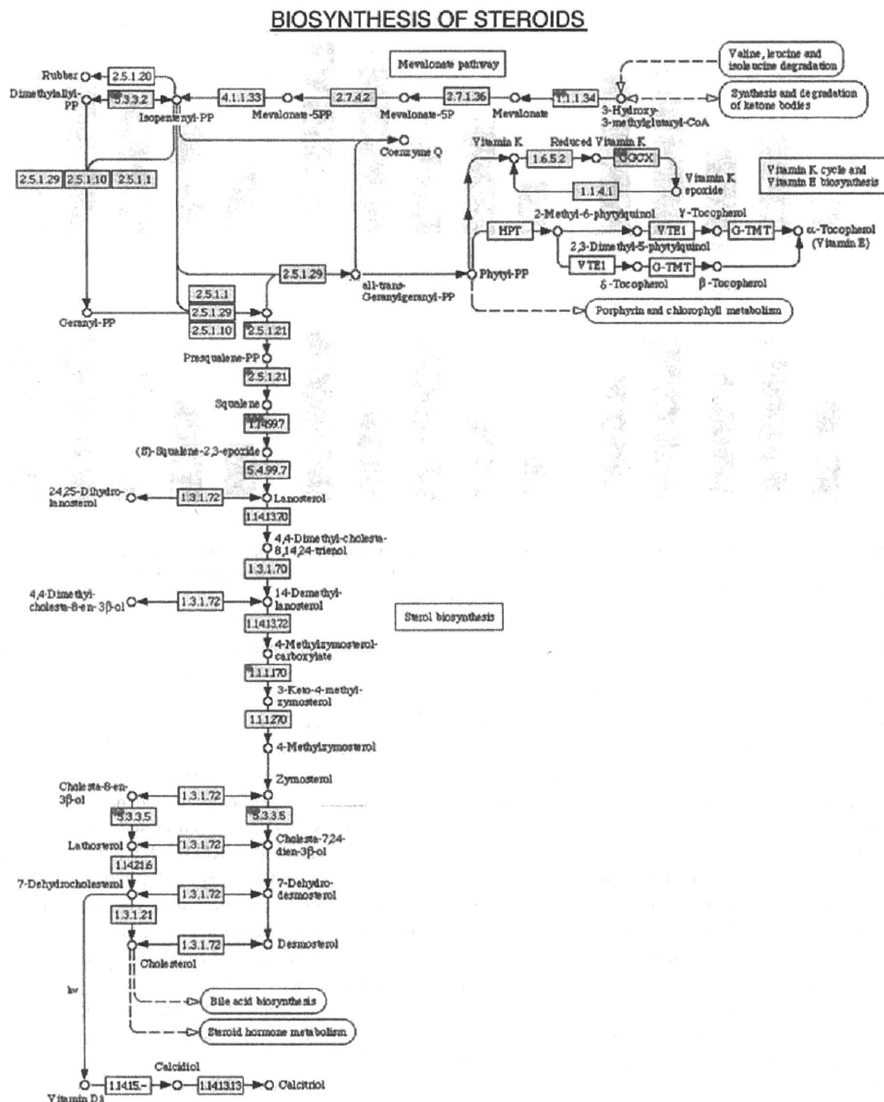

Fig. 5 KEGG Pathway map and array data (biosynthesis of steroids). Gene expression changes were mapped on the pathways. Each *circle* within a *box* represents the corresponding probe set on Human Genome U133 Plus 2.0 array because multiple probe sets are sometimes designed for a single gene. *Red circles* indicate overexpressed genes in *cured* cells compared to parental Huh7 cells. The *dotted numerical code* in each *box* represents the Enzyme Commission (EC) number based on the recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB). Correspondence between the genes that were examined in the microarray analyses and enzymes that are presented in Fig. 5 is shown in Supplementary Table 4

Rep-Feo cells showed that the replication of the HCV replicon was suppressed by clofibrate and fenofibrate in a dose-dependent manner, whereas pioglitazone and troglitazone elevated expression levels of replicon. The MTS assay did not show any effect on cell viability or replication. These results suggest that the decrease or increase in HCV replication is due to specific effects of PPAR-alpha or gamma agonists on HCV replication.

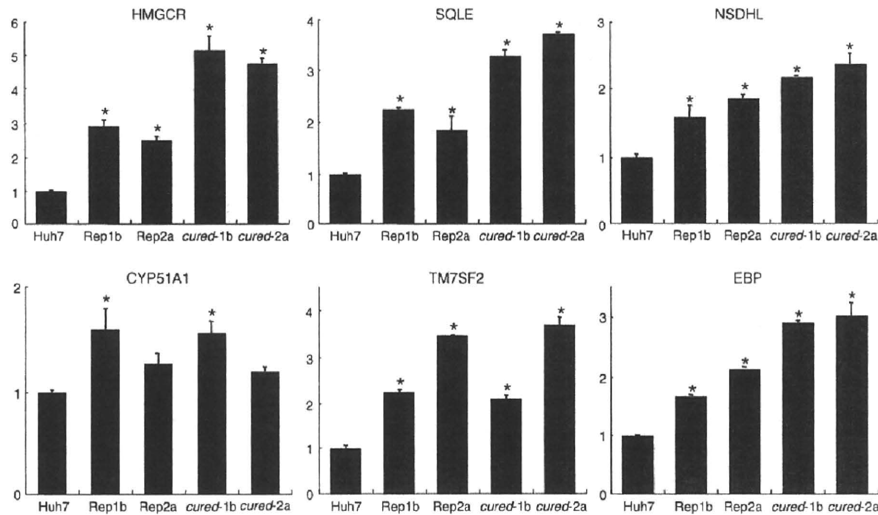

Fig. 6 Real-time detection RT-PCR. Real-time RT-PCR was performed to verify expression levels of genes that were listed in the cholesterol biosynthesis pathway in Fig. 4c and that showed differences in their expression levels by microarray analyses. Assays were done in triplicate, and *asterisks* indicate *P*-values of less than 0.05

Discussion

In our present analyses, we identified MAPK signaling, biosynthesis of steroid related and TGF-beta signaling pathways as significantly changed pathway processes by comparing replicon-expressing and *cured* cells (Supplementary Table 2). The results suggest that these pathways were primarily affected by HCV replication. Comparison of *cured* cells and naïve Huh7 cells identified cell cycle, TGF-beta, sphingolipid metabolism, and biosynthesis of steroids pathways as significantly changed pathways. Interestingly, cholesterol biosynthesis pathways were significantly changed in both comparisons (Supplementary Tables 2, 3). These data suggest that these pathways may positively regulate cellular HCV replication and that cholesterol biosynthesis pathways are primarily activated by HCV replication and may be essential for continuous virus replication.

There are several studies that report gene expression changes in replicon-expressing Huh7 cells as compared with the naïve cells [30–32]. In those studies, however, the changes in gene expression do not only reflect the effect of intracellular HCV replication, but also reflect alteration of host cell clonalities. Indeed, there are inconsistencies among studies. Use of the cured Huh7 cells can minimize the effect of cellular clonal changes because such Huh7 subclones have already been selected through HCV replicon transduction, drug-resistance selection and subsequent HCV elimination [33]. In our study, we have compared gene expression between genotype 1b and 2a replicon cells, respective cured cells and the naïve parental cells, and have identified molecular signaling or metabolic pathways that were differentially up- or down-regulated over different HCV genotypes.

Comprehensive microarray analyses and pathway analyses were very useful for the identification of molecular mechanisms of HCV infection and replication in the host cells. We used the KEGG Pathway database [28], a knowledge-based database of biological systems that integrates genomic, chemical and systemic functional information. KEGG provides a reference knowledge base for linking genome to life through the process of PATHWAY mapping, which is to map, for example, a genomic or transcriptomic content of genes to KEGG reference pathways to infer systemic behavior of the cells or the organism. These pathway databases are free on-line resources. Using these analyses, the close relation between cholesterol metabolism and HCV replication was demonstrated. Moreover, in relation to this, when we examined the pathways of other lipid metabolism, it was shown that fatty acid biosynthesis metabolism-related pathways were significantly changed in *cured* cells, and indeed we found a large number of lipid droplets in the cytosol of replicon cells and *cured* cells.

The HCV-JFH1 strain is the basis of a robustly replicating cell culture system reported recently [5]. We have performed comprehensive gene expression analyses using the HCV-JFH1 and the cured Huh7.5.1 cell line [6]. The

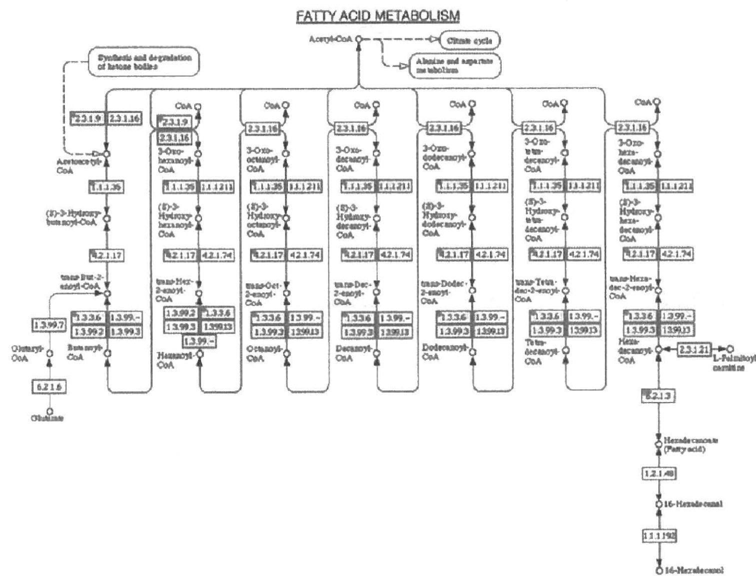

Fig. 7 KEGG Pathway map and array data (fatty acid metabolism). Gene expression changes were mapped on the pathways. Each *circle* within a *box* represents the corresponding probe set on Human Genome U133 Plus 2.0 array because multiple probe sets are sometimes designed for a single gene. *Red circles* indicate overexpressed genes in *cured* cells compared to parental Huh7 cells. The *dotted numerical code* in each *box* represents the Enzyme Commission (EC) number. Correspondence between the genes that were examined in the microarray analyses and enzymes that are presented in Fig. 7 are shown in Supplementary Table 4

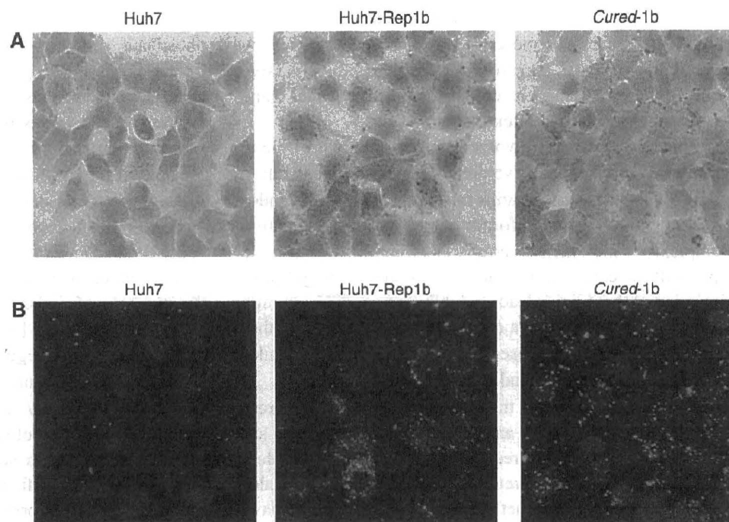

Fig. 8 Detection of intracellular lipid droplets and HCV NS protein. **a** Huh7 cells, replicon cells and *cured* cells were fixed and stained with Oil red O and Mayer's hematoxylin. Intracellular lipid droplets were detected as *red spheres* in the cells. Nuclei are stained in *blue*. **b** Rep1b/Huh7 cells were labeled with antibodies against NS5A (*red*). Lipid droplets and nuclei were stained with BODIPY493/503 (*green*) and DAPI (*blue*), respectively

KEGG Pathway analyses have identified several significantly affected pathways that are involved in the cell cycle, TGF-beta signaling, PPAR signaling and sterol biosynthesis. These findings are consistent with our present results using the HCV subgenomic replicon (see the Supplementary Table 5; Supplementary Figs. 4, 5).

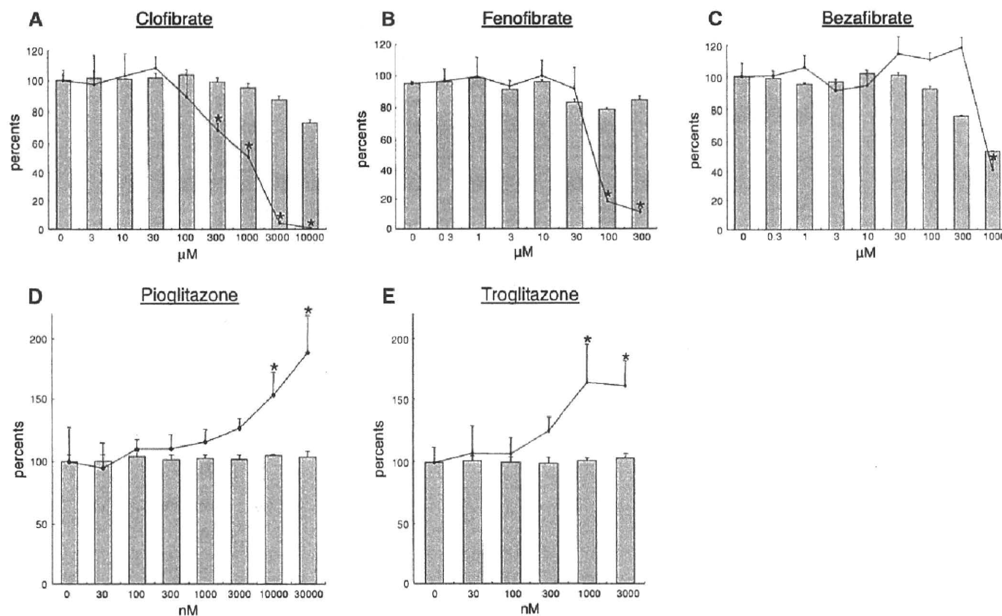

Fig. 9 Results of secondary screening with PPAR-alpha and -gamma agonists. Luciferase activity for HCV replication levels is shown as a percentage of the control. Cell viability is also shown as percentage of the control. Each *bar* represents the average of quadruplicate data points with standard deviation represented as the *error bar*. *Asterisks* denotes a significant difference from the control of at least $P < 0.05$

The JFH1 strain, however, showed substantial cytopathic effects on cultures of more than 5 days accompanied by overall induction of apoptosis-related genes and massive cell death [34]. Thus, it was difficult to conduct gene expression studies consistently.

Lipid metabolism is involved in the life cycle of many viruses. Recent studies have demonstrated the localization of HCV nonstructural proteins in the lipid raft in the endoplasmic reticulum (ER) forming intracellular replication complexes, called membranous webs [35, 36]. Because the lipid raft is enriched in cholesterols and sphingolipids, depletion of these lipids leads to inhibition of HCV genomic replication [19]. Amemiya et al. [37] reported that another serine palmitoyltransferase, myriocin, depleted cellular sphingomyelin contents and inhibited HCV replication.

It has been reported that statins efficiently suppress HCV replication in vitro and in vivo [38–40]. Statins are inhibitors of HMG-CoA reductase and shut down cholesterol biosynthesis by preventing the formation of mevalonate from 3-hydroxy-3-methyl-glutaryl CoA. As we have shown in the results, all enzymes in the cholesterol synthesis pathway were upregulated in the replicon-expressing and the cured Huh7 cells. In addition to lowering intracellular levels of sterols, statins also reduce levels of isoprenoids, which are derived from mevalonate. Isoprenoids such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate serve as lipid attachments for a variety of intracellular signaling molecules. In our results, the cholesterol biosynthesis pathway was also upregulated between cured versus naïve cell lines as well as replicon versus cured cell lines. These results suggest that HCV replication may promote synthesis of lipids including steroids that were essential for the viral efficient replication.

It has been recognized that HCV infection causes hepatic steatosis and subclinical insulin resistance and that they are independent of other risk factors such as obesity or the presence of diabetes mellitus. Similarly, in HCV cell cultures, Yang et al. [41] have reported that cellular fatty acid synthase is upregulated in HCV-infected Huh7 cells and specific inhibition of the enzymatic activity caused suppression of HCV replication. In the present study, although lipid metabolism-related genes were upregulated in cured cells, which supports efficient HCV replication, there was not significant change in lipid-related genes between replicon-expressing as compared with cured cells (Fig. 7). These results suggest that HCV subgenomic replication does not cause steatosis as it did in full-length HCV cell culture [41]. These discrepancies might be due to the absence of the presence of HCV structural genes including core and envelope proteins.

We have shown an increase in lipid droplets in HCV replicon-positive cells and their cured cell lines as a phenotype of the gene expression profiles (Fig. 8). On the other hand, ACOX1, a rate-limiting enzyme of peroxisomal beta-oxidation, was higher in cured cells than parental Huh7 cells (Fig. 7) [42]. We have shown preliminarily that cellular SREBP1 (sterol regulatory element-binding protein 1), which regulates a set of triglyceride synthesis enzymes en bloc, is upregulated in HCV replicon-positive cell lines. These discrepancies might be due to more proficient activation of SREBP1-induced fatty acid biosynthesis pathways. Collectively, our results suggest that the overall fatty acid synthesis pathway, not only fatty acid synthase, is activated by upregulation of a set of responsible enzymes.

We have investigated effects of PPAR agonists to HCV replication. PPAR-alpha agonists, clofibrate and fenofibrate suppressed HCV replication (Fig. 9). PPAR-alpha, not PPAR-gamma, is expressed in hepatocytes, recognizes cellular free fatty acids and leukotriene B4 as a specific ligands, and mediates oxidative degradation of triglyceride and depletion of intracellular fat droplets [43, 44]. These properties of PPAR-alpha agonists suggest that the level of HCV replication is affected by the increased production of fatty acids, but not by the overexpression of their related enzymes. PPAR-gamma agonists, in contrast, amplified HCV replication. Because PPAR-gamma is a regulator of fatty acid metabolism in peripheral tissue and is not expressed in the hepatocytes or in Huh7 cells (data not shown), it is possible that the effects of the PPAR-gamma agonists on HCV replication may be through its pleiotropic side effects such as p38 MAPK activation [45]. Very recently, it has been reported that HCV-NS5A proteins induce expression of PPARgamma [46].

In conclusion, comprehensive gene expression and pathway analyses were useful to study molecular pathways that were involved in HCV pathogeneses and to identify host factors for HCV replication that could constitute antiviral targets.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Mutations in the interferon sensitivity determining region and virological response to combination therapy with pegylated-interferon alpha 2b plus ribavirin in patients with chronic hepatitis C-1b infection

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Abstract

Background Pegylated-interferon-alpha 2b (PEG-IFN) plus ribavirin (RBV) therapy is currently the de-facto standard treatment for hepatitis C virus (HCV) infection. The aims of this study were to analyze the clinical and virological factors associated with a higher rate of response in patients with HCV genotype 1b infection treated with combination therapy.

Methods We analyzed, retrospectively, 239 patients with chronic hepatitis C-1b infection who received 48 weeks of combination therapy. We assessed clinical and laboratory parameters, including age, gender, pretreatment hemoglobin, platelet counts, HCV RNA titer, liver histology, the number of interferon sensitivity determining region (ISDR) mutations and substitutions of the core amino acids 70 and 91. Drug adherence was monitored in each patient. We carried out univariate and multivariate statistical analyses of these parameters and clinical responses.

Results On an intention-to-treat (ITT) analysis, 98 of the 239 patients (41%) had sustained virological responses (SVRs). Patients with more than two mutations in the ISDR had significantly higher SVR rates ($P < 0.01$). Univariate analyses showed that stage of fibrosis, hemoglobin, platelet counts, ISDR mutations, serum HCV RNA level, and adherence to PEG-IFN plus RBV were significantly correlated with SVR rates. Multivariate analysis in subjects with good drug adherence extracted the number of ISDR mutations (two or more: odds ratio [OR] 5.181).

Conclusions The number of mutations in the ISDR sequence of HCV-1b (≥ 2) is the most effective parameter predicting a favorable clinical outcome of 48-week PEG-IFN plus RBV therapy in patients with HCV genotype 1b infection.

Keywords Hepatitis C virus (HCV) · Chronic hepatitis C · PEG-IFN plus RBV therapy · Combination therapy · Interferon sensitivity determining region (ISDR)

Abbreviations

HCV	Hepatitis C virus
IFN	Interferon
PEG	Polyethylene glycol
PEG-IFN	Pegylated-interferon-alpha 2b
RBV	Ribavirin
ISDR	Interferon sensitivity determining region
BMI	Body mass index

M. Nakagawa and N. Sakamoto contributed equally to this work.

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ALT	Alanine transaminase
dM	Double mutant
ITT analysis	Intention-to-treat analysis
PP analysis	Per protocol analysis
SVR	Sustained virological response
ETR	End of treatment response
PKR	Double stranded RNA-dependent protein kinase
TLR	Toll-like receptor
MyD88	Myeloid differentiation primary response gene 88

Introduction

Hepatitis C virus (HCV) is one of the major pathogens causing chronic hepatitis [1, 2] and eradication of the virus by the host occurs infrequently during the natural course of infection once it becomes chronic. Interferon (IFN) has been used widely as the most effective antiviral agent for chronic hepatitis C. Although ribavirin (RBV), a synthetic guanosine analog, alone does not decrease the serum HCV RNA level [3–5], it has been shown that combination therapy with IFN-alpha (given 3 times weekly) and daily RBV gives a higher sustained response rate than IFN monotherapy [6–8]. Pegylation is the process by which an inert molecule of polyethylene glycol (PEG) is covalently attached to a protein, and the addition of PEG to IFN produces a biologically active molecule with a longer half-life and more favorable pharmacokinetics than the natural molecule. These characteristics allow more convenient, once-weekly dosing [9]. Pegylated (PEG)-IFN plus RBV is significantly more effective than IFN plus RBV or PEG-IFN alone for the treatment of chronic hepatitis C, with sustained virological response rates of ~50% in patients infected with HCV genotype 1b [10].

We reported previously a close correlation between the number of mutations in the nonstructural 5A (NS5A) region of the HCV genome encoding amino acids (aa) at positions 2209–2248 [the IFN sensitivity determining region (ISDR)] and IFN efficacy in patients with HCV genotype 1b infection [11–13]. The aims of this study were to analyze clinical and virological factors associated with a higher rate of response by patients with HCV genotype 1b infection who were treated with combination therapy with pegylated-IFN-alpha 2b (PEG-IFN) plus RBV, and to clarify the relationship between ISDR mutations and virological response to the combination therapy.

Methods

Patients and methods

We analyzed, retrospectively, 239 patients with chronic HCV-1b infection who received combination therapy with PEG-IFN plus RBV between December 2004 and April 2008 at Tokyo Medical and Dental University Hospital (Tokyo, Japan) and associated hospitals participating in the Ochanomizu-Liver Conference Study Group. All patients had histologically or clinically proven chronic active hepatitis and were positive for anti-HCV antibodies and serum HCV RNA by reverse transcription polymerase chain reaction (RT-PCR). Patients with a positive test for serum hepatitis B surface antigen, coinfection with other HCV genotypes, coinfection with human immunodeficiency virus, other causes of hepatocellular injury (such as alcoholism, autoimmune hepatitis, primary biliary cirrhosis, or a history of treatment with hepatotoxic drugs), and a need for hemodialysis were excluded.

The following factors were analyzed to determine whether they were related to the efficacy of combination therapy: age; gender; body mass index (BMI); previous IFN therapy; grade of inflammation and stage of fibrosis on liver biopsy; pretreatment biochemical parameters, such as hemoglobin, alanine transaminase (ALT) level, platelet count, low density lipoprotein (LDL) cholesterol, serum HCV RNA level (Log IU/ml); and the amino acid sequence of the IFN sensitivity determining region (aa 2209–2248, ISDR). Liver biopsy specimens were evaluated according to the grade of inflammation and the stage of fibrosis; this was done blindly by an independent interpreter who was not aware of the clinical data. Activity of inflammation was graded on a scale of 0–3: A0 shows no activity, A1 shows mild activity, A2 shows moderate activity, and A3 shows severe activity. Fibrosis was staged on a scale of 0–4: F0 shows no fibrosis, F1 shows moderate fibrosis, F2 shows moderate fibrosis with few septa, F3 shows severe fibrosis with numerous septa without cirrhosis, and F4 shows cirrhosis.

The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the ethics committee of our hospital, and informed written consent was obtained from each patient.

Nucleotide sequencing of the *NS5A* gene

The serum samples were frozen at −80°C until use. Extraction of RNA from serum and RT-PCR were performed as described previously [14]. The PCR and sequencing primers were synthesized with a DNA synthesizer (model 391; Applied Biosystems Japan, Chiba, Japan).

To determine the nucleotide sequence of the *NS5A 2209–2248* region, we amplified nucleotides (nt) 7296–7320 of HCV complementary DNA by using the outer pair of primers [5′ outer primer, 5′-TGG ATG GAG TGC GGT TGC ACA GGT A-3′ (nt 6703–6727 of HC-J4); 3′ outer primer, 5′-TCT TTC TCC GTG GAG GTG GTA TTG C-3′ (nt 7296–7320)]. We transferred 1 μl of the first PCR product to the second PCR reaction along with the nested 5′ and 3′ primers [5′ inner primer, 5′-TGT AAA ACG ACG GCC AGT CAG GTA CGC TCC GGC GTG CA-3′ (nt 6722–6741), with the M13 forward primer sequence underlined; and 3′ inner primer, 5′-CAG GAA ACA GCT ATG ACC GGG GCC TTG GTA GGT GGC AA-3′ (nt 7275–7294), with the M13 reverse primer sequence underlined]. An M13 forward primer and an M13 reverse primer were attached to the 5′ terminal of the 5′ and 3′ inner primers, respectively, to facilitate direct sequencing with an automated DNA sequencer (model 373S; Applied Biosystems Japan).

Both strands of the PCR products were sequenced with the PRISM dye termination kit (Applied Biosystems Japan), according to the manufacturer's instructions. The sequencing primer was the M13 forward primer for the sense strand and the M13 reverse primer for the antisense strand. Deduced aa sequences of *NS5A* 2209–2248 were compared with the *NS5A* 2209–2248 sequences of HCV-J [15], which are prototypic sequences of HCV-1b. The results of the sequencing analysis were confirmed as consistent for each sample by repeating the experiment twice with different PCR products, to rule out the possibility of selection and amplification of minor NS5A quasi species variants in the low-titer specimens.

Nucleotide sequencing of the core gene

Substitutions of amino acids 70 and 91 in HCV-core region were determined according to core sequences obtained as described previously [16, 17]. The pattern of glutamine/histidine (mutant) at aa 70 and methionine (mutant) at aa 91 was evaluated as the double-mutant (dM) type, while the other patterns were non-double-mutant (non dM) type. Two patterns of mutants and competitive were labeled as non-wild. Wild at aa 70 and wild at aa 91 were evaluated as double-wild-type (dW), while the other patterns were considered non-double-wild-type (non dW).

Study design and treatment regimens

Patients were treated with combination therapy with PEG-IFN (Peg-Intron; Schering-Plough Nordic Biotech, Stockholm, Sweden) 1.2–1.5 μg/kg subcutaneously and RBV (Rebetol; Schering-Plough Nordic Biotech) (body weight [b.w.] < 60 kg, 600 mg po daily; b.w. 60–80 kg, 800 mg po daily; b.w. > 80 kg, 1000 mg po daily; in two divided doses). The duration of the combination therapy was set at a standard 48 weeks. Treatment reduction was permitted, to escape side effects, but extended treatment of 72 weeks is not included in this analysis. Achieved rates of PEG-IFN and RBV administration were calculated as the percentage of the actual total dose administered of a standard total dose of 48 weeks according to body weight before therapy. During treatment, patients were assessed as outpatients at weeks 2, 4, 6, and 8, and then every 4 weeks for the duration of treatment and at every 4 weeks after the end of therapy. Biochemical and hematological testing was done by a central laboratory. Serum HCV RNA was measured before treatment, during treatment at 4-weekly intervals, and after therapy at 4-weekly intervals for 24 weeks, by a quantitative PCR assay with a sensitivity of 100 copies/ml (National Genetics Institute, Los Angeles, CA, USA).

Outcomes

The primary end point was a sustained biochemical and virological response. Sustained virological response (SVR) was defined as serum HCV RNA undetectable at 24 weeks after the end of treatment. Secondary end points were end-of-treatment virological responses (HCV RNA undetectable in serum). In addition, tolerability (adverse events) and drug adherence were recorded and factors potentially associated with virological response were explored.

Statistical analysis

SPSS software package (SPSS 12J for Windows; SPSS, Chicago, IL, USA) was used for statistical analysis, which was carried out using the χ^2 or Fisher's exact probability test. Distributions of continuous variables were analyzed by the Mann–Whitney *U*-test. Independent factors possibly affecting response to combination therapy were examined by stepwise multiple logistic-regression analysis. All *P* values were two-tailed and those lessthan 0.05 were considered statistically significant.

Results

Clinical characteristics and response to therapy

The clinical characteristics of the 239 patients are summarized in Table 1. On an intention-to-treat (ITT) analysis, serum HCV RNA levels were undetectable by the end of treatment in 172 of the 239 patients (72%) who were treated with PEG-IFN plus RBV, and among them, 98 of the 239 patients (41%) had an SVR (Table 2). The SVR rate decreased with drug discontinuation and dose

Table 1 Baseline characteristics of participating patients infected with HCV genotype 1b

Total number	239
Age (years)[a]	57 (21–78)
Gender (male/female)	142/97
Body mass index (kg/m^2)[a]	23.3 (15.3–31.0)
Previous interferon therapy (no/yes)	167/72
Histology at biopsy	
Grade of inflammation	
A0/1/2/3	3/65/102/10
Stage of fibrosis	
F0/1/2/3/4	4/73/57/37/9
Hemoglobin (g/dl)[b]	14.3 ± 1.3
ALT (IU/L)[b]	86 ± 67
Platelet count ($\times 10^3/\mu l$)[b]	160 ± 58
LDL cholesterol (mg/dl)[b]	74 ± 19
Serum HCV-RNA level (Log(IU/ml))[b, c]	6.1 ± 0.6
Type of mutations in the core (dM/non dM)	30/166
Type of mutations in the core (dW/non dW)	65/131
Type of ISDR sequence (0/1/2/3/4 or more)	126/45/11/5/18

HCV hepatitis C virus, *LDL* low density lipoprotein, *ALT* alanine transaminase, *ISDR* interferon sensitivity determining region in NS5A $_{2209-2248}$, *dM* double mutant: dual substitutions at amino acids 70 and 91, *non dM* non-double mutant: wild type or substitution at either amino acid 70 or 91, *dW* double wild: wild type at amino acids 70 and 91, *non dW* non-double wild: dual or substitution at either amino acid 70 or 91

[a] Median (range) values are shown

[b] Data are mean ± SD

[c] Data are shown as Log(IU/ml)

Table 2 Sustained response rates to treatment according to drug adherence

Characteristic	Number/total number (%)
Overall	
End of treatment	172/239 (72)
End of follow up	98/239 (41)
PEG-interferon-α2b adherence	
End of treatment	
>80%	131/154 (85)
60–80%	19/27 (70)
<60%	22/58 (38)
End of follow up	
>80%	86/154 (56)
60–80%	7/27 (26)
<60%	5/58 (9)
Ribavirin adherence	
End of treatment	
>80%	113/134 (84)
60–80%	37/46 (80)
<60%	22/59 (37)
End of follow up	
>80%	74/133 (56)
60–80%	18/47 (38)
<60%	6/59 (10)

PEG pegylated

reduction. The SVR rates of patients who received a total cumulative treatment dose of PEG-IFN of more than 80% were almost twice as high as the rates of patients who received less than 80% (56%, 26%, and 9% with >80%, 60%–80% and <60% of the PEG-IFN dose, $P < 0.001$). The SVR rates did not decrease with RBV reduction, as long as the cumulative treatment dose of RBV was more than 60%, but when the RBV reduction fell below 60%, the SVR rates were significantly lower (56%, 38%, and 10% with >80%, 60%–80%, and <60% of the RBV dose, $P < 0.001$).

Factors associated with sustained virological response

Seven parameters that influenced the SVR rate were identified by univariate analysis, including stage of fibrosis at liver biopsy, hemoglobin, platelet count, serum HCV RNA level, the type of ISDR sequence, and adherence to PEG-IFN plus RBV (Table 3). On the other hand, the SVR rate was not related to gender ($P = 0.07$), age or BMI. The amino acid substitution pattern was not significant in the overall analysis, but female patients with dual substitutions at amino acids 70 and 91 had a low tendency to achieve SVR. As shown in Table 4, gender differences existed in the mutations in ISDR and core regions based on therapeutic responses. Because there were rather fewer female than male patients, the type of ISDR sequence did not significantly influence the SVR in females. We also analyzed types of mutations in the core, and the amino acid substitution pattern was not significant in the male patients, but female patients with dual substitutions at amino acids 70 and 91 had a low tendency to achieve an SVR, as mentioned above. We also compared results between treatment-naïve patients and those who had failed previous IFN therapy (Table 5). As there were some differences in stage of fibrosis, platelet count, grade of inflammation, and gender in univariate analysis, treatment was comparably effective in both groups.

Finally we performed multivariate analysis in subjects with good drug adherence (Table 6), which identified only one parameter that influenced the SVR rate independently by variable selection: the number of mutations in the ISDR sequence (two or more: odds ratio [OR] = 5.181, $P < 0.05$). This regression model was always obtained regardless of the variable selection method used, including conditional parameter estimation, Wald statistic, and

Table 3 Clinical and virological characteristics of 239 patients treated with PEG-IFN plus RBV therapy, based on therapeutic response

	SVR ($n = 98$)	Non-SVR ($n = 141$)	P value
Age (years)[a]	56 (27–69)	58 (23–72)	NS
Gender (male/female)	65/33	77/64	0.070
Previous interferon therapy (no/yes)	68/30	99/42	NS
Grade of inflammation (A0–1/2–3)	31/50	37/62	NS
Stage of fibrosis (F0–2/3–4)	68/13	67/33	*0.009*
Body mass index (kg/m^2)[a]	23.3 (15.5-28.1)	23.3 (15.3-31.0)	NS
Pretreatment Hemoglobin (g/dl)[b]	14.6 ± 1.1	14.0 ± 1.4	*<0.001*
Pretreatment ALT (IU/ml)[b]	87 ± 68	86 ± 67	NS
Pretreatment platelet count ($\times 10^3/\mu l$)[b]	178 ± 63	148 ± 51	*<0.001*
Pretreatment LDL cholesterol (mg/dl)[b]	78 ± 21	72 ± 18	NS
Pretreatment serum HCV-RNA level (Log(IU/ml))[b, c]	5.9 ± 0.7	6.2 ± 0.4	*<0.001*
No. of mutations in the ISDR (0–1/2 or more)	66/23	105/11	*0.002*
Type of mutations in the core (dM/non dM)	9/76	21/90	NS
Type of mutations in the core (dW/non dW)	31/54	34/77	NS
PEG-interferon adherence (>80/60–80/<60%)	85/7/6	68/20/53	*<0.001*
Ribavirin adherence (>80/60–80/<60%)	72/19/7	60/28/53	*<0.001*

IFN interferon, *RBV* ribavirin, *SVR* sustained virological response, *NS* not significant, *ALT* alanine transaminase, *ISDR* interferon sensitivity determining region in NS5A $_{2209-2248}$, *core* substitution of amino acids 70 and 91, *dM* double mutant: dual substitutions at amino acids 70 and 91, *non dM* non-double mutant: wild type or substitution at either amino acid 70 or 91, *dW* double wild: wild type at amino acids 70 and 91, *non dW* non-double wild: dual or substitution at either amino acid 70 or 91

[a] Median (range) values are shown

[b] Data are mean ± SD

[c] Data are shown as Log(IU/ml)

Table 4 Mutations in the ISDR and core regions analyzed separately for gender based on therapeutic response

	SVR ($n = 98$)	Non-SVR ($n = 141$)	P value
No. of mutations in the ISDR (0–1/2 or more)			
Male	36/21	56/8	*0.002*
Female	30/2	49/3	NS
Type of mutations in the core (dM/non dM)			
Male	8/46	11/48	NS
Female	1/30	10/42	*0.026*
Type of mutations in the core (dW/non dW)			
Male	18/36	16/43	NS
Female	13/18	18/34	NS

likelihood ratio statistic in combination with forward or backward variable selection methods.

Comparison of SVR rates according to the number of mutations in the ISDR sequence

We analyzed first the percentage of patients with more than two mutations in the ISDR among 762 patients who received IFN therapy between December 2000 and April 2008 at Tokyo Medical and Dental University Hospital and associated hospitals. The percentage of patients with more than two mutations in the ISDR was between about 20% and 30% for all ages (Fig. 1a).

Secondly, we analyzed responses to PEG-IFN plus RBV treatment and serum levels of HCV RNA in relation to the number of mutations in the ISDR. In Fig. 1b, patients with SVR are indicated by open circles and those with non-SVR, by closed circles. Although the rate of SVR tended to be higher in patients with increasing numbers of mutations in the ISDR, 5 patients with more than two mutations in the ISDR who experienced drug discontinuation and dose reduction resulted in non-SVR.

We confirmed changes over time in VR rates in patients treated with PEG-IFN plus RBV (Fig. 1c). Patients with more than two mutations in the ISDR are indicated in the figure by open circles and those with none or one mutation in the ISDR, by closed circles. The VR rates tended to be high early in the treatment in patients with more than two mutations in the ISDR.

Finally we compared the PEG-IFN plus RBV treatment efficacy in two groups, divided based on ISDR mutations. Patients with more than two mutations in the ISDR had a significantly higher tendency to achieve SVR in both ITT and per-protocol (PP) analyses ($P < 0.01$) (Fig. 1d), and

Table 5 Clinical and virological characteristics of 239 patients treated with PEG-IFN plus RBV therapy, based on previous interferon therapy

Previous interferon therapy	No ($n = 167$)	Yes ($n = 72$)	P value
Sustained response rates	68/167 (41)	30/72 (42)	NS
Age (<65/≥65)	127/40	57/15	NS
Gender (male/female)	93/74	49/23	0.074
Grade of inflammation (A0–1/2–3)	55/72	13/40	*0.018*
Stage of fibrosis (F0–2/3–4)	103/24	32/21	*0.003*
Pretreatment hemoglobin (<14.5/≥14.5)	93/74	41/31	NS
Pretreatment platelet count (<160/≥160 $\times 10^3$)	84/83	50/22	*0.006*
Pretreatment Serum HCV RNA level[a] (<6/≥6)	54/112	25/46	NS
No. of mutations in the ISDR (0–1/2 or more)	116/22	55/12	NS
PEG-interferon adherence (>80/60–80/<60%)	110/18/39	43/9/20	NS
Ribavirin adherence (>80/60–80/<60%)	97/30/40	35/17/20	NS

[a] Data are shown as Log(IU/ml)

Table 6 Multivariate analysis for the clinical and virological factors related to sustained response to PEG-IFN plus RBV therapy in 104 patients who were not intolerant to PEG-IFN plus RBV therapy

Factor	Category	Odds ratio (95% CI)	P value
(a) Five-factor model			
Number of mutations in the ISDR	0 or 1	1	
	2 or more	4.486 (0.922–21.74)	0.063
Pretreatment Hemoglobin (g/dl)		1.250 (0.853–1.833)	NS
Pretreatment Serum HCV RNA level[a]		0.510 (0.224–1.159)	NS
Stage of fibrosis	F 0/1/2	1	
	F 3/4	0.460 (0.153–1.382)	NS
Pretreatment Platelet count ($\times 10^3/\mu l$)		1.022 (0.949–1.101)	
(b) Step-wise variable selection			
Number of mutations in the ISDR	0 or 1	1	
	2 or more	5.181 (1.129–23.81)	*0.034*

CI confidence interval, *ALT* alanine transaminase, *ISDR* interferon sensitivity determining region in NS5A $_{2209-2248}$

[a] Data are shown as Log(IU/ml)

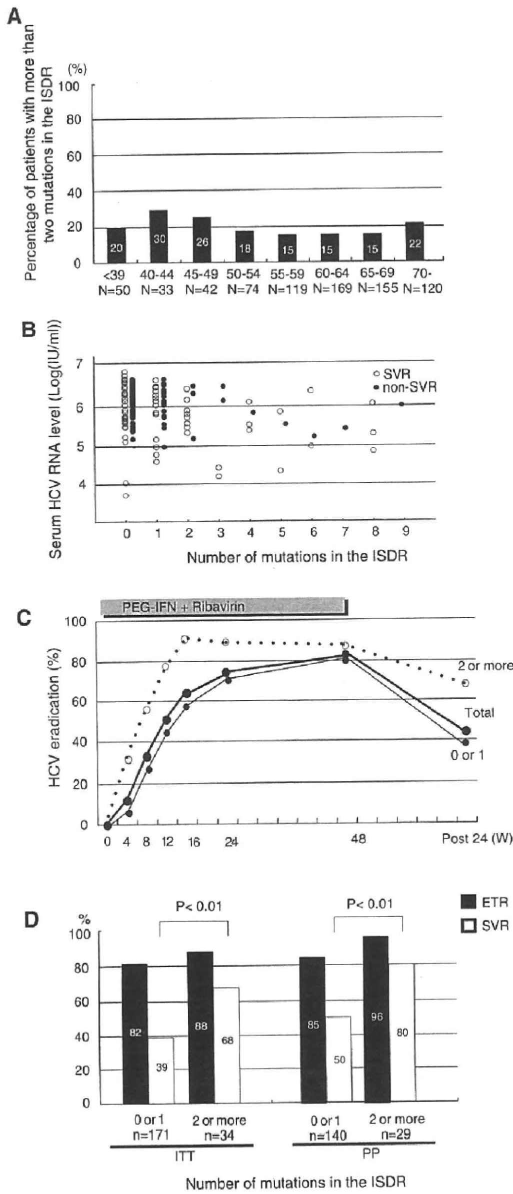
the SVR rates of the patients with good drug adherence was 80%.

Side effects

Side effects leading to treatment discontinuation occurred in 53 patients (22%). Overall, 109 patients (46%) required reduction of the dose of one or both drugs during the treatment regimens (23% required PEG-IFN reduction and 35% required RBV reduction). The most common events leading to drug withdrawal were general fatigue and appetite loss ($n = 15$), hematologic abnormalities ($n = 6$), dermatological symptoms ($n = 5$), retinopathy ($n = 5$), neuro-psychiatric events ($n = 4$), and interstitial pneumonia, including severe cough ($n = 4$).

Discussion

Although the relationship between ISDR mutations and the clinical efficacy of IFN has been conflicting in Western countries [18–24], our results support previous studies reporting a close correlation between the number of mutations in the ISDR and IFN efficacy in patients with chronic HCV-1b infection [11–13]. Because most patients with 4 or more mutations in the ISDR (hereafter classified as the mutant type) experienced SVR with conventional IFN monotherapy, we reported previously that the number of amino acid substitutions in the ISDR was an independent predictor of the response to IFN therapy [12]. In the present study, we demonstrate that ISDR mutations are the most effective predictors of treatment outcome of 48-week



◀ **Fig. 1** **a** The percentages of patients with more than two mutations in the interferon sensitivity determining region in NS5A $_{2209\text{–}2248}$ (*ISDR*), according to age (*horizontal axis*) among 762 patients who received interferon (IFN) therapy between December 2000 and April 2008 at Tokyo Medical and Dental University Hospital and associated hospitals. **b** Responses to pegylated (PEG)-IFN plus ribavirin (RBV) treatment and serum levels of hepatitis C virus (HCV) RNA in relation to the number of mutations in the ISDR. Patients with sustained virological response (*SVR*) are indicated by *open circles* and those with non-SVR by *closed circles*. **c** Changes over time in VR rates in patients treated with PEG-IFN plus RBV. Patients with more than two mutations in the ISDR are indicated by *open circles* and those with no or one mutation in the ISDR by *closed circles*, *W* weeks. **d** PEG-IFN plus RBV treatment efficacy divided into two groups based on ISDR mutations. End-of-treatment response (*ETR*) and SVR are shown in both intention-to-treat (ITT) analysis (*left*) and per-protocol (PP) analysis (*right*)

PEG-IFN plus RBV therapy in patients with HCV genotype 1binfection.

In the present study, the SVR rate was not related to gender, age, or previous IFN therapy by univariate analysis. First of all, in regard to gender ($P = 0.07$), as male patients had a higher tendency to achieve SVR than female patients, further validation in larger-scale studies is required to clarify the significance of gender. Secondly, in regard to age, there was no relation to SVR in overall analysis with continuous variables, but younger patients, aged less than 65 years, had a higher rate of response than those aged more than 65 years ($P < 0.05$, data not shown). Actually there are some reports suggesting the relationship of age and SVR [25, 26]. Finally, in regard to previous IFN therapy, as shown in Table 5, treatment was comparably effective in both groups; previous IFN therapy did not affect the SVR rate. The reasons for equivalent response rates in subjects with prior IFN history, which was not expected, are unclear. In our study, the group with prior IFN history had more advanced liver fibrosis and a low platelet count, and stage of fibrosis was one of the factors extracted by univariate analysis as a useful pretreatment marker predicting SVR. We also analyzed the other three parameters extracted by univariate analysis. Although there was no difference in pretreatment hemoglobin, or number of ISDR mutations, the group with prior IFN history tended to have a low serum HCV-RNA level. Further, the group with prior IFN history had a high proportion of male patients. Although the SVR rate was not related to gender, male subjects had a higher tendency to achieve SVR than female subjects.

In our present study, the SVR rate was not related to core mutations. As described in previous reports [17, 27, 28], amino acid substitutions in the core region are regarded as predictors of response to PEG-IFN plus RBV therapy in Japanese patients infected with HCV genotype 1b. In the present study, the SVR rate was not related to the pattern of amino acid substitution in the overall analysis. The reasons for these discrepant results are unclear, but females with dual substitutions at amino acids 70 and 91 had a lower tendency to achieve SVR. Further studies are necessary to clarify the mechanism of action for amino acid substitutions in the core region of HCV.

Recent studies suggest that the mutations in the ISDR are associated with response to combination therapy with IFN and RBV [29–32]. Most recently, it has been reported

that amino acid substitutions in the core and mutations in the ISDR are predictive of virological response to the combination therapy in patients with HCV genotype 1b and a high viral load [28]. There are some reports suggesting that the mutations in the ISDR may not serve as a predictor for treatment outcome [33, 34], but as the numbers of subjects in these studies were around 30, a number which is not sufficient to evaluate the results, this factor may explain these discrepant results.

The mechanisms of IFN sensitivity in relation to the sequence of the HCV $NS5A_{2209-2248}$ region are not clear. However the "mutant-type" ISDR correlates with a low viral load, as reported previously [12, 35, 36]; most patients in the present study with two or more mutations in the ISDR had high levels of virus. Furthermore, stepwise multiple logistic regression analysis of the factors, including substitution of the ISDR and the viral load, revealed that both of them were independent predictive variables of SVR, and the odds ratio of the number of mutations in the ISDR was the highest in the pretreatment factors associated with SVR by multivariate analysis. The precise mechanism involved must be elucidated in further in vitro studies.

There have been several reports that suggest biological roles of the ISDR in the response to IFN and in HCV infection. Double-stranded RNA-dependent protein kinase (PKR) is a critical component of the cellular antiviral responses induced by IFN. Gale et al. [37, 38] have reported that mutations within the PKR-binding region of NS5A, including ISDR, can disrupt the NS5A–PKR interaction, possibly rendering HCV sensitive to the antiviral effects of IFN. Toll-like receptor (TLR) has also been reported to play various roles in many viral infections, and it has been reported that NS5A bound MyD88, a major adaptor molecule of TLR-mediated signaling, and inhibited the TLR–MyD88 signaling pathway by a direct interaction with the death domain of MyD88 through the ISDR [39]. Furthermore, it has been reported that the lipid droplet is an important organelle for HCV production, and NS5A is a key protein that recruits replication complexes to lipid droplets for the production of infectious viral particles [40]. While the mechanism of action of the ISDR in the response to IFN or viral replication remains to be proven, these findings suggest new aspects of HCV infections.

In our previous report [12], patients with 4 or more mutations in the ISDR experienced SVR with conventional IFN monotherapy, but in more effective therapy with PEG-IFN plus RBV combination therapy, the number of mutations as a predictor of SVR decreased from 4 to 2. Watanabe et al. [41] have also reported that the number and position of mutations in the ISDR correlated with IFN efficacy in HCV-1b infection. Moreover, it has been reported that patients with viruses mutated at positions 2209, 2216, or 2227 more frequently experienced SVR than did those without these mutations. Another group has also reported regarding statistical analysis, using a database of 675 individual ISDR sequences in HCV-NS5A and the IFN response [42]. They have shown that IFN-sensitive viruses contain a larger and more diverse collection of substitutions than IFN-resistant viruses. While it remains unknown how the numbers of mutations are involved in the biological role of ISDR, or which sites of mutation and changes of amino acid are also important for the response to IFN-based treatment, it is thought that the functional importance of numbers or sites of mutations can be explained in terms of interaction between NS5A and some target molecules such as PKR, MyD88, and lipid droplets.

In vitro studies have shown that the introduction of NS5A mutations enables an HCV replicon to replicate efficiently [10, 43, 44]. In our previous report, site-specific mutation of the ISDR also modulated HCV replication [45]. The ISDR was identified originally as the site that determines the sensitivity of HCV to IFN [12]. This indicates that the ISDR mutations are not lethal in vivo. Furthermore, mutations in the ISDR are closely associated clinically with decreased serum HCV RNA levels [42], whereas ISDR mutations in the HCV replicon enhance replication. While the explanation for this paradox has not become clear, a big difference between the environment of cultured cells and that in the human liver is thought contribute to this phenomenon.

We found that the percentage of patients with more than two mutations in the ISDR was between 20% and 30% for all ages; thus, around one-fifth of patients are thought likely to experience SVR. Indeed, the SVR rate among patients with two or more mutations in the ISDR sequence was 68% (ITT) and 80% (PP) compared to 39% (ITT) and 50% (PP) among those patients with no or one mutation in the present study. Furthermore, predictive factors such as serum HCV RNA level, stage of fibrosis, and hemoglobin also aid in the assessments of treatment, and we can use these parameters to develop a treatment strategy.

Several prospective randomized trials have shown that 72-week extended therapy improves SVR by 7.5%–12% in late viral responders [46, 47]. One cohort study showed that 72-week treatment for late viral responders achieved an even higher SVR, of 67.1%, which was 21% higher than the SVR achieved with 48-week treatment [48]. These reports demonstrate that tailoring of treatment duration by on-treatment viral response can further improve the outcomes of antiviral therapy. In our 48-week based treatment, 90% of patients with more than 2 ISDR mutations cleared the virus within 12 weeks of treatment (early viral response; EVR) and consequently achieved 30% higher SVR than those with 1 or no ISDR mutation. These results

suggest that ISDR mutations will remain a significant predictor of good response to IFN therapies, including 72-week extension.

In conclusion, ISDR mutations are the most effective predictors of treatment outcomes in multivariate analysis. The number of mutations in the ISDR sequence of HCV-1b (≥2) is the most effective parameter which will facilitate further the selection of patients with a high likelihood of response to PEG-IFN plus RBV treatment.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Nonalcoholic fatty liver disease in Japanese junior high school students: its prevalence and relationship to lifestyle habits

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Abstract

Background Despite the increase in nonalcoholic fatty liver disease (NAFLD) in Japanese adults, its prevalence in adolescents remains unclear. This prompted us to evaluate the incidence and clinical characteristics of NAFLD among junior high school students.

Methods A population-based cross-sectional study was conducted among students in a single junior high school in Nagano prefecture. Serum alanine aminotransferase (ALT) and γ-glutamyltransferase (γGT) measurements and abdominal ultrasonography were performed in 249 and 288 students in 2004 and 2007, respectively. In the latter survey, student lifestyle habits were also assessed, using questionnaires.

Results The prevalence of NAFLD was 4.4% and 4.5% in 2004 and 2007, respectively, which was lower than that of obesity (10.0% and 5.9%). Body mass index and ALT and γGT levels increased significantly with hepatic steatosis severity. Multivariate logistic regression analysis demonstrated that the presence of obesity and an ALT level of 30 U/L or more were independent predictors of NAFLD (odds ratio 16.9, $P < 0.001$ and odds ratio 16.6, $P = 0.001$, respectively). The ratios of students commuting to and from school by car and not doing sports outside of school were higher in NAFLD students compared with non-NAFLD ones. Such tendencies were observed independently of the presence of obesity. Additionally, one obese student with severe steatosis and liver dysfunction was diagnosed as having nonalcoholic steatohepatitis (NASH).

Conclusions Approximately 4% of junior high school students had NAFLD that was primarily associated with obesity and reduced daily physical activity. Serum ALT measurement during school check-ups is recommended for the early detection of young adolescent NAFLD/NASH.

Keywords Obesity · ALT · Physical activity · Skipping breakfast · Nonalcoholic steatohepatitis

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Introduction

Due to increasing sedentary lifestyles and the rising prevalence of obesity, nonalcoholic fatty liver disease (NAFLD) has become a common cause of chronic liver disease. NAFLD encompasses a spectrum of histological findings that range from macrovesicular steatosis alone (simple

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幹細胞維持にかかわるシグナル伝達 —幹細胞の機能とWntシグナル—

柿沼 晴*,** 渡辺 守*

はじめに

幹細胞とは，多種類の細胞系譜への分化が可能である「多分化能」と，その多分化能を保持しつつ，自己複製して増殖する能力である「自己複製能」の2つを大きな特徴として定義される細胞である．近年，幹細胞の形質について，多くのことが明らかになってきた．幹細胞維持にかかわる分子生物学的なメカニズムは，神経系細胞や造血系細胞で解明が先行し，ついで上皮系細胞でも研究が進んできている．さらに，2007年に報告された，京都大学・山中教授ら[1]の研究グループによるヒト人工多能性幹細胞（induced pluripotent stem cell：iPS細胞）の樹立成功によって，自己の体細胞から誘導された幹細胞を用いて，さまざまな細胞治療が実現しうる可能性も拓けてきた．

本稿ではまず，幹細胞の性質と組織幹細胞システムについて概説する．その幹細胞の機能を維持するためのシグナルとしては，数多くのシグナルについて報告がありすべてを網羅することはむずかしいが，ここではWnt（ウィント）シグナルを例にとって，このシグナルがどのように幹細胞の機能に影響を与えているのか，解説していきたい．

KEY WORDS
◎ 自己複製能
◎ がん幹細胞
◎ β-catenin
◎ PCP経路
◎ カルシウム経路

幹細胞の自己複製と終末分化

生物の臓器は，非常に多数，かつ多種類の細胞が臓器固有の構築をすることによって構成されている．立体的な高次構造形成のために，きわめて精緻な細胞間ネットワークが形成されており，そこに合目的的なシグナル伝達が絶えず交わされることによって，生体の恒常性（ホメオスタシス）が維持されている．そして幹細胞とは，前述のように多分化能と自己複製能を特徴とする細胞であり，臓器形成とその維持の頂点に立ちつつ，何らかの異常時には再生に対しても主たる役割をも果たす細胞でもある．まずは造血幹細胞を一例とし，その自己複製と終末分化のシステムをみることで幹細胞について解説したい．

血液系は10種類以上の多様な形態と機能を有する，赤血球・血小板・白血球（好中球・リンパ球など）の多種類の成熟血液細胞から構成される．これらの成熟細胞には寿命があり，つねに新しい細胞が供給されつづけなければならない．とくに，血小板や好中球の体内での寿命は数日と短く，絶えず新しい血球を大量に産生する必要がある．造血幹細胞はこれらの成熟細胞を産出するシステ

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