Table 2. Risk factors for the development of HCC by chronic hepatitis C patients treated with PegIFNα2b and RBV.

Characteristic	All patients n = 1013	HCC n = 47	non-HCC n = 966	*p* value*
Age (yr)	58 (50-65)	67 (58-71)	58 (49-65)	<0.001
Male, n (%)	498 (49.2)	32 (68.1)	466 (48.2)	0.007
Body mass index (kg/m²)	23.0 (21.1-25.2)	23.6 (21.6-25.7)	23.0 (21.1-25.2)	0.15
ALT (IU/L)	54 (35-89)	74 (46-100)	54 (34-89)	0.008
Albumin (g/L)	41 (39-44)	40 (37-42)	44 (41-46)	0.002
Platelet count (x10⁹/L)	159 (120-199)	110 (88-132)	161 (123-201)	<0.001
Hemoglobin (g/L)	136 (127-147)	136 (128-149)	136 (127-147)	0.89
Ferritin (ng/ml)	165 (84-376)	187 (80-462)	167 (80-306)	0.68
α-fetoprotein (ng/ml)	4.9 (3.0-9.3)	11.7 (6.8-32.7)	4.8 (3.0-8.7)	<0.001
Hemoglobin A1c (%)	5.5 (5.3-5.9)	5.8 (5.4-6.3)	5.5 (5.3-5.9)	0.96
HCV genotype (1/2), n (%)	710/303 (70.1/29.9)	38/9 (80.9/19.1)	672/294 (69.6/30.4)	0.09
Non-cirrhosis/cirrhosis, n	863/150 (85.2/14.8)	19/28 (40.4/59.6)	844/122 (87.4/12.6)	<0.001
Treatment duration (wk)	47 (24-48)	43 (23-48)	47 (24-48)	0.58
Virological response (SVR/TVR/NVR), n (%)	557/304/152 (55.0/30.0/15.0)	13/13/21 (27.7/27.7/44.7)	544/291/131 (56.3/30.1/13.6)	<0.001

Data are expressed as number (%) or median (first-third quartiles).
All demographic and clinical data are those at the start of antiviral treatment.
HCV, hepatitis C virus; HCC, hepatocellular carcinoma; SVR, sustained virological response; TVR, transient virological response; NVR, non-virological response; ALT, alanine aminotransferase.
*Comparison between HCC and non-HCC.

Overall cumulative incidence of HCC classified by treatment outcome

The 5-year cumulative incidence rates of HCC of the SVR (3.1%) and TVR groups (5.8%) were significantly lower than those of the NVR group (18.8%) (both $p < 0.001$), and the rate of the SVR group was lower, but not significantly, than that of the TVR group ($p = 0.21$).

Cumulative incidence of HCC classified by treatment outcome in the non-cirrhosis group

The Kaplan–Meier curves for the incidence of HCC classified by treatment outcome in the non-cirrhosis group are shown in Fig. 1A ($p = 0.009$ by log-rank test). The 5-year cumulative incidence rates of HCC in the SVR (1.7%) and TVR groups (3.2%) were significantly lower than those of the NVR group (7.6%) ($p = 0.003$ and $p = 0.03$, respectively), and the rate of the SVR group was lower, but not significantly, than that of the TVR group ($p = 0.47$).

Cumulative incidence of HCC classified by treatment outcome in the cirrhosis group

The Kaplan–Meier curves for the incidence of HCC classified by treatment outcome in the cirrhosis group are shown in Fig. 1B ($p = 0.03$ by log-rank test). The 5-year cumulative incidence rates of HCC in the SVR (18.9%) and TVR groups (20.8%) were significantly lower than those of the NVR group (39.4%) ($p = 0.03$ and $p = 0.04$, respectively), and the rate of the SVR group was lower, but not significantly, than that of the TVR group ($p = 0.94$).

Adjusted rates of HCC incidence classified by treatment outcome of non-cirrhotic patients under 60 years of age

The Kaplan–Meyer curves of the estimation of the incidence of HCC by non-cirrhosis patients under 60 years of age, classified by treatment outcome, are shown in Fig. 2A ($p = 0.51$ by log-rank test). The 5-year cumulative incidence rates of HCC in the SVR (0.9%) and TVR groups (1.7%) were lower, but not significantly, than those of the NVR group (2.6%) ($p = 0.25$ and $p = 0.45$, respectively).

Table 3. Multivariate logistic regression analysis of possible predictors of HCC development.

Parameter	Hazard ratio	95% CI	*p* value
Age			
<60 yr	1		
≥60 yr	2.81	1.39-5.69	0.004
Sex			
Female	1		
Male	2.98	1.46-6.05	0.003
Platelet count			
≥150 x 10⁹/L	1		
<150 x 10⁹/L	4.04	1.57-10.44	0.004
α-fetoprotein			
<10 ng/ml	1		
≥10 ng/ml	2.50	1.09-5.78	0.03
Liver pathophysiology			
Non-cirrhosis	1		
Cirrhosis	3.22	1.28-8.13	0.01
Treatment outcome			
SVR	1		
TVR	1.50	0.65-3.44	0.34
NVR	3.72	1.69-8.18	0.001

HCC, hepatocellular carcinoma; SVR, sustained virological response; TVR, transient virological response; NVR, non-virological response.

Adjusted rates of HCC incidence classified by treatment outcome of non-cirrhotic patients aged 60 years and over

The Kaplan–Meyer curves of the estimation of the incidence of HCC in non-cirrhosis patients, aged 60 years and over classified by treatment outcome, are shown in Fig. 2B ($p = 0.05$ by log-rank test). The 5-year cumulative incidence rates of HCC in the SVR (3.5%) and TVR groups (4.2%) were significantly lower than those

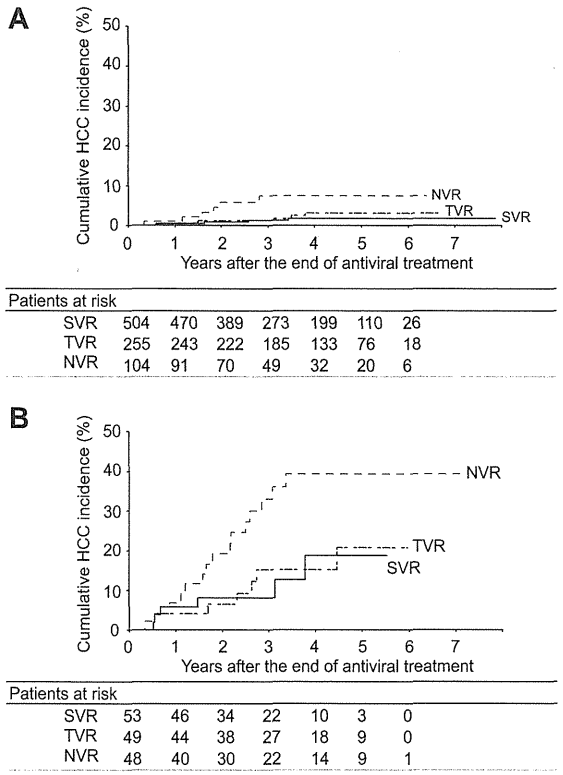

Fig. 1. Cumulative incidence of HCC after PegIFNα2b and RBV treatment stratified by treatment outcome (SVR: continuous line, TVR: long dashed-dotted line, NVR: dashed line). (A) Non-cirrhosis group (overall: $p = 0.009$; SVR *vs.* TVR: $p = 0.47$; SVR *vs.* NVR: $p = 0.003$; and TVR *vs.* NVR: $p = 0.03$ by log-rank test). (B) Cirrhosis group (overall: $p = 0.03$; SVR vs. TVR: $p = 0.94$; SVR *vs.* NVR: $p = 0.03$; and TVR *vs.* NVR: $p = 0.04$ by log-rank test).

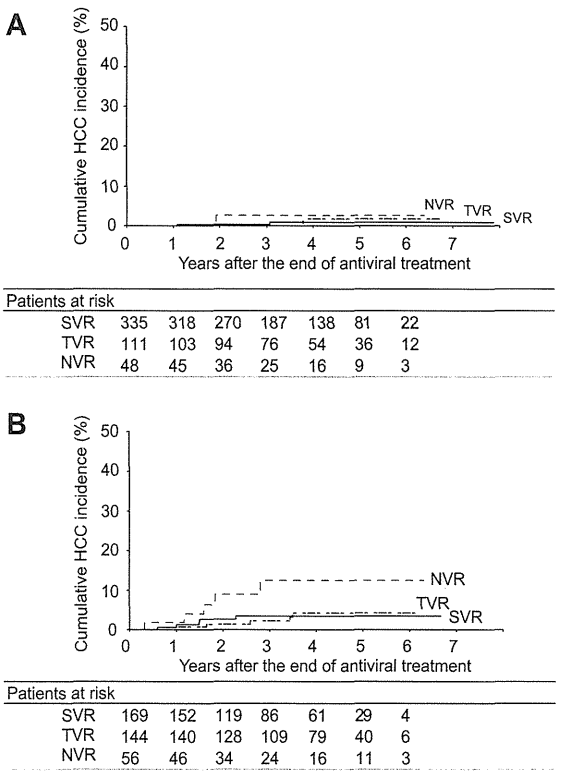

Fig. 2. Cumulative incidence of HCC after PegIFNα2b and RBV treatment stratified by treatment outcome of the non-cirrhosis group (SVR: continuous line, TVR: long dashed-dotted line, NVR: dashed line). (A) Under 60 years of age (overall: $p = 0.51$; SVR *vs.* TVR: $p = 0.94$; SVR *vs.* NVR: $p = 0.25$; and TVR *vs.* NVR: $p = 0.45$ by log-rank test). (B) Aged 60 years and over (overall: $p = 0.05$; SVR *vs.* TVR: $p = 0.96$; SVR *vs.* NVR: $p = 0.04$; and TVR *vs.* NVR: $p = 0.03$ by log-rank test).

of the NVR group (12.4%) ($p = 0.04$ and $p = 0.03$, respectively), and the rate of the SVR group was slightly lower, but not significantly, than that of the TVR group ($p = 0.96$).

The development of HCC by SVR patients

Thirteen patients who achieved SVR (2.3%) (6 non-cirrhotic and 7 cirrhotic patients) developed HCC during the follow-up period. Their individual pretreatment characteristics are shown in Table 4. Of these patients, 3 (patients 1–3) under 55 years of age had liver cirrhosis and the period from the end of antiviral treatment to the diagnosis of HCC was over 3 years. Of the remaining 10 patients (patients 4–13) aged 55 years and over, 6 did not have cirrhosis and the period from the end of antiviral treatment to the diagnosis of HCC was under 2.5 years.

Discussion

We here report the results of a prospective, long-term follow-up study done to evaluate the effect of treatment outcome on the development of HCC in a large cohort of Japanese patients with chronic hepatitis C, who were treated with PegIFNα2b and RBV. We found that those patients who achieved SVR or TVR had a lower risk of developing HCC within 5 years after the end of PegIFNα2b and RBV treatment when compared with NVR, in both cirrhosis and non-cirrhosis groups. Although SVR patients have been reported to have little risk of HCC incidence, a small number of our patients who achieved SVR did develop HCC, showing the necessity of a continued screening of patients with SVR.

Previously, the likelihood of HCC development by PegIFNα- and RBV-treated patients was difficult to determine because of the paucity of adequate long-term prospective studies. Based on the results of this prospective study, sex, age, platelet count, AFP level, and treatment outcome are significant, independent factors for the development of HCC. In addition to our present data, the incidence rate of HCC has been shown to be significantly lower for patients with TT genotype at rs8099917 and CC genotype at rs12979860 near the *IL28B* gene, which are associated with good response to antiviral treatment (data not shown). Of particular interest, the adjusted cumulative incidence of HCC was not significantly different between SVR and TVR for the 5 years after the end of treatment. Two randomized studies of maintenance therapy with low-dose PegIFNα to prevent hepatic decompensation and HCC have been recently reported [25,26]. However, maintenance therapy did not prevent HCC in presence of HCV viremia for at least 5 years, regardless of the degree of viral suppression. Our results showed that complete HCV sup-

Table 4. Individual characteristics of SVR patients who developed HCC.

Patient number	Age (yr)	Sex	Liver pathophysiology	Time to HCC* (yr)	HCV genotype	ALT (IU/L)	Albumin (g/L)	Platelet count (x10^9/L)	AFP (ng/ml)	HbA1c (%)
1	47	F	Cirrhosis	3.1	1	44	40	134	3.3	7.1
2	53	M	Cirrhosis	3.1	2	105	42	68	31.0	6.1
3	54	M	Cirrhosis	3.8	1	86	36	88	13.9	5.9
4	59	M	Non-cirrhosis	1.1	2	227	44	131	4.4	6.6
5	63	F	Cirrhosis	1.5	2	81	33	130	16.3	5.3
6	64	F	Non-cirrhosis	1.5	2	72	38	120	6.6	6.8
7	64	M	Non-cirrhosis	1.5	1	29	46	124	20.7	5.1
8	66	F	Cirrhosis	0.7	2	169	42	105	106.0	6.4
9	66	M	Non-cirrhosis	0.6	1	36	35	147	6.2	5.5
10	71	M	Cirrhosis	0.6	2	80	32	106	10.6	5.5
11	71	M	Non-cirrhosis	1.0	1	47	42	108	4.3	5.7
12	74	M	Non-cirrhosis	2.3	1	47	43	143	12.9	6.9
13	77	M	Cirrhosis	0.5	1	73	30	124	11.6	5.4

All data are those at the start of antiviral treatment.
SVR, sustained virological response; HCC, hepatocellular carcinoma; F, female; M, male; HCV, hepatitis C virus; ALT, alanine aminotransferase; AFP, α-fetoprotein; HbA1c, hemoglobin A1c.
*The time frame for HCC incidence starts from the end of antiviral treatment.

pression during antiviral treatment played an important role in preventing the development of HCC.

A recent prospective study that included Caucasian, Hispanic, and Black patients treated with PegIFNα2a and RBV reported that the adjusted mortality from any cause or liver transplantation, or of any liver-related outcome, was significantly lower in TVR patients than in NVR patients [13]. Similarly, the risk of decompensated liver disease, HCC and liver-related death was also lower in TVR patients than in NVR patients, although these differences did not reach statistical significance [13]. Therefore, the significantly low incidence rate of HCC, for the patients of this study with TVR in comparison with NVR, is an original finding, but the trend was true for cirrhotic patients of all ages and for non-cirrhotic patients aged 60 years and over. One possible explanation for this difference may be related to the rising incidence of HCC for NVR patients aged 60 years and over. Our results indicate that the duration of clinical benefit may outlast the period of actual viral suppression in the 5 years after treatment, however, it remains unclear how older age would explain why TVR resulted in a lower incidence of HCC that matched the incidence in SVR. Therefore, it will be necessary to investigate the development of HCC in SVR and TVR patients beyond five years.

Recently, a number of direct-acting antivirals (DAAs) have been designed and developed. Among them, telaprevir and boceprevir, non-structural 3/4A protease inhibitors, have shown promising results in various clinical trials and have led to an increased SVR rate when given in combination with PegIFNα and RBV, as compared with PegIFNα and RBV alone [27,28]. Furthermore, several IFN-free clinical trials, using regimens that combine several potent DAAs, are ongoing. As a result of advances in antiviral treatment, almost all patients can experience complete HCV suppression during treatment. We showed that TVR patients had a lower incidence rate of HCC than did NVR patients. It will be necessary to study the impact of virological response on the development of HCC by patients who undergo DAAs with and without IFN antiviral treatment.

Findings on the effect of SVR on liver-related preferable clinical outcomes have been reported in many previous reports [13,29–31], however, the analysis of the effect of SVR on the development of HCC is statistically difficult, because the number of events is too small to draw meaningful conclusions. In fact, there were only 13 patients with SVR who developed HCC during the observation period, reducing the validity of the analysis. Additional prospective studies that include a larger number of patients with SVR will be necessary to evaluate the relationship between SVR and the development of HCC.

Risk factors for HCV-related HCC have been reported previously, such as older age, male sex, obesity, diabetes mellitus, alcohol consumption, HCV genotype 1b, insulin resistance, complicated hepatic steatosis, and co-infection with hepatitis B virus or HIV [32,33]. Unfortunately, this study lacks data on insulin resistance and hepatic steatosis. Homeostasis Model Assessment of Insulin Resistance value is also related to a profound effect on PegIFNα2b and RBV treatment outcome [34], thus, there may be a significant difference in HbA1c level between the SVR, TVR and NVR non-cirrhotic groups, indicating differences in glucose metabolism. Moreover, it is known that hepatic steatosis occurs in about 40% of the chronic hepatitis C patients, when all common factors of fatty liver, such as alcohol abuse, obesity, and diabetes, have been excluded [35]. Therefore, it remains unclear whether or not there is a significant bias due to different rates of patients with insulin resistance or hepatic steatosis. Another limitation is the generalizability of the extremely high cumulative incidence rate of HCC, especially for cirrhotic NVR patients. The reasons for this exceedingly high rate are not well understood, although it may be explained by the increasing number of aging chronic hepatitis C patients in Japan, earlier than other countries [14]. Our results, therefore, may not be generalized to other ethnic groups that do not have such high rates of HCC.

In summary, this prospective study demonstrated that SVR and TVR patients had a significantly lower rate than NVR patients of HCC incidence within five years after the end of treatment, both for patients with and without cirrhosis. Because the risk of developing HCC remains present even after HCV eradication, long-term screening of patients with SVR is important.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Acknowledgments

We are grateful to Drs. Masayuki Murata, Mosaburo Kainuma, Kyoko Okada, Kazuhiro Toyoda, Haru Mukae, Kunimitsu Eiraku, Hiroaki Ikezaki, Takeshi Ihara, Takeo Hayashi, Satoshi Hiramine, Fujiko Mitsumoto, and Koji Takayama from the Department of General Internal Medicine, Kyushu University Hospital for their assistance with data collection for this study. We also thank Takeru Abe from the Medical Information Center, Kyushu University Hospital for statistical analysis support.

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Journal of Viral Hepatitis

Effect of vitamin D supplementation on pegylated interferon/ribavirin therapy for chronic hepatitis C genotype 1b: A randomized controlled trial

Journal:	*Journal of Viral Hepatitis*
Manuscript ID:	Draft
Manuscript Type:	Original Paper
Date Submitted by the Author:	n/a
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Keywords:	vitamin D, interferon, hepatitis C

Effect of vitamin D supplementation on pegylated interferon/ribavirin therapy for chronic hepatitis C genotype 1b: A randomized controlled trial

Running title: Effects of vitamin D on PEG-IFN for HCV

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Abstract

Background: The current most effective treatment for patients chronically infected with hepatitis C virus (HCV) genotype 1 consists of pegylated interferon (PEG-IFN), ribavirin (RBV) and a protease inhibitor. Patients who experience severe side effects are treated with PEG-IFN/RBV. Chronic HCV-infected patients tend to have vitamin D deficiency, suggesting that vitamin D supplementation may enhance the effects of PEG-IFN/RBV. We therefore assessed the effects of vitamin D supplementation on viral response to PEG-IFN/RBV. Methods: Eighty-four patients were randomized, 42 to oral vitamin D (1000 IU/day) and 42 to non-supplementation (control), from week 8 to the end of PEG-IFN/RBV therapy. The primary endpoint was negative HCV at week 24 (viral responder [VR]).

Results: VR rate at week 24 was significantly higher in the vitamin D than in the control group (78.6% vs 54.8% p=0.037). Adverse events were similar in both groups. When patients were sub-divided by IL28B SNP rs8099917 genotype, those with the TT genotype group showed significantly higher VR rate at week 24 with than without vitamin D supplementation (86.2% vs. 63.3% vs. p=0.044). Although patients with the genotype TG/GG, who were relatively resistant to PEG-IFN treatment, had similar VR rates at week 24 with and without vitamin D, their decline in viral load from week 8 to week 24 was significantly greater with than without vitamin D. Multivariate analysis showed that rs8099917 genotype and vitamin D supplementation

contributed significantly to VR at week 24.

Conclusion: Vitamin D supplementation can enhance the effects of PEG-IFN/RBV in HCV genotype 1-infected patients.

Key words: chronic hepatitis C, pegylated interferon, ribavirin, vitamin D

Abbreviations: PEG-IFN/RBV, pegylated interferon plus ribavirin; HCV: hepatitis C virus; SVR, sustained virologic response

Introduction

Worldwide, about 170 million people are thought to be hepatitis C virus (HCV) carriers [1,2], about 30% of whom develop serious liver diseases such as decompensated cirrhosis and hepatocellular carcinoma (HCC) [3,4]. Eradication of the virus is necessary to prevent the development of such serious conditions. The recent development of triple combination therapy, consisting of pegylated interferon (PEG-IFN), ribavirin (RBV) and a protease inhibitor, telaprevir or boceprevir, has improved the eradication rate of genotype 1 HCV [5-12]. However, the side effects of these triple therapies may be too severe for patients with comorbid conditions such as anemia and depression. Furthermore, many patients develop skin rash and appetite loss, resulting in premature termination of treatment [5-12]. These patients, as well as those infected with genotypes other than genotype 1, are therefore treated with PEG-IFN plus RBV.

Blood concentrations of the vitamin D metabolite 25 (OH) vitamin D3 are relatively low in patients with chronic hepatitis, especially those with advanced fibrosis, and may be related to

poor responsiveness to interferon based therapy [13]. Vitamin D supplementation in HCV genotype 1-infected patients treated with PEG-IFN plus RBV has been reported to enhance sustained virologic response (SVR) rates compared with PEG-IFN/RBV alone (86% vs 42%) [14]. In that study, however, vitamin D supplementation was started at the beginning of PEG-IFN/RBV therapy. Some patients, however, become negative for HCV RNA after only 4 weeks of treatment. Patients with a rapid virologic response (RVR), defined as undetectable HCV-RNA after 4 weeks of PEG-IFN/RBV treatment of therapy, have an 80–100% likelihood of achieving SVR, whereas those who do not achieve an early virological response (EVR), defined as undetectable HCV-RNA at week 12 of therapy or ≥2-log decrease in RNA compared with baseline, have only an 8% chance of achieving SVR [15-17]. Several studies have evaluated the effects of extending therapy in slow responders [18-20]. The proportion of patients who had undetectable plasma HCV RNA at 24 weeks has been suggested as a surrogate for SVR. To rigorously evaluate the antiviral effects of vitamin D supplementation in HCV genotype-1 infected patients being treated with PEG-IFN/RBV, we randomized patients who did not achieve RVR to vitamin D or placebo, beginning 4 weeks after the start of PEG-IFN/RBV, and assessed the proportions of patients with undetectable serum HCV RNA after 24 weeks of treatment.

Patients and Methods

Patients

Patients at Hiroshima University Hospital and 13 other hospitals and clinics in Hiroshima Prefecture, Japan, were enrolled. Patients were included if they were aged ≥20 years, were chronically infected with HCV genotype 1, and had plasma HCV RNA concentrations ≥100 log IU/ml. Patients were excluded if they had any other type of liver disease, decompensated cirrhosis, liver cancer, HBV or HIV infection, renal insufficiency, history of heart disease or cerebral infarction, and were pregnant or currently breastfeeding.

Study design

This was an intension-to-treatment prospective randomized study. All experimental procedures were approved by our institutional review board, and informed consent was obtained from all participants. The study was designed to compare the "add on" effects of vitamin D combined with PEG-IFN/RBV with those of PEG-IFN/RBV alone. PEG-IFN was administered subcutaneously at a dose of 1.5 μg per kilogram of body weight once weekly, along with weight-based oral RBV, at 600 to 1200 mg per day. To assess the effects of vitamin D, patients who achieved RVR at week 4 were excluded. The remaining patients were stratified according to viral load (decline <1 log IU/ml or ≥1 log IU/ml in HCV RNA at week 4) and randomized to the vitamin D or control group using a sealed envelope. Patients randomized to the vitamin D group received 1,000 IU vitamin D once daily from week 8 until the end of treatment. The duration of treatment was determined according to response; i.e., patients with undetectable HCV RNA at week 12 were continued on treatment until week 48, whereas patients with detectable HCV RNA at week 12 were continued on treatment until week 72 (Fig. 1).

SNP genotyping and quality control

Because the two reported significant *IL28B* SNPs (rs8099917 and rs12979860) are in strong linkage disequilibrium, we examined only rs8099917. Genotypes in some samples were determined using the Illumina HumanHap610-Quad Genotyping BeadChip (San Diego, CA, USA), whereas the remaining samples were genotyped using the Invader assay (Third Wave Technologies, Madison, WI, USA), as described.

Efficacy assessments

Plasma HCV RNA concentrations were measured using the COBAS TaqMan HCV RNA 2.0 assay (Roche Diagnostics), with lower limits of detection and quantification of 10 IU/ml and 25 IU/ml, respectively. HCV RNA concentrations were measured on day 1; on weeks 2, 4, 8, 12, 16, 20, and 24, every 4 weeks until the end of treatment, and every 4 weeks for 6 months after the end of treatment.

Vitamin D3

Patients randomized to the vitamin D group were administered 1000 IU/day vitamin D once a day after breakfast, beginning 8 weeks after the start of PEG-IBN/RBV until the end of treatment at 48 or 72 weeks.

Serum vitamin D measurements

Serum concentrations of the vitamin D3 metabolite, 25(OH) vitamin D_3 concentration, were determined by 125-I-radioimmunoassay.

Safety assessment

Biochemical and hematologic assessments were performed at the same time as the efficacy assessments during and following treatment. Adverse events were recorded at physical examinations. PEG-IFN dose was reduced to 1.0 μg/kg body weight when neutrophil count was <750/ml and was withdrawn temporarily when neutrophil count was <500/ml. RBV dose was reduced in accordance with product labeling. The adverse effects of vitamin D such as hypercalcemia and phosphorus blood symptoms were carefully monitored.

End points

The primary end point was the proportion of patients who had undetectable plasma HCV RNA at 24 weeks.

Statistical analysis

HCV RNA negative conversion rates for the first 12 weeks were observed in 96% (26/27) of patients treated with vitamin D plus PEG-IFN/RBV and 48% (15/31) treated with PEG-IFN/RBV alone[14]. The HCV RNA negative conversion rates for the first 4 weeks were 44% (12/27) and 18% (5/31), respectively. We assumed that negative conversion occurred between weeks 5 and 12, rather than between weeks 5 and 24, because HCV RNA concentrations were unchanged between weeks 12 and 24 week. Assuming negative conversion rates within 12 weeks of 70% in the vitamin D group and 36% in the control group, and with an α=0.05, β=0.2 and power=0.8, we estimated that at least 33 patients per group would be required. Assuming a 30% drop-out rate, we enrolled 45 patients per group. Baseline characteristics and serum concentrations were compared using Pearson chi-square tests and Wilcoxon signed-rank tests,

as appropriate. The cumulative disappearance of HCV RNA was analyzed by the Kaplan-Meier method, with differences between curves tested by the log rank test. P values < 0.05 were considered statistically significant. Logistic regression analysis was used to assess independent factors contributing to response to therapy. Factors with a P-value less than 0.1 were included in a multiple logistic regression model and analyzed using the forward stepwise method. All analyses were performed using R version 2.16.0.

Results

Study patients

Of the 89 patients enrolled in the study, two discontinued treatment within the first 4 weeks because of the side effects of PEG-IFN. In three patients, HCV RNA became negative within the first 4 weeks of treatment.

Of the remaining 84 patients, 11 showed a decrease in HCV RNA <1 log IU/ml at week 4, and 73 showed a decrease >1 log IU/ml; these 84 patients were randomly assigned to vitamin D supplementation or placebo (Fig. 2). The vitamin D group consisted of 42 patients, 37 with a >1 log IU/ml HCV RNA decrease and five with a <1 log IU/ml decrease at week 4. The control group of 42 patients consisted of 36 with a >1 log IU/ml HCV RNA decrease and six with a <1 log IU/ml decrease at week 4. A comparison of the baseline characteristics of these two groups showed that platelet counts and serum vitamin D concentrations at baseline were significantly higher in the control group, but there were no other significant between group differences (Table 1). In the control group, one patient died of lung cancer during week 7, and one could not

continue treatment because of general malaise. In the vitamin D group, one patient discontinued at week 13 due to neurosis. Overall, 41 patients in the vitamin D group and 40 in the control group completed 24 weeks of treatment.

Serum vitamin D concentration

Compared with baseline concentrations, the mean serum vitamin D concentrations at 12 and 24 weeks increased 9.8 ng/ml and 19.6 ng/ml, respectively, in the vitamin D group. In contrast, serum vitamin D concentrations at 12 and 24 weeks, decreased 3.6 ng/ml and 4.1 ng/ml, respectively, in the control group.

Efficacy

The viral response (VR) rate at week 24 was significantly higher in the vitamin D than in the control group (78.6% [33/42] vs 54.8% [23/42]; P=0.037; Fig. 3). We also assessed VR rates relative to IL28B genotype (Fig. 4). Of patients with the TT genotype, those in the vitamin D group had a significantly higher VR rate than those in the control group (86.2% [25/29] vs 63.3% [19/30]; P=0.044), but there was no between group difference in patients with the TG and GG genotypes. However, the median decline in viral load from week 8 to week 24 in patients with the TG and GG genotypes was significantly greater in those who did (2.65 log IU/ml [range, 0-2.7 log IU/ml]) than did not (1.1 log IU/ml [range, 0.1-5.0 logIU/ml]) receive vitamin D supplementation (p=0.022)

In assessing factors associated with HCV RNA negativity within 24 weeks, we compared the 56 patients with undetectable HCV RNA and the 28 positive for HCV RNA at week 24 (Table 2). We

found that IL28B genotypes (TT vs TG/GG p=0.003) and vitamin D supplementation (p=0.029) differed significantly between these two groups.

Finally, to assess factors contributing to VR rate at week 24, we performed stepwise logistic regression analyses. Factors included IL28B genotype and vitamin D supplementation, together with γGTP and AST, which both had P-values <0.1 in univariate analysis. We found that IL28B genotype (TT/TG vs GG: adjusted odds ratio [OR]=3.12, 95% confidence interval [CI]=1.03-9.5) and vitamin D supplementation (yes vs. no: adjusted OR=5.85, 95% CI=1.75-9.57) were significant predictors of VR rate at week 24.

Safety

The adverse effects in both groups were those common to PEG-IFN/RBV, including fever, headache, general malaise, nausea, insomnia, myalgia, anemia, neutropenia, and thrombopenia. No serious side effects were observed. One patient in the control group discontinued treatment because of anxiety neurosis, and one in the vitamin D group discontinued because of general fatigue. Adherence to PEG-IFN/RBV did not differ significantly in the two groups, with no differences in dose reduction for adverse effects of PEG-IFN/RBV. There were no adverse effects associated with vitamin D supplementation.

Discussion

Vitamin D is supplied by food, as well as being synthesized within the body. Vitamin D consists of vitamin D_2 and D_3, with the latter dominant in mammals. Vitamin D_3 is synthesized in skin from 7-dehydrocholesterol through the action of ultraviolet rays and heat. Vitamin D_3 is

metabolized by the liver to 25(OH) vitamin D_3, which is carried to the kidney and converted to $1,25(OH)_2$ vitamin D_3, an active form of vitamin D_3 [21-23]. Vitamin D_3 derived from food is absorbed in the body through lymphatic vessels, and is similarly metabolized in the liver and kidneys. Although we thought that the ability to convert to 25(OH) vitamin D3 would have declined in the liver of patients with chronic hepatitis, serum vitamin D3 concentrations after 24 weeks of vitamin D supplementation were elevated, whereas those in the control group were reduced. The latter may be due to reduced outdoor activities and a decline in dietary intake during PEG-IFN/RBV treatment.

The mechanism by which vitamin D enhances the antiviral effects of PEG-IFN/RBV remain unclear. An in vitro study showed that 25(OH) vitamin D_3, but not vitamin D_3 or $1,25(OH)_2$ vitamin D_3, dose-dependently reduced the intra- and extra-cellular concentrations of HCV core antigen, suggesting that 25(OH) vitamin D_3 may be more effective than other forms of vitamin D_3 during interferon treatment [24]. Furthermore, both vitamin D_3 and $1,25(OH)_2$ vitamin D_3 were found to reduce infectious virus production [25]. The effects have been ascribed to 1,25(OH) vitamin D_3 enhancement of IFNβ expression and induction of IFN-stimulated genes (ISGs). Although type 1 IFN has been reported to induce approximately 300 ISGs, which express effector molecules with antiviral activities. Another study, however, found that 25(OH) vitamin D_3 did not induce ISGs [26] or HCV entry or replication, but did affect the assembly of selective components [24]. These differences may have been due to differences in viral sequences examined. Recently, vitamin D was recognized as not only playing an important role in the

formation and maintenance of bone, but in defenses against autoimmune diseases and cancer as well. Vitamin D deficits increase the risk of malignancies, particularly of the colon, breast and prostate gland, and of chronic inflammatory and autoimmune diseases [27]. Low vitamin D concentrations associated with severe fibrosis in chronic HCV-infected patients.[13] Improvements of vitamin D deficiency may be due to the effects of interferon treatment.

Our randomized trial assessed whether vitamin D supplementation of chronic HCV-infected patients treated with PEG-IFN/RBV improved VR rate at week 24. Our primary end point, VR rate at week 24, was better and more simply able to assess the antiviral effects of vitamin D. VR rate at week 24 was significantly higher in the vitamin D than in the control group (78.6% vs. 54.8%, p=0.037). Serum concentrations of 25(OH) vitamin D_3 increased during vitamin D supplementation, along with HCV eradication, suggesting an antiviral effect of vitamin D. Vitamin D deficiency has been reported in HCV-infected patients without advanced fibrosis [28], with a functional CYP27B1-1260 polymorphism associated with diminished active $1,25(OH)_2$ vitamin D_3 concentrations, resulting in a poor response to IFN-based therapy. Moreover, the polymorphism (rs10877012) was associated with response to IFN-based therapy in patients [29].Serum vitamin D concentration and the IL28B (rs12979860 C/T) polymorphism were reported to be two independent factors for SVR in intractable patients [30]. Rather than evaluating rs12979860, we evaluated but rs8099917, because of the strong linkage disequilibrium between these two IL28B polymorphisms in Japanese HCV patients. When patients were divided by IL28B SNP rs8099917 genotype, those with the TT genotype, who are more

sensitive to IFN, had a significantly higher VR rate at week 24 with than without vitamin D (86.2% vs. 63.3%, p=0.044). In contrast, patients with the TG and GG genotypes, who are relatively resistant to IFN treatment, had similar VR rates at week 24, regardless of vitamin D supplementation, although viral load showed a significantly greater decrease after week 8 in patients who did than did not receive vitamin D. These findings suggest that the antiviral effects of vitamin D may not be strong enough to eradicate the virus in poor responders to IFN. Indeed, our multivariate analysis showed that the rs8099917 genotype and vitamin D supplementation were factors significant for VR at week 24.

PEG-IFN/RBV treatment may be prolonged in patients showing a slow decline in serum HCV RNA, so-called late viral responders, to increase the likelihood of SVR. Japanese guidelines recommend that PEG-IFN/RBV be continued for 72 weeks in patients remaining positive for HCV RNA at week 13 or later. Since it is difficult to compare SVR rates in patients treated with PEG-IFN/RBV for 48 or 72 weeks, our primary end point was not SVR.

Our study had several limitations. First, we did not assess final SVR rate. As explained above, however, VR rate at week 24 may be a better assessment factor. Second, our sample size was insufficiently large, and all patients were Japanese, so our results may not be replicated in other ethnic populations.

In conclusion, vitamin D supplementation improved VR rate at week 24, suggesting that vitamin D supplementation be included in HCV patient being treated with PEG-IFN/RBV.

ACKNOWLEDGEMENTS

The authors thank Dr. Keitarou Yamashina and Dr. Michihiro Nonaka for the cooperation.

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