Average patient age has been increasing in Japan [4], and such older patients have a lower virological response with PEG-IFN/RBV treatment compared to younger patients [5–7]. Anemia and other adverse events also occur more frequently in older patients [8, 9].

Recently telaprevir (TVR) has been approved for clinical use in several countries. Patients with high viral load of genotype 1 are treated with a three-drug combination therapy of TVR and PEG-IFN/RBV for 24 weeks. Since TVR is a selective inhibitor of HCV NS3/4A protease activity, marked improvement in sustained virological response (SVR) rates are expected [10–14]. Furusyo et al. [15] examined the efficacy of triple therapy among older Japanese patients, but the effects of TVR dose reduction on treatment response and adverse events for older patients are unknown.

Recent genome-wide association studies have shown that common single nucleotide polymorphisms (SNPs) rs8099917 and rs12979860 near the *interleukin (IL)-28B* gene on chromosome 19 are strongly associated with outcomes of both PEG-IFN plus RBV [16–19] dual therapy and TVR, PEG-IFN and RBV triple therapy [20, 21]. More recently, a polymorphism (ss469415590) within the gene that encodes a novel interferon-lambda 4 (IFNL4) protein has been found to be more strongly associated with HCV clearance and outcome of PEG-IFN plus RBV combination treatment compared to rs12979860 [22]. This IFNL4 protein can be produced by individuals who carry the ΔG allele of the ss469415590 variant (IFNL4-ΔG) but not by individuals who are homozygous for the IFNL4-TT allele because of a frameshift in exon 1 caused by the insertion variant [22]. The rs12979860 variant is located within intron 1 of IFNL4. Linkage disequilibrium is strong between the IFNL4-ΔG allele and the unfavorable rs12979860-T allele in individuals of European or Asian ancestry, whereas this linkage disequilibrium is moderate in individuals of African ancestry. Compared to rs12979860, ss469415590 is more strongly associated with HCV clearance in individuals of African ancestry, although it provides comparable performance in Europeans and Asians.

In the present study, we assessed the efficacy of the IFNL4 polymorphism and the effect of TVR dose reduction on treatment response and adverse events in TVR, PEG-IFN plus RBV triple therapy in older Japanese patients.

Methods

Patients

A total of 313 patients with chronic genotype 1 HCV infection who were treated with TVR, PEG-IFNα2b, and RBV triple therapy between November 2011 and July 2013 at Hiroshima University Hospital and hospitals belonging to the Hiroshima Liver Study Group were enrolled. Inclusion criteria for the therapy included remaining positive for genotype 1 HCV RNA for 6 months and having an HCV RNA level ≥5.0 log IU/mL, as determined by the COBAS TaqMan HCV test (Roche Diagnostics KK). Patients with cirrhosis were excluded.

Patients were classified according to response to prior interferon therapy. Non-responders never became HCV RNA-negative during prior therapy, whereas relapsers became HCV RNA-negative before the end of treatment but reverted to being HCV RNA-positive after treatment was discontinued.

HCV RNA levels

HCV RNA levels were measured using the TaqMan reverse transcription polymerase chain reaction (PCR) test. The linear dynamic range was 1.2–7.8 log IU/mL. Samples that exceeded the measurement range were diluted with phosphate-buffered saline and reanalyzed. Amino acid substitutions at position 70 in the HCV core protein (core70) were determined using direct sequencing of PCR products after extraction and reverse transcription of HCV RNA as in Akuta et al. [23, 24].

Single-nucleotide polymorphism (SNP) genotyping

Each patient was genotyped for rs8099917 in the IL28B locus, ss469415590 in the IFNL4 locus, and rs1127354, an inosine triphosphate pyrophosphate (ITPA) SNP reported to be associated with ribavirin-induced anemia [25–27]. Samples were genotyped using the Illumina HumanHap610-Quad Genotyping BeadChip or with the Invader or TaqMan assays, as described previously [28, 29].

Therapeutic protocol

In this study, 750 mg of TVR (Telavic; Mitsubishi Tanabe Pharma, Osaka, Japan) were administered 3 times a day at 8-h intervals after meals (2,250 mg/day) in 174 patients and twice per day (1,500 mg/day) in 139 patients. The TVR dose was determined by each physician according to age, sex, body weight, and hemoglobin level. PEG-IFNα2b (PEG-Intron, MSD, Tokyo, Japan) was injected subcutaneously at a median dose of 1.5 μg/kg once per week (50–150 μg/week). 200–600 mg of RBV (Rebetol, MSD) was administered after breakfast and dinner. The RBV dose was adjusted by body weight (600 mg for <60 kg; 800 mg for 60–80 kg; and 1,000 mg for >80 kg). Triple therapy with PEG-IFNα2b, RBV, and TVR was continued for 12 weeks and then switched to PEG-IFNα2b and RBV dual therapy for an additional 12 weeks. Completion of the treatment was defined as completion of both the 12 weeks of TVR and the 24 weeks of PEG-IFN/RBV treatment.

Efficacy of the treatment

Sustained virological response (SVR) was defined as undetectable serum HCV RNA at 24 weeks after the end of treatment. Rapid virological response (RVR) was defined as undetectable HCV RNA at week 4 of treatment.

Statistical analysis

Continuous variables are presented as median and range and were analyzed using the Mann–Whitney *U*-test. Categorical variables were compared using the Chi square or Fisher exact test, as appropriate. Multivariate analysis was conducted with a Cox proportional hazard model using the stepwise selection of variables or two logistic analyses. All statistical analyses were performed using the SPSS software package (version 12.0 for Windows, SPSS Inc., Chicago, IL, USA), with $P < 0.05$ denoting statistical significance.

Results

Baseline patient characteristics

Patients are grouped according to age (younger: ≤65 years, $n = 226$; older: >65 years, $n = 87$) in Table 1. As IFNL4 SNP ss469415590 is in strong linkage disequilibrium with IL28B SNPs rs8099917 and rs12979860 [22], only 4 out of 313 patients (1.3 %) showed discrepant haplotypes in this study. In older patients, hemoglobin levels were lower and serum creatinine levels were higher than in younger patients. More older patients received a reduced initial dose of TVR (1,500 mg) compared to younger patients. The older group included 17 treatment-naïve patients, 40 prior relapsers, and 30 previous non-responders. The younger group included 96 treatment-naïve patients, 94 prior relapsers, and 63 previous non-responders ($P = 0.138$).

The initial average dosage of TVR based on weight was similar between older and younger patients (32.6 ± 0.97 vs. 32.0 ± 0.54 mg/kg); however, the initial PEG-IFNα2b dosage was significantly larger, and the RBV dosage was significantly smaller in older patients compared to younger patients (1.49 ± 0.02 vs. 1.46 ± 0.01 μg/kg, $P = 0.04$ and 10.1 ± 0.3 vs. 11.0 ± 0.14 mg/kg, $P = 0.012$, respectively).

Sustained virological response by age

Outcome of therapy could be determined for 154 out of the 226 younger patients and 58 out of the 87 older patients. The SVR rate for older patients was slightly lower than that for younger patients (69 vs. 82 %, $P = 0.043$) (Fig. 1).

Table 1 Patient characteristics by age

Variables	≤65 years ($n = 226$)	>65 years ($n = 87$)	*P* value
Sex (male/female)	128/98	43/44	0.251
Age (years)	59 (20–65)	69 (66–79)	<0.001
Body weight (kg)	63 (37–100)	55 (36–81)	<0.001
Body mass index (kg/m^2)	23.3 (14.7–37.0)	22.3 (16.1–27.3)	0.022
Aspartate aminotransferase (IU/L)	38 (16–145)	36 (16–111)	0.110
Alanine aminotransferase (IU/L)	40 (13–286)	37 (10–174)	0.001
γ-Glutamyl-transpeptidase (IU/L)	32 (9–442)	29 (10–221)	0.190
Serum creatinine (mg/dL)	0.69 (0.44–1.3)	0.71 (0.44–1.8)	0.032
eGFR (mL/min/1.73 m^2)	78.0 (43.9–145.8)	72.6 (30.6–119)	0.016
Leukocyte count (/mm^3)	4,990 (2,400–11,830)	4,700 (3,100–7,804)	0.406
Hemoglobin (g/dL)	14.1(10.0-17.4)	13.7(10.4-17.6)	0.017
Platelet count (×R10^4/μL)	17.0(5.2-30.0)	14.0(6.7-22.1)	0.022
Previous treatment response			
Naïve/relapser/NR	96/94/63	17/40/30	0.138
Initial TVR dose (2,250/1,500 mg/day)	141/85	33/54	<0.001
Level of viremia (log IU/mL)	6.6 (5–7.8)	6.6 (5–7.4)	0.182
Core70 amino acid substitutions			
Wild/mutant/ND	74/51/101	34/25/28	0.840
IL28B genotype			
rs8099917 (TT/TG+GG/ND)	134/65/27	54/31/2	0.535
IFNL4 genotype			
ss469415590 (TT/TT/TT/ΔG+ΔGΔG/ND)	112/65/49	49/30/8	0.848
ITPA genotype			
rs1127354 (CC/CA+AA/ND)	147/52/27	62/23/2	0.871

Categorical data are represented as numbers of patients, and continuous data are represented as median and range.

TVR telaprevir, *NR* non-responder, *HCV* hepatitis C virus, *IL28B* interleukin 28B, *Core70* HCV core protein amino acid 70, *IFNL4* interferon lambda 4, *ITPA* inosine triphosphate pyrophosphatase, *ND* not determined

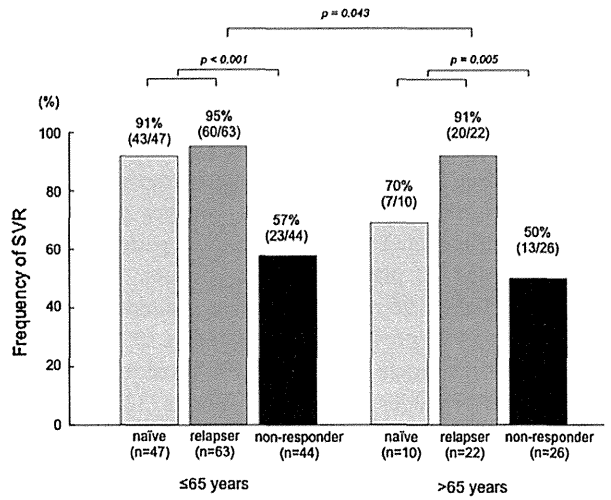

Fig. 1 SVR rates for triple therapy grouped by response to prior interferon treatment and age. *SVR* sustained virological response 24 weeks after the end of therapy. Patients were grouped by age (younger group, ≤65 years and older group, >65 years) and response to prior interferon treatment.

SVR was more likely to be achieved in treatment-naïve patients and prior relapsers than in prior non-responders in both younger (94 vs. 57 %, $P < 0.001$) and older patients (84 vs. 50 %, $P = 0.005$). Prior relapsers achieved higher SVR rates regardless of age.

Predictive factors associated with SVR in older and younger patients

In older patients, significant univariate predictors for SVR included clinical factors (platelet count, rs8099917 and ss469415590 genotype), response to prior treatment (naïve or relapse), and on-treatment factors (RVR) (Table 2). In the multivariate model, RVR (OR 36.601 for non-RVR, $P = 0.002$) and ss469415590 TT/TT genotype (OR 19.502 for TT/ΔG+ΔG/ΔG genotype, $P = 0.009$) were identified as significant independent predictors for SVR for older patients. In younger patients, multiple logistic regression analysis identified three independent predictive factors for SVR: platelet count

Table 2 Univariate and multivariate analyses of host and viral factors associated with SVR during triple therapy in older patients

	SVR ($n = 40$)	Non-SVR ($n = 18$)	Univariate analysis *P* value	Multivariate analysis	
				OR (95 % CI)	*P* value
Sex (male/female)	22/18	8/10	0.631		
Age (years)	68 (66–76)	68 (66–73)	0.760		
Body weight (kg)	58.4 (44–70)	58.1 (44–77)	0.204		
Body mass index (kg/m^2)	22.3 (19.2–26.1)	22.6 (19.1–27.3)	0.713		
Aspartate aminotransferase (IU/L)	27.0 (19–146)	40.5 (23–78)	0.158		
Alanine aminotransferase (IU/L)	26.0 (10–141)	38.5 (15–71)	0.444		
eGFR (mL/min/1.73 m^2)	72.6 (43–119)	70.8 (58–103)	0.989		
Leukocyte count (/mm^3)	4,620 (3,200–7,450)	4,200 (3,100–6,400)	0.453		
Hemoglobin (g/dL)	13.8 (10.4–15.4)	13.4 (11.4–15.9)	0.342		
Platelet count (× 10^4/μL)	14.5 (7.6–21.9)	11.2 (6.7–24.8)	0.016		
Platelet count (≥12/<12 × 10^4/μL)	35/5	10/8	0.007		
Previous treatment response (naïve or relapse/NR)	27/13	5/13	<0.001		
Initial TVR dose (2,250 mg/1,500 mg)	19/21	7/11	0.542		
Rapid viral response (with/without/ND)	32/3/5	5/9/4	<0.001	36.601 (3.562–376.148)	0.002
Completion/discontinuation of the treatment	30/10	9/9	0.076		
Core70 amino acid substitutions (wild/mutant/ND)	17/11/12	4/6/8	0.463		
IL28B genotype rs8099917 (TT/TG+GG/ND)	30/10	6/12	0.009		
IFNL4 genotype ss469415590 (TT/TT/TT/ΔG+ΔGΔG/ND)	27/9/4	6/12/0	0.012	19.502 (2.104–180.812)	0.009
ITPA genotype rs1127354 (CC/CA+AA)	28/12	16/2	0.219		

Categorical data are represented as numbers of patients, and continuous data are represented as median and range.

SVR sustained virological response, *NR* non-responder, *TVR* telaprevir, *HCV* hepatitis C virus, *Core70* HCV core protein amino acid 70

Table 3 Univariate and multivariate analyses of host and viral factors associated with SVR during triple therapy in younger patients

	SVR (n = 126)	Non-SVR (n = 28)	Univariate analysis P value	Multivariate analysis	
				OR (95 % CI)	P value
Sex (male/female)	70/56	15/13	0.913		
Age (years)	58 (24–65)	61 (27–65)	0.203		
Body weight (kg)	60.5 (37–100)	64.3 (48–81)	0.471		
Body mass index (kg/m^2)	23.3 (14.7–34.7)	22.9 (19.1–34.0)	0.230		
Aspartate aminotransferase (IU/L)	31 (18–133)	46 (21–145)	0.002		
Aspartate aminotransferase ($\leq$35/>35 $\times$ 10^4/μL)	64/62	6/22	0.005		
Alanine aminotransferase (IU/L)	36 (13–286)	41 (21–137)	0.078		
eGFR (mL/min/1.73 m^2)	81 (43–120)	76 (51–145)	0.516		
Leukocyte count (/mm^3)	5,250 (2,400–11,830)	4,900 (2,400–7,660)	0.161		
Hemoglobin (g/dL)	14.2 (10.3–17.4)	13.7 (10.8–16.8)	0.029		
Hemoglobin ($\geq$14/<14 $\times$ 10^4/μL)	73/53	10/18	0.033		
Platelet count ($\times$10^4/μL)	17.2 (5.2–40.4)	12.1 (5.4–24.4)	<0.001		
Platelet count ($\geq$12/<12 $\times$ 10^4/μL)	110/16	13/15	<0.001	24.841 (2.030–303.947)	0.012
Previous treatment response (naïve or relapser/NR)	103/23	7/21	<0.001		
Initial TVR dose (2,250 mg/1,500 mg)	81/45	19/9	0.682		
Rapid viral response (with/without/ND)	99/18/9	13/13/2	0.001		
Completion/discontinuation of the treatment	114/12	15/13	<0.001	47.776 (2.286–998.663)	0.013
Core70 amino acid substitutions (wild/mutant/ND)	46/23/57	2/15/11	<0.001		
IL28B genotype rs8099917 (TT/TG+GG/ND)	84/24/8	6/22/0	<0.001	71.692 (4.617–1,113.33)	0.002
IFNL4 genotype ss469415590 (TT/TT/TT/ΔG+ΔGΔG/ND)	74/26/26	4/23/1	<0.001		
ITPA genotype rs1127354 (CC/CA+AA/0)	80/28/8	20/8/0	0.860		

Categorical data are represented as numbers of patients, and continuous data are represented as median and range.

SVR sustained virological response, *NR* non-responder, *TVR* telaprevir, *HCV* hepatitis C virus, *Core70* HCV core protein amino acid 70

$\geq$12 $\times$ 10^4/μL (OR 24.841 for <12 $\times$ 10^4/μL, P = 0.012), completion of the treatment (OR 47.776 for discontinuation of the treatment, P = 0.013), and IL28B TT genotype (OR 71.693 for TG+GG genotypes, P = 0.002) (Table 3).

Effect of IFNL4 genotype and response to previous interferon treatment in older patients

We assessed the relationship between IFNL4 genotype and SVR in older patients. Patients with the ss469415590 TT/TT genotype were significantly more likely to achieve SVR compared to those with the TT/ΔG or ΔG/ΔG genotypes (82 vs. 43 %, P = *0.012*) (Table 2). According to the response to previous IFN treatment, the SVR rate for treatment-naïve patients and prior relapsers with the ss469415590 TT/TT genotype was significantly higher than for prior non-responders (92 vs. 56 %, P = 0.017) (Fig. 2a).

Relationship of IFNL4 to SVR and RVR in previously treated non-responders

Rapid virological response was achieved in 37 of 49 (76 %) older patients. SVR rates were significantly higher for RVR than in non-RVR in patients with both ss469415590 TT/TT (96 vs. 50 %, P = *0.003*) and TT/ΔG or ΔG/ΔG (64 vs. 0 %, P = 0.011) genotypes (Fig. 2b). No older patient with ss469415590 TT/ΔG or ΔG/ΔG who was non-RVR achieved SVR.

Adverse events

TVR and PEG-IFN/RBV combination treatment is associated with a high frequency of adverse events such as anemia and renal dysfunction [10–14]. Pre-treatment hemoglobin levels were significantly lower in older patients than younger patients, and serum creatinine levels

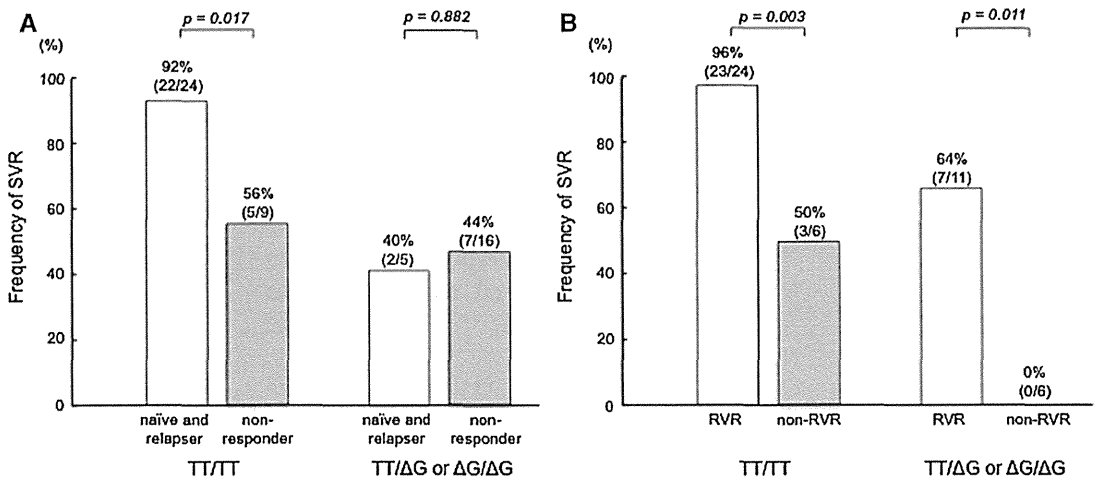

Fig. 2 Relationship between IFNL4 genotype and treatment response. **a** SVR rates for triple therapy grouped by ss469415590 genotype (TT/TT and TT/ΔG or ΔG/ΔG) and response to prior interferon treatment. **b** Relationship between rapid virological response (RVR) and SVR according to IFNL4 genotype. *RVR* undetectable HCV RNA at week 4 of therapy

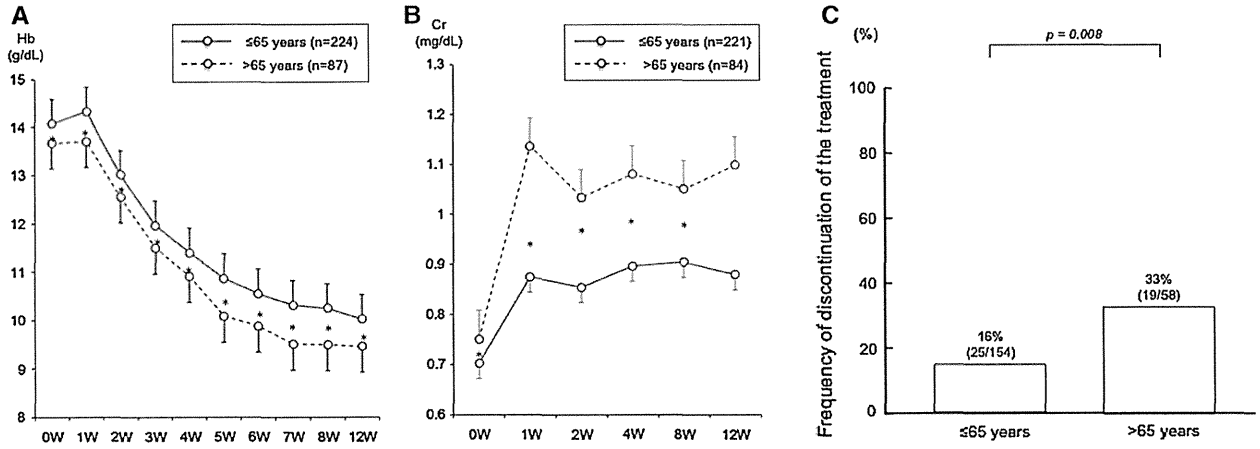

Fig. 3 Relationship between age and adverse events. Time courses of hemoglobin (**a**) and serum creatinine (**b**) levels grouped by age. $*P < 0.05$. **c** Frequency of discontinuation of the treatment by age

were significantly higher (Table 1). Similarly, treatment-related decreases in hemoglobin levels (Fig. 3a) and increases in serum creatinine levels (Fig. 3b) were higher in older patients compared to younger patients. These adverse events resulted in a significantly higher frequency of discontinuation of the treatment in older patients (33 vs. 16 %, $P = 0.008$) (Fig. 3c).

The total average dosages of TVR and PEG-IFNα2b during the treatment were based on body weight and tended to be larger in younger patients than older patients (2,286.5 ± 68.8 vs. 2,076.6 ± 99.0 mg/kg and 31.1 ± 1.1 vs. 28.0 ± 1.5 μg/kg, respectively); those of RBV were significantly larger in younger patients than older patients (1,068.0 ± 37.9 vs. 903.6 ± 88.8 mg/kg, $P = 0.004$). The difference of the total dosages of agents may be one of the contributing factors for the higher SVR rate in younger patients compared to older patients.

Effect of initial TVR dose on adverse events and treatment response in older patients

33 out of 87 (38 %) older patients were treated with an initial TVR dose of 2,250 mg, and the remaining 54 patients (62 %) were administered a reduced initial dose of 1,500 mg (Table 1). The average dosage of total TVR based on body weight was significantly larger in patients treated with an initial TVR dose of 2,250 mg than patients with an initial TVR dose of 1,500 mg. ($P = 0.006$)

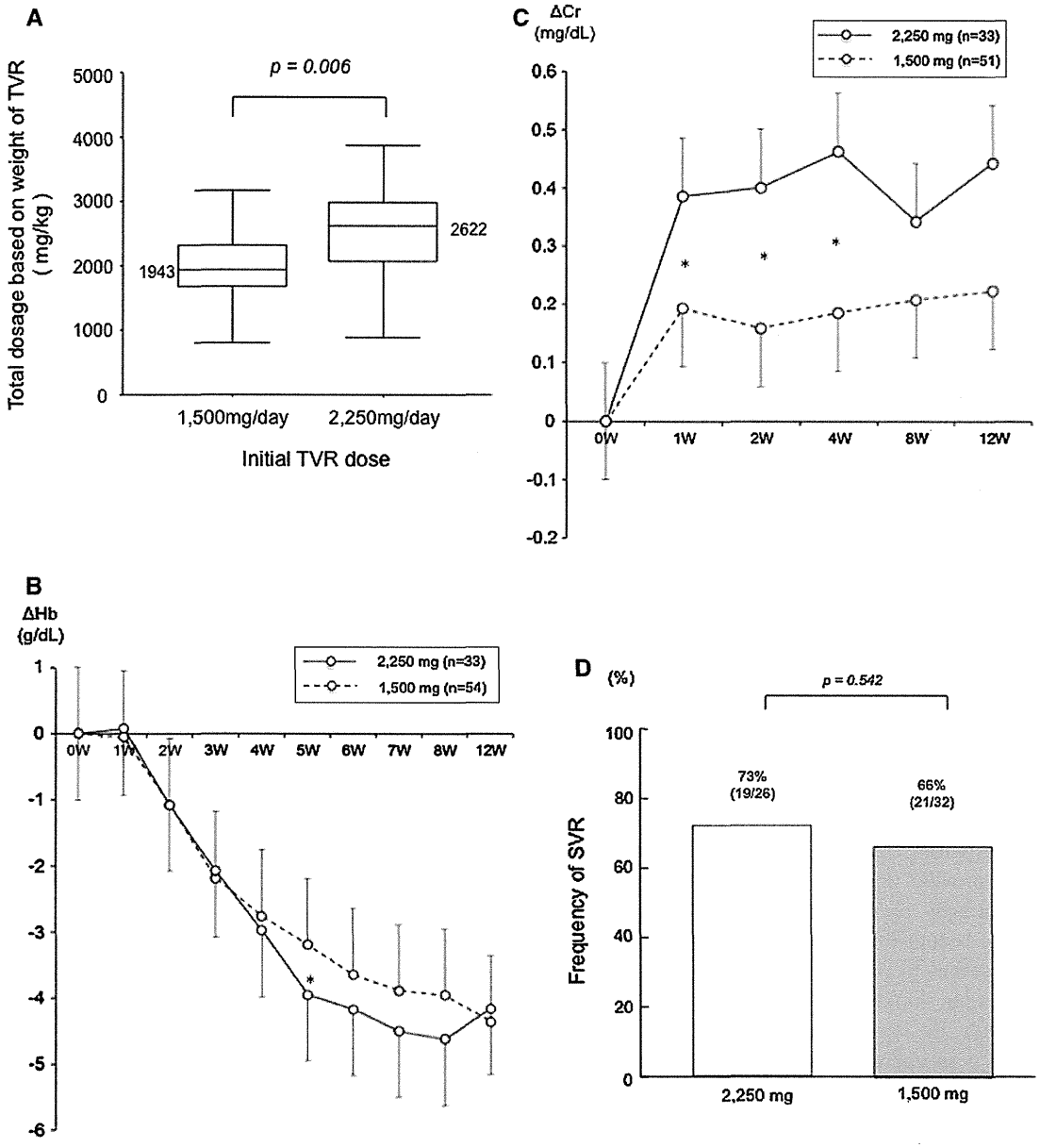

Fig. 4 Relationship between TVR dose and adverse events, and treatment response in older patients. **a** Differences in the average dosage of total TVR based on body weight between initial TVR doses. Medians are shown as *horizontal bars*. *Boxes* cover the interquartile range, and tails show the minimum and maximum values. Time courses of hemoglobin (B), serum creatinine (C) levels, and SVR rates (D) are grouped by initial TVR dose in older patients. $^*P < 0.05$

(Fig. 4a). Both the decrease of hemoglobin levels (Fig. 4b) and the increase of creatinine levels (Fig. 4c) in older patients treated with the 1,500 mg TVR dose were smaller than in patients who received the 2,250 mg dose. However, initial TVR dose was not associated with the rate of SVR (Fig. 4d). The oldest patient in this study, an 80-year-old male, was treated with the reduced initial dose of TVR. The patient was able to complete the 24-week treatment and achieved SVR.

Discussion

Elimination of HCV after IFN therapy significantly reduces the risk of hepatocellular carcinoma (HCC) and death in older patients [30]; however, the best way to treat older patients with genotype 1 HCV infection is controversial because of the lower viral response rate and higher frequency of adverse events in this group [7, 31, 32]. In fact, this study also showed that adverse events such as anemia

and renal dysfunction were more serious (Fig. 3a, b) and SVR rate was significantly lower (Fig. 1) in older patients than in younger patients.

In this study, SVR rates were higher in treatment-naïve patients and prior relapsers than prior non-responders in both younger and older patients (Fig. 1). This suggests that treatment-naïve patients and prior treatment relapsers are suitable candidates for triple therapy regardless of age.

SNP ss469415590 in the IFNL4 gene is strongly associated with HCV clearance through both the innate immune reaction and by PEG-IFN plus ribavirin therapy [22]. IFNL4 genotype is also an important predictor of the probability of eradicating the virus by triple therapy. While the SVR rate of older patients with IFNL4 genotype TT/TT was 82 %, the eradication rate was 56 %, even in older patients with non-response to prior therapy (Fig. 2A). In contrast, patients with IFNL4 TT/ΔG or ΔG/ΔG, especially among non-responders to prior therapy, had poor response. Therefore, older patients who have IFNL4 genotype TT/TT should be treated by triple therapy. Older patients with IFNL4 genotype TT/ΔG or ΔG/ΔG are expected to have poor response to the therapy.

IFNL4 SNP ss469415590 is in strong linkage disequilibrium with IL28B SNP rs8099917, especially in Asians [22]. In this study, only 4 out of 313 patients (1.3 %) showed discrepant haplotypes. Further analysis with many more patients is needed to determine whether rs8099917 or ss469415590 is more important for prediction of the treatment response. However, to a large extent the polymorphisms provide equivalent information, and patients who have already been genotyped for rs8099917 would probably not benefit from genotyping for ss469415590.

It is important to predict the effect of the therapy as early as possible in older patients.

In this study, the rapid change in HCV RNA levels early in treatment is an important predictor of treatment success. More than 85 % of patients who achieved RVR also achieved SVR. In contrast, patients who did not achieve RVR showed poor response to the therapy, especially in patients with IFNL4 TT/ΔG or ΔG/ΔG genotypes (Fig. 2b). Accordingly, termination of the therapy should be considered when older patients with IFNL4 genotypes associated with poor response fail to show RVR.

The dose of TVR for use in triple therapy was determined based on a dose-finding study conducted in the United States and Europe, which found that the 2,250 mg regimen achieved the greatest reduction of HCV RNA [33]. However, average body weights of Japanese patients are lower than American and European patients. It was reported that the anti-HCV effect of triple therapy was similar when patients were given TVR at 1,500 mg/day compared with those given at 2,250 mg/day in the Japanese patients [34]. In the present study, dose reduction of the initial TVR dose alleviated adverse events such anemia and renal dysfunction (Fig. 4b, c) without affecting the virological response rate in older patients (Fig. 4d), suggesting that reduction of TVR might be possible for older patients.

In conclusion, older patients who received triple therapy with TVR and PEG-IFN/RBV showed a lower viral response than younger patients. Analysis of IFNL4 polymorphism is a valuable predictor in older patients receiving telaprevir triple therapy. TVR dose reduction could alleviate adverse events without compromising the treatment response in older patients.

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Case Report

Two patients treated with pegylated interferon/ribavirin/telaprevir triple therapy for recurrent hepatitis C after living donor liver transplantation

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It is difficult to use protease inhibitors in patients with recurrent hepatitis C virus (HCV) infection after liver transplantation (LT) due to interaction with immunosuppressive drugs. We report our experience with two patients treated with telaprevir (TVR) combined with pegylated interferon/ribavirin (PEG IFN/RBV) for recurrent HCV genotype 1 infection after LT. The first was a 63-year-old man with HCV-related liver cirrhosis, who failed to respond to IFN-β plus RBV after LT. Treatment was switched to PEG IFN-α-2b plus RBV and TVR was started. The donor had TT genotype of interleukin (IL)-28 single nucleotide polymorphisms (SNP) (rs8099917). The recipient had TT genotype of IL-28 SNP (rs8099917). Completion of 12-week triple therapy was followed by PEG IFN-α-2b plus RBV for 36 weeks. Finally, he had sustained viral response. The second was a 70-year-old woman with HCV-related liver cirrhosis and hepatocellular carcinoma. She failed to respond to PEG IFN-α-2b plus RBV after LT, and was subsequently switched to PEG IFN-α-2b/RBV/TVR. Genotype analysis showed TG genotype of IL-28 SNP for the donor, and TT genotype of IL-28 SNP for the recipient. Serum HCV RNA titer decreased below the detection limit at 5 weeks. However, triple therapy was withdrawn at 11 weeks due to general fatigue, which resulted in HCV RNA rebound 4 weeks later. Both patients were treated with cyclosporin, starting with a small dose to avoid interactions with TVR. TVR is a potentially suitable agent for LT recipients who do not respond to PEG IFN-α-2b plus RBV after LT.

Key words: hepatitis C virus, liver transplantation, telaprevir

INTRODUCTION

THE HEPATITIS C virus (HCV) has infected 170 million people worldwide, which progresses in some patients to liver cirrhosis and/or hepatocellular carcinoma (HCC).[1] The current treatment for patients infected with HCV genotype 1 is the combination of pegylated interferon-α and ribavirin (PEG IFN/RBV) for 48 weeks.[2] However, this treatment produces sustained viral response (SVR) in only approximately 50% of patients with genotype 1 HCV infection. In 2011, the first direct-acting antiviral agent (DAA) for the treatment of HCV genotype 1, telaprevir (TVR), was approved and treatment with this agent improved SVR to approximately 70–80% of patients with genotype 1 HCV infection.[3,4]

Recurrence of HCV infection after liver transplantation (LT) is one of the major causes of morbidity and allograft loss after LT.[5,6] Because the outcome of post-LT therapy with the classic antiviral agents PEG IFN/RBV are at most moderate with respect to SVR, LT patients constitute one of the classic difficult-to-treat groups.[7–9] The newly introduced triple therapy of protease inhibitors (PEG IFN/RBV/TVR) offers promising perspectives

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for the management of LT patients, although TVR is not yet approved for use in LT patients.

Although there is urgent need for effective treatment of HCV recurrence after LT, significant concern has been expressed about the safety and efficacy of HCV protease inhibitors in this setting because of the side-effect profile and the potential for drug–drug interactions with immunosuppressive agents.[10] Both cyclosporin and tacrolimus are substrates of cytochrome P450 3A and P-glycoprotein. Thus, co-administration of TVR, a potent cytochrome P450 3A4 substrate and inhibitor with the potential to saturate or inhibit intestinal P-glycoprotein, substantially increases the blood levels of cyclosporin and tacrolimus.[11] Consequently, the blood concentration of tacrolimus increased 78-fold, and that of cyclosporin increased fourfold by interaction with TVR.[11] In their recent pilot study, Werner *et al.*[10] described the response to 12-week treatment with TVR plus tacrolimus, cyclosporin or sirolimus in nine patients. Pungpapong *et al.*[12] also reported the preliminary data of 35 patients treated with TVR plus cyclosporin and those of another group of 25 patients treated with boceprevir. Here, we report our preliminary data on protease inhibitors used in combination with PEG IFN/RBV for the treatment of recurrent HCV genotype 1 infection after LT.

CASE REPORT

Case 1

THIS PATIENT WAS a 63-year-old man with HCV-related liver cirrhosis. Living donor LT (LDLT) was performed after obtaining informed consent at May 2009. In August 2009, the patient was started on IFN-β (600 μg) plus RBV (200 mg) due to depression. Because serum HCV RNA titer never fell below the detection limit (1.2 log IU/mL) over the 48-month treatment period, tacrolimus was switched to cyclosporin. In April 2012, treatment was changed to PEG IFN-α-2b (100 μg) plus RBV (200 mg, due to anemia) and TVR (1500 mg) because of depression. At the start of triple therapy, the platelet count was 24.6×10^4/μL, alanine aminotransferase (ALT) was 45 IU/L, genotype was 1b and HCV RNA was 6.8 log IU/mL. Further analysis showed six amino acid (a.a.) substitutions in interferon sensitivity-determining region (ISDR), and mutant- and wild-type amino acids at a.a.70 and a.a.91 in the core region, respectively. The donor had TT genotype of IL-28 single nucleotide polymorphisms (SNP) (rs8099917) and TT/TT genotype of λ4 (ss469415590). The recipient had TT genotype of interleukin (IL)-28 SNP (rs8099917) and TT/TT genotype of λ4 (ss469415590) (Table 1, Fig. 1). Cyclosporin was started at 10 mg/day after triple

Table 1 Laboratory data of patient 1 at start of triple therapy after LT

CBC		LDH	219 IU/L	Tumor marker	
WBC	4630/μL	ALP	357 IU/L	AFP	4.8 ng/mL
RBC	4.01×10^6/μL	γ-GT	20 IU/L		
Hb	12.4 g/dL	TP	7.3 g/dL	HCV virus markers	
Ht	37.8%	Alb	4.0 g/dL	HCV RNA	6.8 KIU/mL
Plt	24.6×10^4/μL	TC	164 mg/dL	Genotype	1b
		TTT	12 U		
Blood coagulation test		ZTT	15 U		
PT	120%	BUN	24.6 mg/dl	a.a. substitution in ISDR	6
		Cr	1.07 mg/dl	a.a.70 in the core region	Mutant
Blood chemistry		CRP	0.10 mg/dl	a.a.91 in the core region	Wild
T-Bil	0.5 mg/dL	NH_3	32 μg/mL	*IL-28B* donor	TT genotype
AST	30 IU/L			*IL-28B* recipient	TT genotype
ALT	45 IU/L			ss469415590 donor	TT/TT genotype
FBS	98 mg/dL			ss469415590 recipient	TT/TT genotype
HbA1c	5.5%			AUC of telaprevir	103 μgh/mL

γ-GT, γ-glutamyltransferase; a.a. substitution in ISDR, amino acid substitutions in the interferon sensitivity-determining region; AFP, α-fetoprotein; Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under curve; BUN, blood urea nitrogen; CBC, complete blood count; Cr, creatinine; CRP, C-reactive protein; FBS, fasting blood sugar; Hb, hemoglobin; HbA1c, hemoglobin A1c; Ht, hematocrit; LDH, lactate dehydrogenase; LT, liver transplantation; RBC, red blood cells; Plt, platelets; PT, prothrombin time; T-Bil, total bilirubin; TC, total cholesterol; TP, total protein; TTT, thymol turbidity test; WBC, white blood cells; ZTT, zinc sulfate turbidity test.

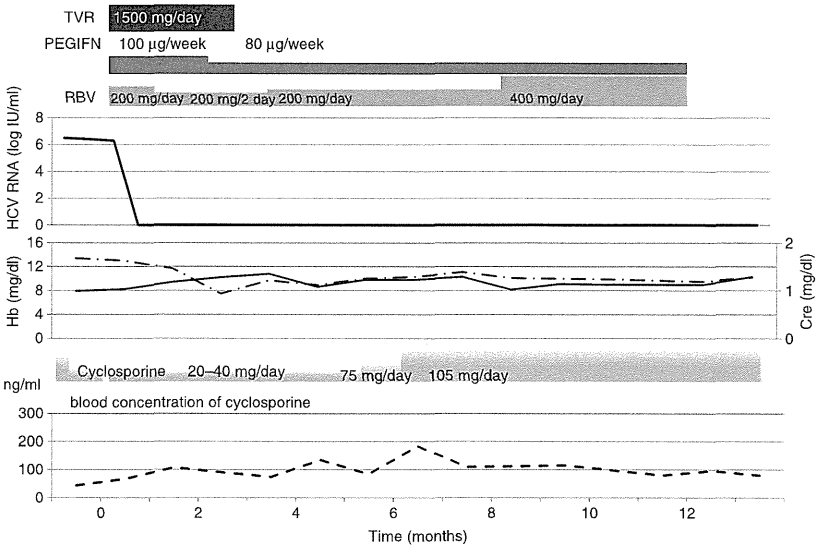

Figure 1 Clinical course of patient 1. Cre, creatinine; Hb, hemoglobin; HCV, hepatitis C virus; PEG IFN, pegylated interferon; RBV, ribavirin; TVR, telaprevir. —, Cre; – –, Hb.

therapy, but subsequently increased (based on measurement of its level in the peripheral blood during follow up) to 105 mg/day. The area under the curve (AUC) of TVR was 103 μgh/mL. Serum HCV RNA titer fell below the detection limit (1.2 log IU/mL) at 2 weeks after triple therapy. After 12-week triple therapy, PEG IFN-α-2b and RBV were continued for 36 weeks until April 2013. Finally, he achieved SVR.

Case 2

The patient was a 70-year-old woman with HCV-related liver cirrhosis and HCC. LDLT was performed in May 2006 after obtaining informed consent. Postoperatively, the patient was treated with PEG IFN-α-2b (80 μg) plus RBV (200 mg, due to anemia), which commenced in August 2006. Because serum HCV RNA titer never decreased below the detection limit (1.2 log IU/mL) in the subsequent 48 months, tacrolimus was changed to cyclosporin, and PEG IFN-α-2b plus RBV was changed to the combination of PEG IFN-α-2b (100 μg), RBV (200 mg, due to anemia) and TVR (1500 mg). At the start of triple therapy, platelet count was $19.8 \times 10^4/\mu L$, ALT was 15 IU/L, genotype was 1b, and HCV RNA was 6.2 log IU/mL. Further analysis showed no a.a. substitutions in the ISDR, but mutant- and wild-type a.a. at a.a.70 and a.a.91 in the core region, respectively were detected. The donor had TG genotype of IL-28 SNP (rs8099917) and TT/ΔG genotype of λ4 (ss469415590), while the recipient had TT genotype of IL-28 SNP (rs8099917) and TT/TT genotype of λ4 (ss469415590) (Table 2, Fig. 2). Cyclosporin was started at 10 mg/day, and based on measurement of its concentration in peripheral blood, the dose was increased gradually to 40 mg/day. Subsequent analysis showed a rise in serum creatinine and uric acid, but parameters improved following transfusion. Skin rashes of grade 2 appeared during the triple therapy, which was successfully treated with steroid cream. On the other hand, serum HCV RNA titer decreased below the detection limit (1.2 log IU/mL) at 5 weeks. However, triple therapy was stopped at 11 weeks due to general fatigue. HCV RNA rebounded 4 weeks later.

DISCUSSION

THE SVR RATE has improved since the introduction of PEG IFN/RBV for patients who undergo LT for HCV-related end-stage liver disease. The current estimated SVR rate for LT patients with history of HCV genotype 1 infection is 30–50%.[13–18] These results are much better than those reported in the 1990s and early 2000s, however, more than half of recipients still suffer from recurrent chronic hepatitis C.

It is often difficult to use protease inhibitors for HCV recipients after LT due to potential interaction with immunosuppressive drugs. We reported here our experience with two patients treated with protease inhibitors combined with PEG IFN/RBV for the treatment of recurrent post-LT hepatitis caused by genotype 1 HCV.

A recent study that examined the effect of TVR on the pharmacokinetics of cyclosporin and tacrolimus

Table 2 Laboratory data of Patient 2 at start of triple therapy after LT

CBC		LDH	241 IU/L	Tumor marker	
WBC	7530/μL	ALP	294 IU/L	AFP	5.6 ng/mL
RBC	4.23×10^6/μL	γ-GT	17 IU/L		
Hb	13.3 g/dL	TP	6.4 g/dL	HCV virus markers	
Ht	39.7%	Alb	3.5 g/dL	HCV RNA	6.2 log IU/mL
Plt	17.8×10^4/μL	TC	219 mg/dL	genotype	1b
		TTT	7 U		
Blood coagulation test		ZTT	12 U		
PT	121%	BUN	12.6 mg/dL	a.a. substitution in ISDR	0
		Cr	0.50 mg/dL	a.a.70 in the core region	Mutant
Blood chemistry		CRP	0.11 mg/dL	a.a.91 in the core region	Wild
T-Bil	0.7 mg/dL	FBS	106 mg/dL	*IL-28B* donor	TG genotype
AST	20 IU/L	HbA1c	6.9%	*IL-28B* recipient	TT genotype
ALT	15 IU/L	NH_3	57 μg/mL	ss469415590 door	TT/⊿G genotype
				ss469415590 recipient	TT/TT genotype

γ-GT, γ-glutamyltransferase; a.a. substitution in ISDR, amino acid substitutions in the interferon sensitivity-determining region; AFP, α-fetoprotein; Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under curve; BUN, blood urea nitrogen; CBC, complete blood count; Cr, creatinine; CRP, C-reactive protein; FBS, fasting blood sugar; Hb, hemoglobin; HbA1c, hemoglobin A1c; Ht, hematocrit; LDH, lactate dehydrogenase; LT, liver transplantation; RBC, red blood cells; Plt, platelets; PT, prothrombin time; T-Bil, total bilirubin; TC, total cholesterol; TP, total protein; TTT, thymol turbidity test; WBC, white blood cells; ZTT, zinc sulfate turbidity test.

reported a 78-fold increase in tacrolimus blood concentration and fourfold rise in cyclosporin blood concentration through interaction with TVR.[11] For this reason, we changed tacrolimus to cyclosporin before triple therapy. We also started cyclosporin using a small dose and checked the blood concentration of cyclosporin on a daily basis. Based on these measures, cyclosporin blood concentration remained at approximately 100 ng/mL. Considered collectively, it is important to change the dose of immunosuppressive drugs and frequently monitor cyclosporin blood concentrations.

It is noteworthy that the blood concentration of TVR also increased by interaction with cyclosporin. The AUC of TVR in patient 1 was 103 μgh/mL, while the AUC of TVR of 10 chronic hepatitis C patients treated with PEG IFN/RBV was 52 μgh/mL in our hospital (data not shown). These findings highlight the need for awareness of the potential side-effects of TVR. In fact, various side-

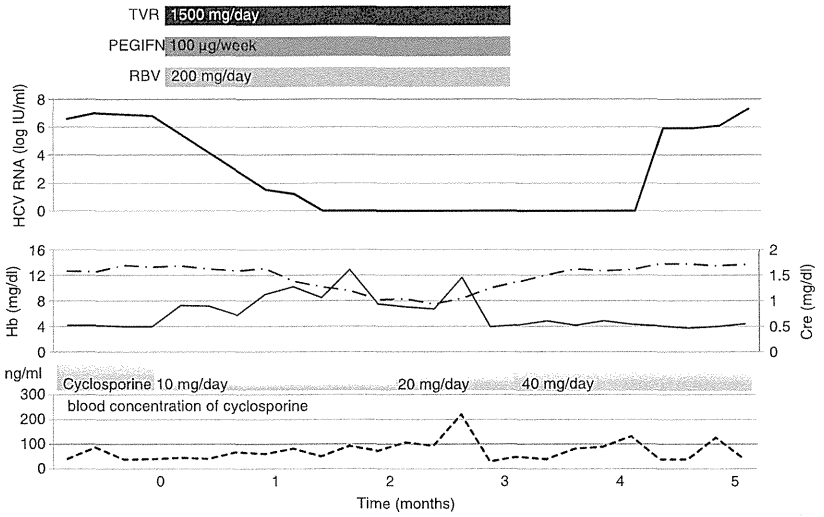

Figure 2 Clinical course of patient 2. Cre, creatinine; Hb, hemoglobin; HCV, hepatitis C virus; PEG IFN, pegylated interferon; RBV, ribavirin; TVR, telaprevir. ——, Cre; — ·, Hb.

effects were reported by patient 2, including anemia, renal dysfunction and skin rashes. Consequently, the triple therapy was discontinued at 11 weeks in this patient.

What are the indications for triple therapy? While there are no standardized rules for the initiation of this mode of treatment, we believe that triply therapy should be used under the following conditions: (i) laboratory tests should show normal hemoglobin and serum creatinine levels to avoid potential side-effects of TVR; and (ii) recipients who develop HCV RNA relapse while receiving PEG IFN/RBV dual therapy after LT. In naïve cases, we recommend PEG IFN/RBV therapy. There are some reports of triple therapy for recipients after LT.[19–21] However, there is no evidence in safety of triple therapy for recipients. Furthermore, Coilly *et al.* recommends PEG IFN/RBV dual therapy for naïve cases in review.[22]

Third, both the donor and recipient must have good SNP (IL28B or λ4). On the other hand, we recommend withholding triple therapy for patients who fail to respond to PEG IFN/RBV and those who have minor SNP (IL28B or λ4) of donor and recipient. In this regard, several groups have reported that *IL28B* of both recipients and donors influenced the SVR to PEG IFN/RBV in patients with recurrent hepatitis C after LT.[23–26]T.[19–22]

Another important question regarding treatment of recurrent post-LT HCV infection is the duration of IFN therapy. The answer to this question is difficult and currently there are no data on the ideal duration of triple therapy. However, we recommend long-term PEG IFN/RBV therapy following triple therapy from 12 to 36 weeks, with a total duration of treatment of 48 weeks. This is based on our previous finding that the majority of patients with genotype 1b in whom HCV RNA reached undetectable levels were able to achieve SVR (87.5%; 7/8).[23] Eradication of HCV by triple therapy should increase the SVR rate. In fact, Pungpapong *et al.* used 12-week triple therapy followed by 36-week PEG IFN/RBV therapy and reported an SVR rate associated with this regimen of 100% (7/7) for genotype 1b recipients.[12]

On the other hand, for such hard-to-treat patients after LT, DAA will become a standard therapy in the future. Because SVR rate and safety of DAA therapy is more higher than triple therapy.[27–29] However, there is a problem of mutation of HCV against DAA therapy.[30,31] In these instances, it may be necessary to recommence triple therapy. The experience of the present study provides a good reference for such an occurrence (e.g. dose of TVR and dose of immunosuppressive agents).

In conclusion, we reported our experience with two patients who developed recurrent HCV genotype 1 infection after LT and were treated with protease inhibitors combined with PEG IFN/RBV. The results point to possible achievement of SVR by triple therapy; however, more studies are needed to evaluate the clinical benefits and side-effects of triple therapy for recurrent post-LT HCV infection.

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Human microRNA hsa-miR-1231 suppresses hepatitis B virus replication by targeting core mRNA

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SUMMARY. Pathogen-specific miRNA profiles might reveal potential new avenues for therapy. To identify miRNAs directly associated with hepatitis B virus (HBV) in hepatocytes, we performed a miRNA array analysis using urokinase-type plasminogen activator (uPA)–severe combined immunodeficiency (SCID) mice where the livers were highly repopulated with human hepatocytes and human immune cells are absent. Mice were inoculated with HBV-infected patient serum samples. Eight weeks after HBV infection, human hepatocytes were collected from liver tissues, and miRNAs were analysed using the Toray 3D array system. The effect of miRNAs on HBV replication was analysed using HBV-transfected HepG2 cells. Four miRNAs, hsa-miR-486-3p, hsa-miR-1908, hsa-miR-675 and hsa-miR-1231 were upregulated in mouse and human livers with HBV infection. These miRNAs were associated with immune response pathways such as inflammation mediated by chemokine and cytokine signalling. Of these miRNAs, hsa-miR-1231, which showed high homology with HBV core and HBx sequences, was most highly upregulated. In HBV-transfected HepG2 cells, overexpression of hsa-miR-1231 resulted in suppression of HBV replication with HBV core reduction. In conclusion, a novel interaction between hsa-miR-1231 and HBV replication was identified. This interaction might be useful in developing new therapeutic strategies against HBV.

Keywords: HB core, hepatitis B virus, hsa-miR-1231, human hepatocyte chimeric mouse, microRNA.

INTRODUCTION

Hepatitis B virus (HBV) is a member of the *Hepadnaviridae* family, which contains a group of hepatotropic small DNA viruses that infect their respective animal hosts [1–3]. Once HBV infects human hepatocytes, the HBV genome translocates into the nucleus. Some genome copies are converted into a covalently closed circular DNA (cccDNA) form and organized into a minichromosome with histone and nonhistone proteins [4–8]. HBV cccDNA utilizes the cellular transcriptional machinery to produce all viral RNAs including the pregenomic RNA [9], and these gene products regulate viral replication and pathogenesis by regulating host gene expression [10,11].

MicroRNAs (miRNAs) are small noncoding RNAs of 21-25 nucleotides in length, processed from hairpin-shaped transcripts [12]. MiRNAs can bind the 3′-untranslated regions (UTRs) of messenger RNAs and downregulate gene expression by cleaving messenger RNA or inhibiting translation. Several miRNAs associated with HBV infection, HBV replication and hepatocarcinogenesis have recently been identified [13–19]. However, the direct influence of HBV infection on miRNA expression is still unclear.

MicroRNAs are currently being investigated for their therapeutic potential in antiviral therapy. As several studies have demonstrated that hsa-miR-122, which is specifically and abundantly expressed in hepatocytes, supported hepatitis C virus (HCV) replication by improving RNA

Abbreviations: HBc, hepatitis B core; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; miRNA, microRNA; RI, replication intermediates.

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stability [20–24], small molecules or siRNAs which are able to knock down miR-122 expression have been explored as a new therapeutic agent for HCV eradication.

A similar microRNA-based antiviral approach is also sought for the treatment of chronic hepatitis B, as it is difficult to eradicate HBV genomes converted into cccDNA or minichromosomes under present antiviral therapies. To develop new strategies for complete eradication of the viral genome from hepatocytes, it is important to clarify the direct associations between hepatic miRNAs and HBV infection.

In this study, miRNA microarray analysis was performed using human hepatocyte chimeric mouse livers to assess the direct impact of HBV infection on miRNA expression. We successfully demonstrated that HBV infection attenuated the expression of miRNAs under immunodeficient conditions to protect early viral propagation. A novel interaction between hsa-miR-1231 and HBV replication was identified.

MATERIALS AND METHODS

Human serum inoculum

Serum samples were obtained from a carrier infected with HBV genotype C after obtaining written informed consent for the donation and evaluation of blood samples. Inoculum was positive for HBs and HBe antigens with high-level viremia (HBV DNA: 7.1 log copies/mL). The experimental protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the Hiroshima University Hospital ethical committee (Approval ID: D08-9).

Human hepatocyte chimeric mice experiments

Human hepatocyte chimeric mice (PXB mice), in which human hepatocytes were transplanted into uPA$^{+/+}$/SCID$^{+/+}$ mice, were purchased from Phoenix Bio (Hiroshima, Japan). Mouse experiments were performed in accordance with the guidelines of the local committee for animal experiments at Hiroshima University.

Six chimeric mice, in which more than 90% of the liver tissue was replaced with human hepatocytes, were divided into two experimental groups. Group A contained three uninfected mice. Group B consisted of three mice that were inoculated via the mouse tail vein with human serum containing 6×10^6 copies of HBV. Serum HBV DNA titres were quantified every 2 weeks by real-time PCR, and human albumin levels were measured using the Human Albumin ELISA Quantitation kit (Bethyl Laboratories Inc., Montgomery, TX, USA) as described previously [25]. Eight weeks after inoculation, all three infected mice were sacrificed. Infection, extraction of serum samples and sacrifice were performed under ether anaesthesia as described previously [26].

miRNA microarray analysis

Human hepatocytes were finely dissected from the mouse livers and stored in liquid nitrogen after submerging in RNA*later*® solution (Applied Biosystems, Foster City, CA, USA). Experimental sample RNAs were isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and analysed using TORAY 3-D Gene Chip human miRNA ver. 12.1 (TORAY, Chiba, Japan).

Data analysis

Gene expression profiles were analysed using GeneSpring GX 10.0.2 software (Tomy Digital Biology, Tokyo, Japan). Expression ratios were normalized per chip to the 50th percentile. To determine whether there were miRNAs differentially expressed among samples, we performed two Welch's *t*-tests ($P < 0.01$) on this prescreened list of miRNAs with Benjamini and Hochberg's correction. Complete linkage hierarchical clustering analysis was applied using Euclidean distance.

Pathway analysis

The miRNA target genes were predicted by the online database miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html). Target prediction was performed using 3′-UTR sequences of mRNAs, and the probability distributions were calculated using the Poisson distribution [27]. The mRNAs with *P* values <0.01 were considered significant. To improve the accuracy of target gene selection, the predicted genes were screened using other prediction programs, including miRanda (August 2010 release), miRDB (April 2009 release) and TargetScan version 5.1 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). Genes that were predicted by at least two alternate programs were selected. Pathway analysis was performed by PANTHER version 8.1 (http://www.pantherdb.org/) to determine the effects of the predicted target genes on pathways.

Quantification of miRNAs

Small RNAs were extracted from liver tissues or HepG2 cells with mirVana™ miRNA Isolation Kit (Applied Biosystems) and reverse-transcribed according to the manufacturer's instructions. The selected miRNAs were quantified with TaqMan® MicroRNA Assays (Applied Biosystems) using the 7300 Real-Time PCR System (Applied Biosystems), and the expression of RNU6B served as a control.

Quantification of mRNAs

Total RNA was extracted from HepG2 cells transfected with control miRNA or miR-1231 expression plasmid using

RNeasy Mini Kit and reverse-transcribed (RT) using ReverTra Ace (TOYOBO, Osaka, Japan) with random primer according to the manufacturer's instructions. The selected cDNAs were quantified by real-time PCR. Differences between groups were examined for statistical significance using Student's *t*-test. The primer sequences were as follows: GAPDH forward 5′-ACAACAGCCTCAAGATCATCAG-3′ and reverse 5′-GGTCCACCACTGACACGTTG-3′; Mx1 forward 5′- TTCGGCTGTTTACCAGACTCC-3′ and reverse 5′- CAAAGCCTGGCAGCTCTCTAC-3′; 2′–5′ oligoadenylate synthetase 1 (OAS1) forward 5′- ACCTGGTTGTCTTCCTCA GTCC-3′ and reverse 5′- GAGCCTGGACCTCAAACTTCAC-3′; double stranded RNA dependent protein kinase (PKR) forward 5′- TGGCCGCTAAACTTGCATATC-3′ and reverse 5′- AGTTGCTTTGGGACTCACACG-3′; and SOCS1 forward 5′-ACGAGCATCCGCGTGCACTT-3′ and reverse 5′-AAGAGG CAGTCGAAGCTCTC-3′.

Plasmid construction

The construction of wild-type HBV 1.4 genome length, pTRE-HB-wt, was described previously [25]. The nucleotide sequence of the cloned HBV genome was deposited into GenBank AB206817. The HBc and HBx genes, amplified from pTRE-HB-wt, were cloned into pcDNA3 and p3xFLAG-CMV10 vectors and designated pcDNA-HBc and p3FLAG-HBx, respectively. The human miR-1231 precursor expression plasmid (HmiR0554-MR04) and the control miRNA plasmid (CmiR0001-MR01), which was a miRNA-scrambled control clone, were commercially produced (GeneCopoeia™, Rockville, MD, USA).

Transfection of HepG2 cell lines with the plasmids

The HBV expression plasmid was transfected into HepG2 cells with control miRNA or miR-1231 expression plasmid using *Trans*IT-LT1 (Mirus, Madison, WI, USA) reagent according to the manufacturer's instructions. 24–48 h after transfection, core-associated HBV DNA and HBV RNA were extracted and quantified by real-time PCR or RT real-time PCR, respectively [28]. For identifying targets within the HBV genome, HBc or HBx expression plasmids were transiently transfected with miR-1231 expression plasmid into HepG2 cells. Twenty-four hours after transfection, the cells were harvested to perform Western blot analysis.

Analysis of HBV replication intermediates

Quantitative analysis of HBV replication intermediates was performed as described previously [29]. The HBV-specific primers used for amplification were 5′-TTTGGGCATGGAC-ATTGAC-3′ and 5′-GGTGAACAATGTTCCGGAGAC-3′. The lower detection limit of this assay was 300 copies.

Western blot analysis

Cell lysates, prepared with RIPA like buffer [50 mM Tris-HCl (pH 8.0), 0.1% SDS, 1% NP-40, 150 mM sodium chloride, and 0.5% sodium deoxycholate] containing protease inhibitor cocktail (Sigma-Aldrich, Tokyo, Japan), were separated on 5–20% (wt/v) SDS-polyacrylamide gels (Bio-Rad Laboratories, Inc., Tokyo, Japan). Immunoblotting was performed with anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) or anti-HBV core monoclonal antibody HB91 (Advanced Life Science Institute Inc., Saitama, Japan) or anti β-actin monoclonal antibody (Sigma-Aldrich) followed by incubation with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin (GE Healthcare, Buckinghamshire, UK). Expression of HBc protein was quantified based on the densities of the immunoblot signals by Quantity One® software (Bio-Rad Laboratories, Inc.).

RESULTS

miRNA expression alterations associated with HBV infection

To analyse the influence of HBV infection on human hepatocytes, miRNA microarray expression profiles were compared between groups A (mice without HBV infection) and B (mice with HBV infection). Among the 900 miRNAs on the microarray, 10 miRNAs showed a more than 2.0-fold change with HBV infection. Five of the 10 miRNAs were upregulated, and the remaining five were downregulated (Fig. S1). Because immunity was severely suppressed in the chimeric mice, changes in miRNA expression are thought to be closely associated with HBV infection, and the upregulated miRNAs might play a protective role against HBV infection. Thus, we focused on these 5 upregulated miRNAs.

Comparison of expression of the 5 upregulated miRNAs in human liver tissues

To verify the microarray results, quantitative analysis of miRNAs was performed using liver tissues from the chimeric mice. Three of the 5 miRNAs were significantly upregulated by HBV infection (Fig. 1). Expression changes in the other 2 miRNAs (hsa-miR-675 and hsa-miR-1908) showed a similar trend but were not significant due to individual variation. Therefore, further quantitative analysis was performed using human liver tissues. Nine liver tissue samples were obtained from patients with chronic hepatitis B ($N = 3$), chronic hepatitis C ($N = 2$) or alcoholic liver dysfunction ($N = 4$), and miRNA expression levels were compared. Expressions of all miRNAs except for miR-886-5p were significantly higher in liver tissues with chronic hepatitis B than in those with other liver diseases (Fig. 2).

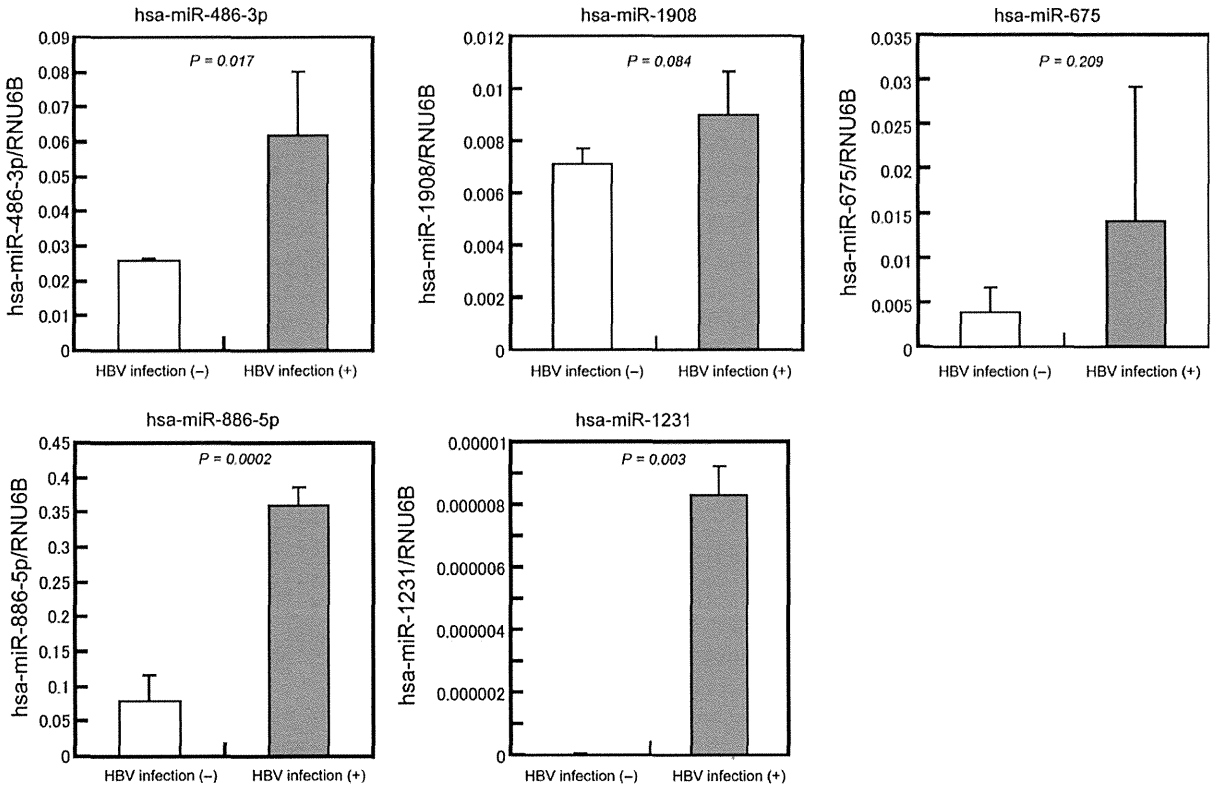

Fig. 1 Upregulation of microRNA by HBV infection. Signal intensities of five upregulated miRNAs were compared between HBV-infected and noninfected mouse livers. All 5 miRNAs were significantly upregulated by HBV infection. *P* values were calculated by the Mann–Whitney *U*-test.

Associations between signalling pathways and the upregulated miRNAs

To analyse the influence of miRNA upregulation on signalling pathways, pathway analysis was performed. However, there are several obstacles in analysing the association between miRNAs and pathways, such as the lack of reliable miRNA target prediction algorithms, differences in the results among target prediction systems, and the small number of validated target genes. To improve the reliability of the targets, we performed the pathway analysis in combination with four prediction tools (miRWalk, TargetScan, miRanda and miRDB). After this operation, 482 targets were predicted (hsa-miR-1231: 203 targets, hsa-miR-1908: 3 targets, hsa-miR-486-3p: 251 targets, hsa-miR-675: 25 targets), and these 482 targets were submitted to the PANTHER classification system for pathway analysis. As shown in Table 1, several immunological pathways such as inflammation mediated by chemokine and cytokine signalling pathway, and the interleukin signalling pathway were identified, but it was difficult to identify characteristic pathways.

Suppression of HBV replication with miR-1231 overexpression

Because hsa-miR-1231 was most the highly upregulated among these four miRNAs and had a high homology with the HBV genome, we focused on hsa-miR-1231. Using GENETYX ver. 8.2.1 (GENETYX, Tokyo, Japan), the hsa-miR-1231 sequence was predicted to hybridize at the HB core and X regions of the HBV genome (Fig. 3). To analyse the influence of hsa-miR-1231 on HBV replication, changes in HBV replication intermediates were evaluated using an *in vitro* HBV replication model. As shown in Fig. 4a, HBV replication intermediates were significantly reduced by hsa-miR-1231 overexpression, and the suppression of HBV RNA and HBc proteins were also observed by hsa-miR-1231 overexpression (Figs 4b,c). Thus, HBV replication was concluded to be inhibited by hsa-miR-1231 at the post-transcriptional level.

Specific regulation of HBV-related protein levels with hsa-miR-1231 overexpression

As the preceding results indicated an association between the production of HBV-related protein or HBV particles and

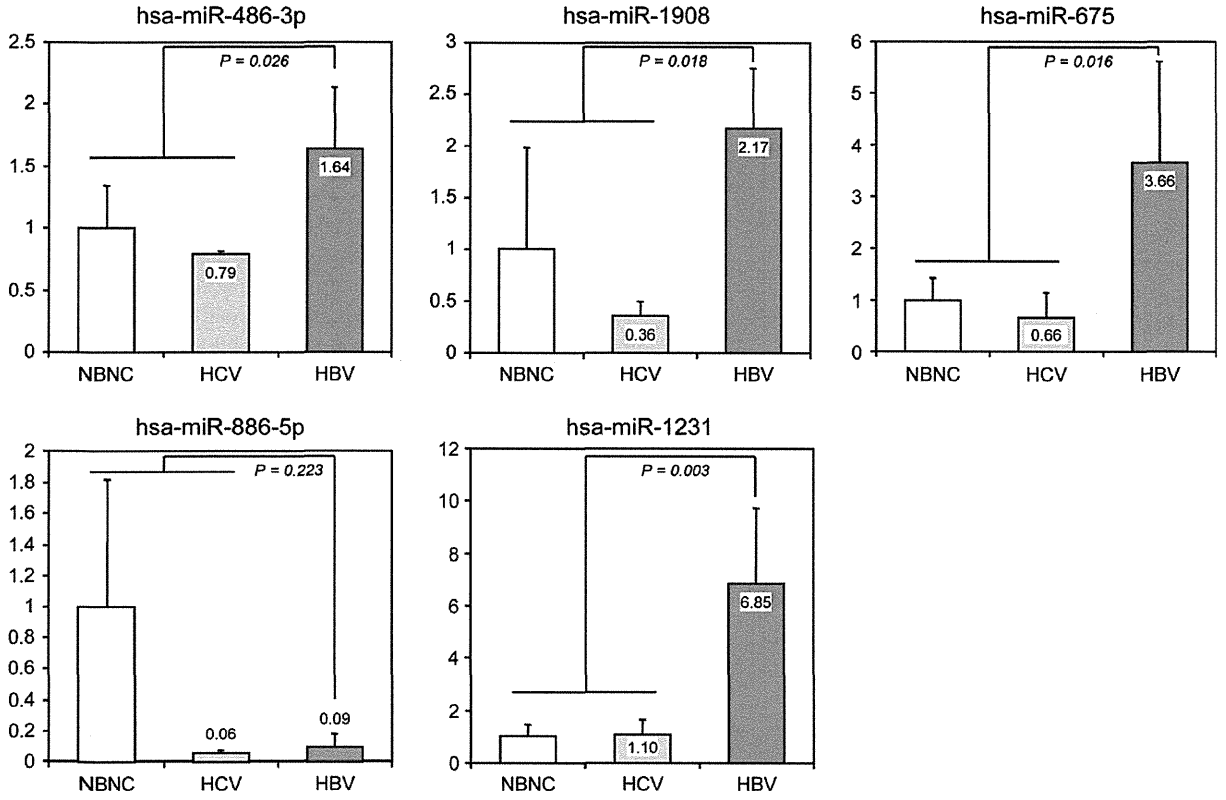

Fig. 2 Comparison of microRNA expression in clinical liver tissues. Quantification of miRNAs was performed by real-time PCR using nine human liver tissues obtained from the patients who had chronic hepatitis B ($N = 3$), C ($N = 2$) or alcoholic liver dysfunction ($N = 4$). Expression levels of four miRNA were significantly higher in the chronic hepatitis B patients than in those of other liver diseases. The results of miR-886-5p levels were not statistically significant. *P* values were assessed by Mann–Whitney *U*-test.

hsa-miR-1231 expression, further analysis was performed to identify the region hybridized by hsa-miR-1231. As shown in Fig. 5, HBc protein expression was remarkably reduced by hsa-miR-1231 expression, but no reduction in HBx protein was observed. These results indicate that hsa-miR-1231 might interact with HBV core mRNA and suppress HBV replication by inhibiting HBV core protein production.

The effects of hsa-miR-1231 on the expression of interferon-stimulated genes

Alternatively, hsa-miR-1231 might suppress HBV replication through activation of the interferon signalling pathway. We thus evaluated mRNA expression of interferon-stimulated genes (ISGs) with or without hsa-miR-1231 overexpression. None of the examined ISGs (MxA, PKR, OAS-1 and SOCS1) were regulated by hsa-miR-1231 expression (Fig. S3). These results suggest that hsa-miR-1231 suppresses HBV replication at the post-transcriptional level but not through the activation of interferon signalling.

DISCUSSION

Previously, we have demonstrated that human hepatocyte chimeric mice can be chronically infected with hepatitis B and C viruses [25,30,31]. This mouse model facilitates analysis of the effect of viral infection under immunodeficient conditions. In the present study, we performed miRNA array analysis using this mouse model and obtained miRNA expression profiles reflecting the direct influence of HBV infection on human hepatocytes. Furthermore, we found a novel mechanism for HBV replication mediated by hsa-miR-1231.

To avoid contamination with mouse tissue, human hepatocyte chimeric mice were used in which liver tissue was largely (>90%) replaced by human hepatocytes. Although it is feasible to use microarray analysis in this chimeric mouse model [32], signals from miRNA array analysis may be influenced by cross-hybridization with mouse miRNA from a small amount of contaminated mouse-derived cells because of the high homology between the human and mouse genomes. To compensate

Table 1 Pathways associated with the 4 miRNAs upregulated by HBV infection

Pathway	Number of gene hits	Ratio of genes %
Inflammation mediated by chemokine and cytokine signalling pathway (P00031)	11	2.60
Angiogenesis (P00005)	10	2.30
Integrin signalling pathway (P00034)	9	2.10
Gonadotropin releasing hormone receptor pathway (P06664)	7	1.60
Wnt signalling pathway (P00057)	7	1.60
Parkinson disease (P00049)	7	1.60
EGF receptor signalling pathway (P00018)	7	1.60
Alzheimer's disease–presenilin pathway (P00004)	6	1.40
PDGF signalling pathway (P00047)	6	1.40
B-cell activation (P00010)	6	1.40
Interleukin signalling pathway (P00036)	5	1.20
Huntington disease (P00029)	5	1.20
FGF signalling pathway (P00021)	5	1.20
Cadherin signalling pathway (P00012)	5	1.20
VEGF signalling pathway (P00056)	4	0.90
Toll receptor signalling pathway (P00054)	4	0.90
T-cell activation (P00053)	4	0.90
Ras pathway (P04393)	4	0.90
Heterotrimeric G-protein signalling pathway-Gi alpha and Gs alpha-mediated pathway (P00026)	4	0.90
Endothelin signalling pathway (P00019)	4	0.90

for contamination, mice that were negative for HBV infection were set up as negative controls.

Only 5 miRNAs showed more than 2.0-fold upregulation with HBV infection under miRNA array analysis using chimeric mouse livers (Fig. S1). Comparing these results with our previous study using patient sera, only hsa-miR-486-3p showed a similar change in sera from chronic hepatitis B patients, but no upregulation of the other 4 miRNAs was observed [15]. These results suggest that miRNA expression in sera from chronic hepatitis B patients might be regulated not only by HBV infection but also by human immune responses. In addition, it might be difficult to analyse changes in expression of miRNAs that are expressed at low levels in human hepatocytes, including hsa-miR-1231, using human serum.

To identify targets of miR-1231, we searched using four prediction systems. Although 632 target genes were identified (data not shown), and involvement of a number of pathways was indicated (Table S1), critical targets associated with human immunity or HBV replication could not be identified. Interferon signalling was also a potential mechanism of HBV suppression, but several ISG mRNAs were not induced by hsa-miR-1231 overexpression *in vitro* (Fig. S2). Therefore, we concluded that hsa-miR-1231 does not suppress HBV replication via interferon signalling.

To examine the possibility that miR-1231 directly regulates HBV replication by interacting with HBV-related mRNAs, we searched for hsa-miR-1231-binding motifs and found two candidate sequences in the HBV core and X genes (Fig. 3). As shown in Fig. 5, one target in the HBV core region could hybridize with hsa-miR-1231, and HBc expression was found to be suppressed by hsa-miR-1231 overexpression. The hsa-miR-1231-binding motif in the HBV core region was conserved in more than 90% of the HBV sequences in GenBank, regardless of HBV genotype (data not shown). Thus, we speculate that hsa-miR-1231 binds to the HBc target region and suppresses HBc production to inhibit HBV replication.

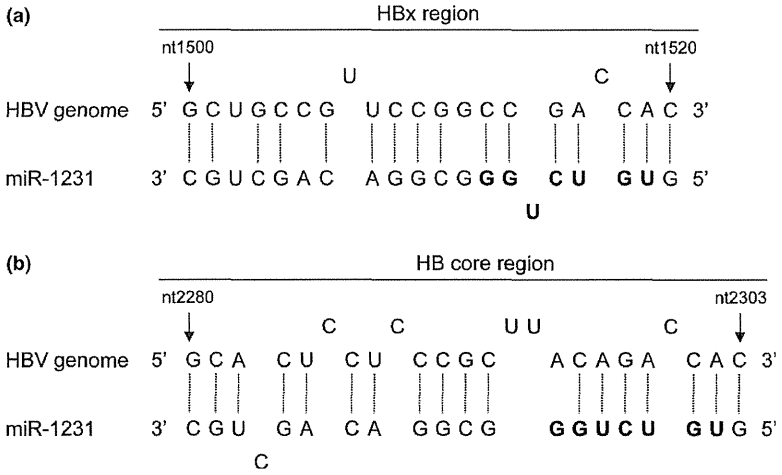

Fig. 3 Alignment of hsa-miR-1231 to HBV genome. Alignment of hsa-miR-1231 to the HBV genome was performed. MiR-1231 sequence was predicted to hybridize at the HBV core **(a)** and HBV X region **(b)**.