283.9 (11.8)	0.313
γ-GTP (IU/L), mean (SE)	63.4 (6.0)	76.0 (6.4)	0.013
Total cholesterol (mg/dL), mean (SE)	177.5 (3.3)	172.3 (3.2)	0.345
LDL cholesterol (mg/dL), mean (SE)	99.0 (2.6)	83.5 (2.8)	<0.001
Fasting blood sugar (mg/dL), mean (SE)	114.1 (4.1)	104.4 (1.9)	0.97
AFP (ng/dL), mean (SE)	9.8 (1.1)	11.5 (1.6)	0.190
HCV RNA (log IU), mean (SE)	6.2 (0.1)	6.1 (0.1)	0.186
s-aa 70 wild type (%)	70/103 (68)	30/81 (37)	<0.001
s-aa 91 wild type (%)	70/103 (68)	41/81 (51)	0.017
ISDR mutation 0–1 point (%)	82/100 (82)	70/81 (86)	0.42

AFP, α-fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region; LDL, low-density lipoprotein; SE, standard error.

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Table 2 Univariate and multivariate analyses of patients with chronic hepatitis C treated with PEG IFN/RBV with respect to VR and NVR

Variable	Univariate analysis			Multivariate analysis	
	VR ($n = 128$)	NVR ($n = 98$)	*P*-value	OR (95% CI)	*P*-value
Sex (% male)	65 (51)	42 (43)	0.237		
Age (years), mean (SE)	55.6 (0.8)	59.6 (0.9)	0.002	1.075 (1.012–1.143)	0.02
rs8099917 (TG or GG genotype) (%)	23/128 (18)	73/98 (74)	<0.001	25.460 (7.436–87.169)	<0.001
Hemoglobin (g/dL), mean (SE)	14.4 (0.3)	13.7 (0.2)	0.053		
Platelet count (/μL), mean (SE)	16.9 (0.5)	15.0 (0.5)	0.01		
ALT (IU/L), mean (SE)	83.9 (6.4)	74.5 (6.2)	0.116		
ALP (IU/L), mean (SE)	274.1 (12.3)	282.9 (11.2)	0.169		
γ-GTP (IU/L), mean (SE)	65.9 (6.4)	72.6 (5.6)	0.013		
Total cholesterol (mg/dL), mean (SE)	180.3 (3.1)	168.4 (3.5)	0.017		
LDL cholesterol (mg/dL), mean (SE)	100.5 (2.7)	83.5 (2.8)	<0.001	0.978 (0.956–0.999)	0.046
Fasting blood sugar (mg/dL), mean (SE)	106.6 (2.9)	114.8 (4.4)	0.058		
AFP (ng/dL), mean (SE)	9.6 (1.1)	12.0 (1.6)	0.021		
HCV RNA (Log IU), mean (SE)	6.2 (0.1)	6.2 (0.1)	0.876		
s-aa 70 wild type (%)	67/102 (66)	33/82 (54)	0.001		
s-aa 91 wild type (%)	67/102 (66)	44/82 (54)	0.097		
ISDR mutation 0–1 point (%)	79/96 (82)	73/85 (86)	0.511		

AFP, α-fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; CI, confidence interval; γ-GTP, γ-glutamyl transpeptidase; HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region; LDL, low-density lipoprotein; NVR, non-virological response; OR, odds ratio; PEG IFN, peginterferon; SE, standard error; RBV, ribavirin; VR, virological response.

significantly higher than that of patients with VR (VR vs NVR: 23/128 [18%] vs 73/98 [74%], $P < 0.001$). The percentage of wild-type s-aa 70 in patients with NVR was significantly lower than that in patients with VR [VR vs NVR: 67/102 [66%] vs 33/82 [54%], $P = 0.001$]. Multivariate analysis showed that older age (odds ratio [OR] = 1.075; 95% confidence interval [CI] = 1.012–1.14; $P = 0.02$), TG or GG genotype of rs8099917 (OR = 25.460; 95% CI = 7.436–87.169; $P < 0.001$) and lower LDL cholesterol levels (OR = 0.978; 95% CI = 0.956–0.999; $P = 0.046$) were independently associated with NVR.

VR to treatment depending on *IL28B* genotype

In the patients with the rs8099917 TT genotype, the rates of SVR, TVR and NVR were 62%, 19% and 19%, respectively. Therefore, 19% patients were NVR, even though rs8099917 represents the TT genotype (predicted as VR). In contrast, in the patients with rs8099917 TG or GG, the rates of SVR, TVR and NVR were 14%, 10% and 76%, respectively. Therefore, 24% patients were VR, even though rs8099917 was TG or GG genotype (predicted as NVR) (Fig. 1).

Factors associated with NVR in patients with the rs8099917 TT genotype

Table 3 shows the factors associated with NVR in patients with the rs8099917 TT genotype (predicted as VR) by univariate and multivariate analyses. Univariate analysis showed that female sex ($P = 0.003$), older age

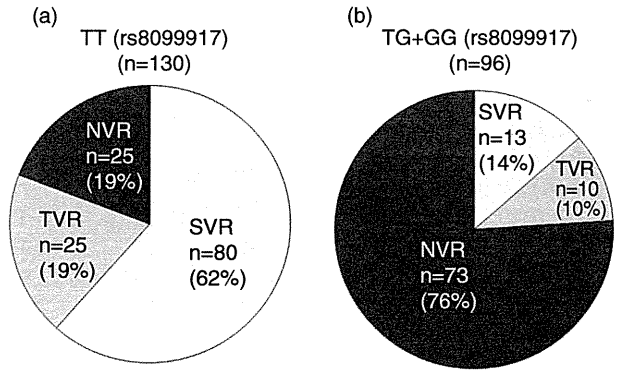

Figure 1 Virological responses to pegylated interferon and ribavirin therapy were shown in patients with rs8099917 TT (a) and TG + GG (b). NVR, non-virological response; SVR, sustained virological response; TVR, transient virological response.

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Table 3 Variables associated with NVR by univariate and multivariate analyses in patients with rs8099917 TT genotype

Variable	Univariate analysis			Multivariate analysis	
	VR ($n = 105$)	NVR ($n = 25$)	*P*-value	OR (95% CI)	*P*-value
Sex (% male)	56 (53)	5 (20)	0.003		
Age (years), mean (SE)	56.1 (0.8)	61.7 (1.6)	0.001	1.142 (1.026–1.271)	0.015
Hemoglobin (g/dL), mean (SE)	14.6 (0.4)	13.1 (0.3)	0.005		
Platelet count (/μL), mean (SE)	16.7 (0.6)	13.8 (1.0)	0.019		
ALT (IU/L), mean (SE)	83.6 (6.3)	61.0 (7.9)	0.053		
ALP (IU/L), mean (SE)	270.6 (13.6)	285.9 (22.3)	0.206		
γ-GTP (IU/L), mean (SE)	66.9 (7.1)	49.2 (7.4)	0.473		
Total cholesterol (mg/dL), mean (SE)	180.2 (3.6)	165.0 (7.6)	0.072		
LDL cholesterol (mg/dL), mean (SE)	101.2 (2.9)	88.5 (5.2)	0.067		
Fasting blood sugar (mg/dL), mean (SE)	108.4 (3.5)	140.0 (15.5)	0.127		
AFP (ng/dL), mean (SE)	9.4 (1.2)	12.2 (3.6)	0.245		
HCV RNA (log IU), mean (SE)	6.2 (0.1)	6.2 (0.1)	0.948		
s-aa 70 wild type (%)	57/83 (66)	13/20 (75)	0.752		
s-aa 91 wild type (%)	55/83 (66)	15/20 (75)	0.452		
ISDR mutation 0–1 point (%)	64/79 (81)	18/21 (86)	0.618		

AFP, α-fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; CI, confidence interval; γ-GTP, γ-glutamyl transpeptidase; HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region; LDL, low-density lipoprotein; NVR, non-virological response; OR, odds ratio; SE, standard error; VR, virological response.

($P = 0.001$), lower Hb levels ($P = 0.005$) and lower platelet counts ($P = 0.019$) were significantly associated with NVR in patients with the rs8099917 TT genotype. Multivariate analysis showed that only older age was independently associated with NVR in patients with the rs8099917 TT genotype (predicted as VR) (OR = 1.142; 95% CI = 1.026–1.27; $P = 0.015$).

Factors associated with VR in patients with the rs8099917 TG or GG genotypes

Table 4 shows the factors associated with VR in patients with the rs8099917 TG or GG genotypes (predicted as NVR) by univariate and multivariate analyses. Younger age ($P = 0.005$), lower γ-GTP ($P = 0.009$) and higher LDL cholesterol levels ($P = 0.032$) were significantly associated with VR by univariate analysis. Multivariate analysis showed that only younger age was independently associated with VR in patients with the rs8099917 TG or GG genotype (predicted as NVR) (OR = 0.926; 95% CI = 0.867–0.990; $P = 0.023$).

Rate of VR depending on the rs8099917 genotype of each age group

We divided patients into four age groups and compared VR rates by the differences in rs8099917 genotype for each group. The rate of VR decreased gradually in the older age groups independent of genotype. In the less than 49 years age group, the rate of VR in patients with the rs8099917 TT genotype was significantly higher than that in patients with the rs8099917 TG + GG genotypes ($P = 0.0002$). Further, in the 50–59 and 60–69 years age groups, the rates of VR in patients with the rs8099917 TT genotype were significantly higher than those in patients with the rs8099917 TG + GG genotypes ($P < 0.0001$, respectively). In the group that included subjects aged older than 69 years, only 50% of patients achieved VR even in those with the rs8099917 TT genotype (predicted as VR). In contrast, 47.6% of patients achieved VR, including those with the rs8099917 TG or GG genotypes (predicted as NVR) in the less than 49 years group (Fig. 2).

DISCUSSION

SINGLE NUCLEOTIDE POLYMORPHISM array analysis employing GWAS technology conducted by our laboratory and others revealed the relationships between SNP associated with the *IL28B* locus or present within the coding sequences for IFN-λ3, or the response to PEG IFN/RBV therapy for CHC.[7–9] Subsequent studies have confirmed that the response to PEG IFN/RBV therapy correlates with the SNP associated with *IL28B*[18,19] and indicates their value for predicting the response to PEG IFN/RBV therapy. Unfortunately, these predictions do not hold for some patients. In an attempt to understand the reasons for this, in the present study,

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Table 4 Variables associated with VR by univariate and multivariate analyses in patients with rs8099917 TG or GG genotypes

Variable	Univariate analysis			Multivariate analysis	
	VR ($n = 23$)	NVR ($n = 73$)	*P*-value	OR (95% CI)	*P*-value
Sex (% male)	9 (40%)	37 (51%)	0.333		
Age (years), mean (SE)	53.2 (1.7)	58.8 (1.1)	0.005	0.926 (0.867–0.990)	0.023
Hemoglobin (g/dL), mean (SE)	13.6 (0.3)	13.9 (0.2)	0.44		
Platelet count (/μL), mean (SE)	17.6 (1.1)	15.5 (0.6)	0.059		
ALT (IU/L), mean (SE)	85.5 (21.6)	78.9 (7.8)	0.767		
ALP (IU/L), mean (SE)	291.9 (28.6)	281.8 (13.0)	0.921		
γ-GTP (IU/L), mean (SE)	62.2 (15.1)	80.4 (6.9)	0.009		
Total cholesterol (mg/dL), mean (SE)	180.5 (6.2)	169.5 (3.7)	0.17		
LDL cholesterol (mg/dL), mean (SE)	97.6 (6.9)	81.9 (3.6)	0.032		
Fasting blood sugar (mg/dL), mean (SE)	98.1 (2.8)	106.3 (2.3)	0.084		
AFP (ng/dL), mean (SE)	10.3 (3.4)	11.9 (1.8)	0.123		
HCV RNA (log IU), mean (SE)	5.9 (0.1)	6.2 (0.1)	0.087		
s-aa 70 wild type (%)	10/19 (53)	20/62 (32)	0.108		
s-aa 91 wild type (%)	12/19 (63)	29/62 (47)	0.211		
ISDR mutation 0–1 point (%)	15/17 (88)	55/64 (86)	0.806		

AFP, α-fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; CI, confidence interval; γ-GTP, γ-glutamyl transpeptidase; HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region; LDL, low-density lipoprotein; NVR, non-virological response; OR, odds ratio; SE, standard error; VR, virological response.

we recruited a new set of patients for further analysis. Here, we confirmed that *IL28B* polymorphism was the most significant predictive factor for NVR with respect to PEG IFN/RBV treatment. Moreover, 19% of patients exhibiting the rs8099917 TT genotype were NVR, although they were predicted as VR. Twenty-four percent of patients with the rs8099917 TG or GG genotypes were VR, although they were predicted as NVR. We were able to determine by multivariate analysis that age was the most likely factor responsible for the discordance

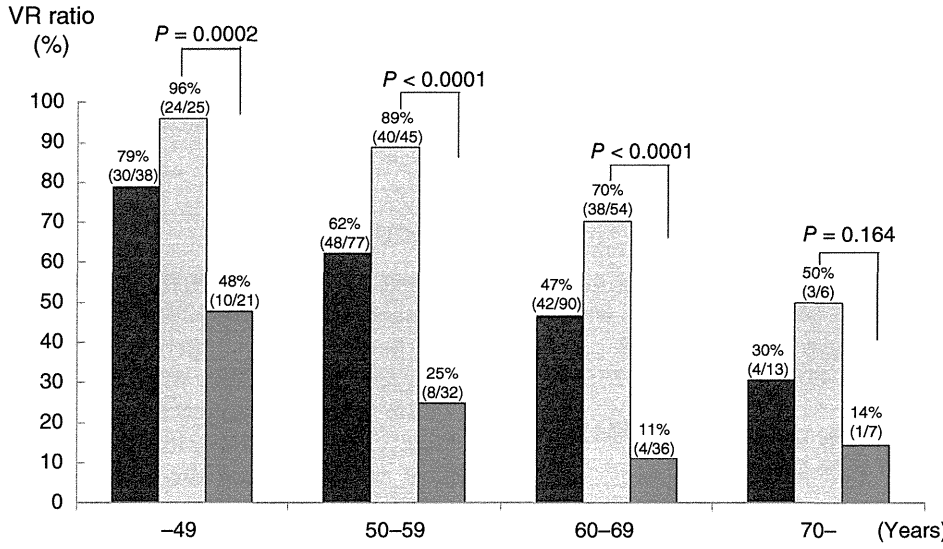

Figure 2 Virological responses (VR) to pegylated interferon and ribavirin therapy were compared between the patients with rs8099917 TT and TG + GG in each generation group. (■) Total patients, (□) TT genotype (rs8099917), (▣) TG + GG genotype (rs8099917).

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between *IL28B* genotype and patients' response to viral infection.

How does age influence the VR to PEG IFN/RBV therapy? First, the lower rate of VR to PEG IFN/RBV therapy in patients with CHC was attributed to lower compliance with the IFN or RBV dose.[20,21] Because lower compliance with PEG IFN or RBV therapy was expected to be associated with a lower rate of VR in older patients, we recruited patients who were administrated over 80% of the prescribed dose of IFN/RBV. Therefore, lower compliance can be discounted as a reason for reduced response. Second, a more advanced stage of fibrosis might have been present in the older group. Platelet counts in patients with NVR were significantly lower than those in patients with VR, and lower platelet counts may be associated with advanced fibrosis.[22] Moreover, advanced fibrosis is associated with lower rates of SVR to IFN-based therapy.[23] Third, epigenetic factors such as DNA methylation induced by aging may be involved in the reduced efficacy of PEG IFN/RBV treatment in older patients. DNA methylation near gene promoters is known to turn off transcription or reduce it considerably,[24] and advanced age is strongly associated with the increased DNA methylation.[25] Therefore, DNA methylation may be increased near or in the *IL28B* promoter as a function of age resulting in suppression of *IL28B* transcription.

Lower LDL cholesterol levels were significantly associated with NVR in patients with CHC. Moreover, LDL cholesterol levels in patients with the rs8099917 TT genotype were significantly higher than those in patients with the TG + GG genotypes. The association between LDL cholesterol and *IL28B* polymorphism as well as the VR to PEG IFN/RBV has been reported.[26] Higher pretreatment levels of LDL cholesterol have been shown to predict increased response to standard PEG IFN/RBV treatment for patients with CHC.[27,28] Although the mechanisms responsible for the association between LDL cholesterol levels and the VR to PEG IFN/RBV are unknown, the *IL28B*-rs8099917 TT responder genotype, which may correlate with an increased likelihood of treatment response and higher LDL cholesterol levels, is associated with either lower IFN-λ3 activity or reduced expression of genes regulated by IFN-mediated signaling pathways.

In conclusion, our studies provide compelling evidence that patient age is most likely responsible for incorrect predictions of VR to PEG IFN/RBV therapy in Japanese CHC patients based on *IL28B* genotypes. Our findings indicated that patients should be treated as soon as they are diagnosed. It will be important to investigate the role of the epigenetic factors associated with *IL28B* expression to develop more effective PEG IFN/RBV-based therapies for patients with CHC.

ACKNOWLEDGMENT

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Short communication

Suppression of hepatitis C virus replicon by adenovirus vector-mediated expression of tough decoy RNA against miR-122a

Fuminori Sakurai[a], Norihisa Furukawa[b], Maiko Higuchi[b], Sayuri Okamoto[a], Kaori Ono[a], Takeshi Yoshida[c], Masuo Kondoh[c], Kiyohito Yagi[c], Naoya Sakamoto[d], Kazufumi Katayama[a], Hiroyuki Mizuguchi[a,b,e,*]

[a] Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan
[b] Laboratory of Stem cell Regulation, National Institute of Biomedical Innovation, Osaka, Japan
[c] Laboratory of Bio-functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan
[d] Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan
[e] The Center for Advanced Medical Engineering and Informatics, Osaka University, Osaka, Japan

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ABSTRACT

Recent studies have demonstrated that the liver-specific microRNA (miRNA) miR-122a plays an important role in the replication of hepatitis C virus (HCV). Antisense nucleotides against miR-122a, including locked nucleic acid (LNA), have shown promising results for suppression of HCV replication; however, a liver-specific delivery system of antisense nucleotides has not been fully developed. In this study, an adenovirus (Ad) vector that expresses tough decoy (TuD)-RNA against miR-122a (TuD-122a) was developed to suppress the HCV replication in the liver hepatocytes. Ad vectors have been well established to exhibit a marked hepatotropism following systemic administration. An in vitro reporter gene expression assay demonstrated that Ad vector-mediated expression of TuD-122a efficiently blocked the miR-122a in Huh-7 cells. Furthermore, transduction with the Ad vector expressing TuD-122a in HCV replicon-expressing cells resulted in significant reduction in the HCV replicon levels. These results indicate that Ad vector-mediated expression of TuD-122a would be a promising tool for treatment of HCV infection.
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Hepatitis C virus (HCV) is a hepatotropic human virus belonging to a member of the family *Flaviviridae* and possessing a 9.6-kb positive-sense RNA genome. HCV infection causes chronic hepatic inflammation and fibrosis, leading to hepatocellular carcinoma (Hoofnagle, 2002). Currently, 170 million people worldwide are infected with HCV, and suffering from or at risk for the diseases described above. In order to suppress the replication of HCV, PEGylated interferon alpha and ribavirin, which is a nucleotide analogue, have been used as standard-of-care therapy; however, the therapeutic efficiency has been limited, in spite of relatively severe side effects, including fever and malaise (Chisari, 2005; Feld and Hoofnagle, 2005). Another therapeutic strategy should be developed to efficiently suppress the HCV infection and HCV-caused diseases.

Among several host factors involved in HCV infection, the abundant liver-specific microRNA (miRNA), miR-122a has been demonstrated to be crucial for efficient replication and/or translation of the HCV genome (Henke et al., 2008; Jopling et al., 2005; Randall et al., 2007). The HCV genome has two closely spaced miR-122a-binding sites in the 5′-untranslated region (UTR), which contains overlapping *cis*-acting signals involved in translation and RNA synthesis (Jopling et al., 2005). Although the mechanism of the miR-122a-mediated enhancement of HCV replication is controversial (Henke et al., 2008; Jopling et al., 2005; Machlin et al., 2011; Roberts et al., 2011; Wilson et al., 2011), antisense oligonucleotides complementary to miR-122a, including locked nucleic acid (LNA) oligonucleotides, have been shown to significantly inhibit miR-122a and reduce the HCV genome, and thereby to exhibit superior therapeutic effects (Henke et al., 2008; Jopling et al., 2005; Krutzfeldt et al., 2005; Lanford et al., 2010). Intravenous administration of LNA oligonucleotides against miR-122a into HCV-infected chimpanzees resulted in the long-lasting suppression of HCV viremia without viral resistance or severe side effects (Lanford et al., 2010). In addition, the 5′-UTR of the HCV genome is composed of highly conserved structural domains, suggesting that a mutant lacking the miR-122a-binding sites in the genome is unlikely to appear. These results indicate that miR-122a is a promising target for the treatment of HCV-related diseases; however, LNA oligonucleotides accumulate in the kidney immediately after intravenous administration and are excreted into the urine (Fluiter et al., 2003).

* Corresponding author at: Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 8185; fax: +81 6 6879 8186.
E-mail address: mizuguch@phs.osaka-u.ac.jp (H. Mizuguchi).

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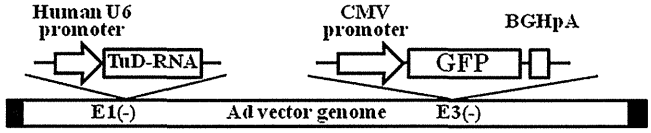

Fig. 1. Structure of Ad vectors used in this study. The human U6 promoter-driven TuD-RNA expression cassette was inserted into the E1-deleted region of the Ad vector genome. The CMV promoter-driven GFP expression cassette was inserted into the E3-deleted region of the Ad vector genome.

Systems which efficiently deliver or express anti-miR-122a drugs in the liver are necessary to efficiently treat HCV-related diseases.

Recently, tough decoy (TuD)-RNAs against miRNAs, which efficiently and specifically inhibit miRNAs, were developed by Haraguchi et al. (2009). TuD-RNAs are composed of two miRNA-binding sequence (MBS) regions and two stem structures with 3-nucleotide linkers. The MBS in the TuD-RNA is considered to tightly bind to miRNAs, leading to the inhibition of miRNAs. The inhibition activity of the TuD-RNA against miRNAs is higher than that of LNA oligonucleotides and miRNA sponges (Haraguchi et al., 2009). Another advantage of the TuD-RNA is that it can be expressed by viral and non-viral vectors. miRNAs can be persistently suppressed by lentivirus vector- and retrovirus vector-mediated expression of the TuD-RNA. Furthermore, liver-specific expression of the TuD-RNA is thought to be achievable by an adenovirus (Ad) vector and adeno-associated virus vector, because these vectors can express transgenes in a liver-specific manner after systemic administration. These properties of the TuD-RNA are highly promising for inhibition of miR-122a in the liver and suppression of HCV replication.

In the present study, we developed an Ad vector expressing the TuD-RNA against miR-122a (TuD-122a) to efficiently inhibit miR-122a and to suppress the HCV replication. Transduction with an Ad vector expressing TuD-122a efficiently inhibited miR-122a in vitro. In HCV replicon-expressing cells, HCV replicon levels were significantly reduced by Ad vector-mediated TuD-122a expression.

First, in order to examine the transduction efficiencies of the Ad vectors constructed in this study in the HCV replicon-expressing cells, Huh-7.5.1 1bFeo cells, which is a genotype 1b HCV replicon cell line (Yokota et al., 2003), were transduced with an Ad vector expressing TuD-122a (Ad-TuD-122a) or the control TuD-RNA (Ad-TuD-NC). Ad-TuD-122a and Ad-TuD-NC were prepared as described in Supplemental materials and methods. Structure of Ad vectors used in this study is shown in Fig. 1. The ratio of particles-to-biological titer was between 6 and 9 for each Ad vector used in this study. Both Ad-TuD-122a and Ad-TuD-NC carry the TuD-RNA expression cassette and the green fluorescence protein (GFP) expression cassette in the E1-deleted and E3-deleted region, respectively (Fig. 1). Both Ad-TuD-NC and Ad-TuD-122a efficiently transduced Huh-7.5.1 1bFeo cells (Fig. 2). More than 80% of the cells were found to be GFP-positive following transduction with Ad-TuD-122a and Ad-TuD-NC, respectively, at a multiplicity of infection (MOI) of 100. The averages of GFP-positive cells following transduction with Ad-TuD-NC were slightly higher than those with Ad-TuD-122a; however, statistically significant differences were not found for either group. Apparent cellular toxicity was not found following transduction with Ad-TuD-122a or Ad-TuD-NC (data not shown). These results indicate that Ad-TuD-122a and Ad-TuD-NC efficiently transduce Huh-7.5.1 1bFeo cells.

Next, in order to examine the inhibitory effects of TuD-122a expressed by the Ad vector on miR-122a, a reporter gene assay using the miR-122a complementary sequence-encoded plasmid, psiCheck-122aT, was performed in Huh-7 cells. Huh-7 cells endogenously express a high level of miR-122a (Suzuki et al., 2008). Huh-7 cells were transduced with the Ad vectors at MOIs of 25 and 100 for 1.5 h. After a 24-h incubation, the cells were transfected with

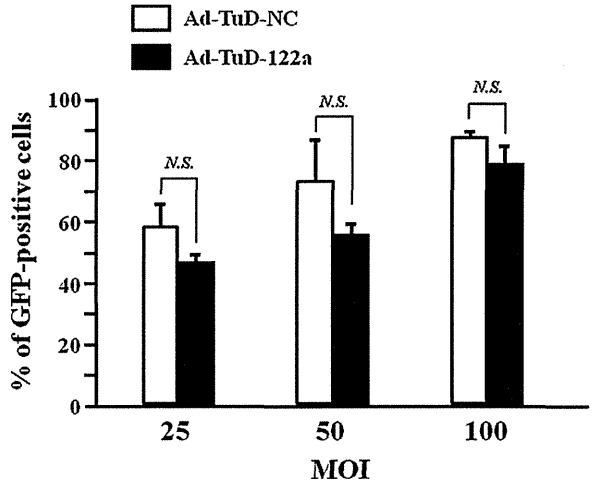

Fig. 2. Transduction efficiencies of Ad-TuD-122a and Ad-TuD-NC in Huh-7.5.1 1bFeo cells. The cells were transduced with Ad-TuD-122a or Ad-TuD-NC at multiplicities of infection (MOIs) of 25, 50, and 100 for 1.5 h. At 48 h after transduction, GFP expression was evaluated by flow cytometry. The data are expressed as the means ± S.D. ($n = 3$). The percentage of GFP-positive cells in the mock-transduced group was less than 0.2%. N.S.: not significant.

psiCheck-2 or psiCheck-122aT. The renilla and firefly luciferase expression was evaluated 48 h after transfection with the plasmid DNA. psiCheck-122aT, plasmid DNA containing the two copies of miR-122a complementary sequences in the 3′-UTR of the renilla luciferase gene, was constructed by ligation of *NotI*/*XhoI*-digested psiCheck-2 (Promega, Madison, WI) with the oligonucleotides 122aT-F and 122aT-R. The sequences of the oligonucleotides 122aT-F and 122aT-R are described in the Supplemental information. In mock-transduced cells, the relative renilla luciferase expression level by psiCheck-122aT was about 5-fold lower than that by the control plasmid psiCheck-2, which does not possess miR-122a target sequences, due to the endogenous expression of miR-122a in Huh-7 cells (Fig. 3). The renilla luciferase expression profiles following transfection with psiCheck-122aT were similar in the mock-transduced cells and Ad-TuD-NC-transduced cells, indicating that expression of the control TuD-RNA does not inhibit the miR-122a. Ad-TuD-122a did not alter the renilla luciferase expression level by psiCheck-2; on the other hand, psiCheck-122aT-mediated renilla luciferase expression was significantly restored by Ad-TuD-122a. The cells transduced with Ad-TuD-122a exhibited 2.8-fold and 3.5-fold higher renilla luciferase expression at MOIs of 25 and 100, respectively, than the mock-transduced cells following transfection with psiCheck-122aT. These results indicate that miR-122a is efficiently inhibited by Ad-TuD-122a. We also performed quantitative RT-PCR analysis for miR-122a following transduction with Ad-TuD-122a and Ad-TuD-NC in Huh-7 cells. No significant differences in the miR-122a expression levels were found in the cells transduced with Ad-TuD-122a and the cells transduced with Ad-TuD-NC (data not shown), probably because TuD-RNA does not induce degradation of miRNA, although TuD-RNA tightly binds to the target miRNA (Haraguchi et al., 2009).

Next, in order to examine whether TuD-122a-mediated inhibition of miR-122a suppresses the HCV replicon, Huh-7.5.1 1bFeo cells were transduced with Ad-TuD-122a and Ad-TuD-NC at the indicated MOIs. Huh-7.5.1 1bFeo cells express an mRNA consisting of the HCV 5′-UTR and the upstream part of the core region, connected in-frame with the firefly luciferase gene, which allows the simple evaluation of the HCV replicon levels by measuring the firefly luciferase activity in the cells (Yokota et al., 2003). Huh-7.5.1 1bFeo cells were transduced with the Ad vectors at MOIs of 25, 50, and 100 for 1.5 h. After a total 48-h incubation,

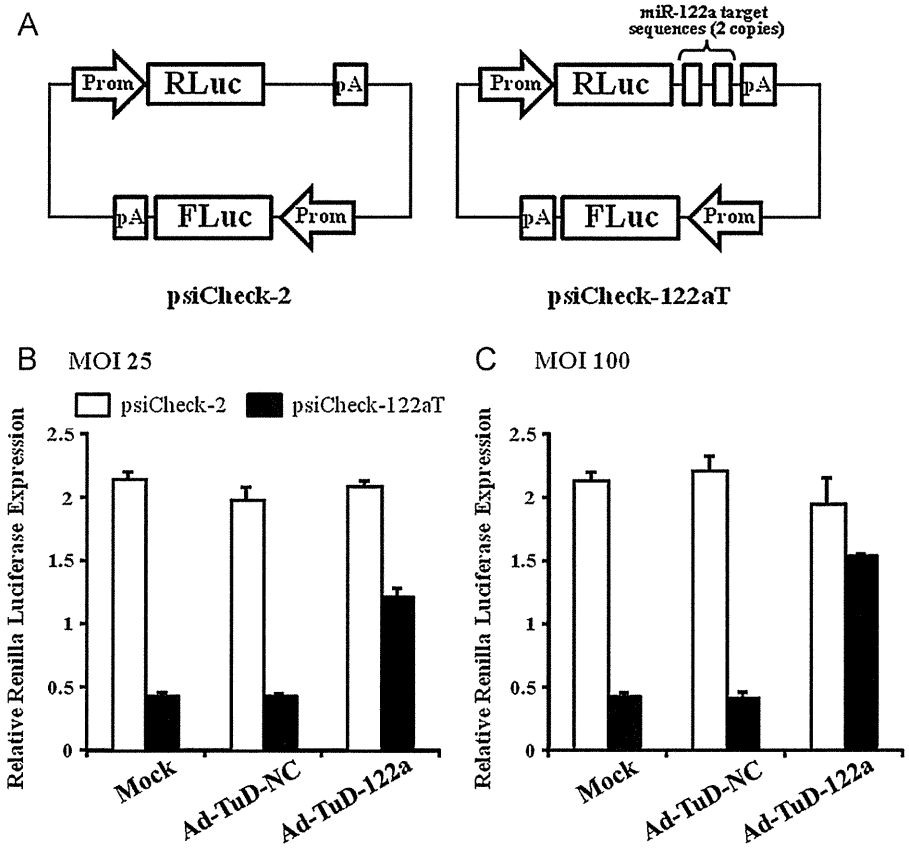

Fig. 3. Inhibition of miR-122a by Ad vector-meditated TuD-122a expression. (A) Structure of the reporter gene-expressing plasmids psiCheck-2 and psiCheck-122aT. (B and C) Relative renilla luciferase expression levels following transduction with Ad-TuD-NC or Ad-TuD-122a at MOIs of 25 (B) and 100 (C). The data are expressed as the means ± S.D. ($n = 4$)..

firefly luciferase expression levels were determined. Ad-TuD-122a significantly reduced the firefly luciferase expression levels in a dose-dependent manner (Fig. 4a). The firefly luciferase expression level was reduced to 29% of that in the cells transduced with Ad-TuD-NC at MOI of 100 by transduction with Ad-TuD-122a at MOI of 100. In contrast, no significant changes in the firefly luciferase expression were found by transduction with Ad-TuD-NC.

To examine whether inhibition of miR-122a by Ad vector-mediated TuD-122a expression leads to a reduction in HCV replicon RNA levels, strand-specific real-time RT-PCR analysis was performed to determine the HCV replicon RNA levels. Briefly, Huh-7.5.1 1bFeo cells were transduced with the Ad vectors as described above, and the total RNA was isolated 48 h after transduction. Real-time RT-PCR analysis for the HCV positive-strand RNA genome was performed as follows. Briefly, 2 μg of total RNA was reverse-transcribed to cDNA using the primer specific for the HCV positive-strand genome (RC21; 5′-ctc ccg ggg cac tcg caa gc-3′). Real-time RT-PCR was performed using the primers (RC21 and RC1; 5′-gtc tag cca tgg cgt tag ta-3′) and SYBR Premix Ex Taq II (Takara Bio Inc., Kyoto, Japan). Similarly to the results for the firefly luciferase expression in Fig. 4A, HCV replicon RNA levels were significantly reduced by Ad-TuD-122a (Fig. 4B). There was an approximately 2.2-fold decline in the HCV replicon RNA level in the cells transduced with Ad-TuD-122a at an MOI of 100, compared with the HCV replicon RNA level in the cells transduced with Ad-TuD-NC at an MOI of 100. Ad-TuD-NC did not apparently decrease the HCV replicon RNA levels. These results indicate that the inhibition of miR-122a by Ad vector-mediated TuD-122a expression efficiently suppresses the replication of the HCV replicon.

The present study demonstrates that Ad vector-mediated TuD-122a expression significantly inhibits the function of miR-122a and replication of the HCV replicon. Replication of the HCV genome is promoted by the direct interaction between miR-122a and the complementary sequences in the 5′-UTR of the HCV genome (Henke et al., 2008; Jangra et al., 2010), indicating that sequestration of miR-122a leads to suppression of the HCV replication. In order to suppress the HCV replicon by inhibiting miR-122a, TuD-RNA was selected as an inhibitor of miRNA in this study, because TuD-RNA potently inhibits miRNA by strongly binding to miRNA (Haraguchi et al., 2009). In addition, TuD-RNA can be expressed by conventional gene delivery vectors, including virus vectors. One drawback of TuD-RNA is that TuD-RNA does not discriminate miRNA members that belong to the same miRNA family (Haraguchi et al., 2009); however, miR-122a does not constitute a family of miRNA, suggesting that TuD-122a would not inhibit other miRNAs.

As described above, an Ad vector is suitable for liver-specific expression of TuD-RNA due to the strong hepatotropism. Previous studies demonstrated that Ad vectors expressing short-hairpin RNA (shRNA) or antisense RNA against the HCV genome successfully exhibited the suppressive effects on HCV infection in vivo (Gonzalez-Carmona et al., 2011; Sakamoto et al., 2008). Another advantage of using an Ad vector for treatment of HCV-related diseases is that in vivo administration of an Ad vector induces type I interferon (IFN) production via innate immune responses (Huarte et al., 2006; Zhu et al., 2007). Our group previously demonstrated that VA-RNA, which is a small non-coding RNA expressed from a replication-incompetent Ad vector as well as wild-type Ad, stimulates type I IFN production in an IFN-β promoter stimulator-1 (IPS-1)-dependent manner (Yamaguchi et al., 2010). Ad vector-induced type I IFN would contribute to suppression of HCV infection. The anti-HCV activity of Ad-TuD-122a can also be up-regulated by insertion of an expression cassette of an

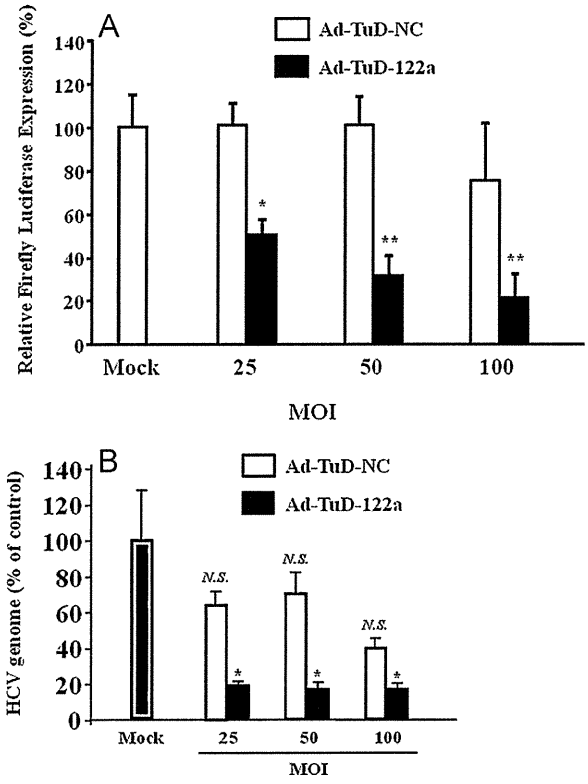

Fig. 4. Suppression of the HCV replicon by Ad vector-mediated TuD-122a expression. (A) Firefly luciferase expression levels and (B) HCV replicon RNA levels in Huh-7.5.1 1bFeo cells following transduction with the Ad vectors. All the data are shown as the means ± S.D. ($n = 3$). N.S.: not significant. $^{*}P < 0.05$, $^{**}P < 0.005$ between mock-transduced cells and cells transduced with Ad-TuD-122a.

anti-HCV gene, including type I IFN genes and short-hairpin RNA (shRNA) or antisense RNA against the HCV genome, into the Ad vector genome. Our group has developed various types of Ad vectors in which two or three transgene expression cassettes can be inserted into a single Ad vector genome (Mizuguchi et al., 2001, 2005, 2003).

Previous studies have demonstrated that lipid droplets, which are lipid-storage intracellular organelles, are crucial for the production of infectious HCV particles (Hinson and Cresswell, 2009; Miyanari et al., 2007). Miyanari et al. demonstrated that HCV capsid proteins recruit the non-structural proteins and the replication complex to the lipid droplet-associated membrane (Miyanari et al., 2007). miR-122a is an important factor that regulates cholesterol and fatty-acid metabolism in the hepatocytes (Esau et al., 2006; Iliopoulos et al., 2010). Intravenous administration of the antisense oligonucleotide against miR-122a resulted in a reduction in the plasma levels of cholesterol and triglycerides (Esau et al., 2006; Lanford et al., 2010). In addition to the enhancement of accumulation and translation of the HCV genome, miR-122a might up-regulate HCV infection by regulating lipid metabolism in the hepatocytes.

Almost similar levels of reduction in the HCV replicon RNA copy numbers were found for Ad-TuD-122a at MOIs of 25, 50, and 100, although there was dose-dependent reduction in the firefly luciferase expression following transduction with Ad-TuD-122a. It remains unclear why dose-dependent reduction in the HCV replicon RNA copy numbers was not found, however, miR-122a plays a crucial role in the enhancement of both translation and stability of HCV genome (Henke et al., 2008; Jopling et al., 2005; Randall et al., 2007; Shimakami et al., 2012). Stability of HCV genome might be more susceptible to inhibition of miR-122a than translation. The averages of HCV replicon RNA levels were also reduced following transduction with Ad-TuD-NC, although statistically significant differences were not found, compared with the mock-transduced cells. Replication-incompetent Ad vectors express non-coding small RNA (VA-RNA), which forms RNA-induced silencing complex (RISC) with argonaute 2 (Ago2) (Xu et al., 2007). Ago2 is an important factor for miRNA processing (Diederichs and Haber, 2007). Processing of miR-122a might be slightly disturbed by Ad vector-expressed VA-RNA, leading to the reduction in the HCV replicon RNA levels.

In summary, we efficiently suppressed the HCV replicon levels by Ad vector-mediated expression of TuD-122a, which blocks the function of miR-122a. This study indicates that Ad vector-mediated expression of TuD-122a in liver hepatocytes would offer an alternative approach for the treatment of HCV infection.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2012.02.003.

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

Hiroe Sejima, Kyoko Mori, Yasuo Ariumi[1], Masanori Ikeda, Nobuyuki Kato*

Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1, Shikata-cho, Okayama 700-8558, Japan

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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.

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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.

* Corresponding author. Tel.: +81 86 235 7385; fax: +81 86 235 7392.
E-mail address: nkato@md.okayama-u.ac.jp (N. Kato).
[1] Present address: Center For AIDS Research, Kumamoto University, Kumamoto 860-0811, Japan.

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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.