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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.

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Figure captions

Figure 1. Study design.

To properly determine the effect of Vitamin D, patients who became HCV RNA negative at week 4 were excluded. The remaining patients were stratified according to viral load (decline in HCV RNA level at week 4 <1 log IU/ml or ≥1 log IU/ml) and randomly assigned to a vitamin D or control group. The patients randomized to the vitamin D group received 1,000 IU /day vitamin D, beginning at week 8 and continuing until the end of treatment.

Figure 2. Enrollment, randomization or assignment, and follow-up of study patients

Forty-two patients were randomized to the vitamin D group and 42 to the control group.

Figure 3. HCV RNA disappearance rate at week 24 in the vitamin D and control groups.

All patients were treated with PEG-IFN plus RBV, with the vitamin D group also receiving vitamin D. IL28B TT: rs8099917 genotype TT patients, who were sensitive to IFN treatment

Figure 4. Differences in HCV RNA disappearance rate at week 24 stratified by IL28B genotype.

VR rates at week 24 were assessed in patients with the IL28B SNP rs8099917 TT and TG/GG genotypes randomized to the vitamin D and control groups. Vitamin D(-): control group, treated with PEG-IFN/RBV. Vitamin D(+): vitamin D group, treated with PEG-IFN/RBV plus vitamin D. IL28b TT: IL28B SNP rs8099917 genotype TT, which is sensitive to IFN therapy. IL28B TG/GG: IL28B SNP rs8099917 genotypes TG/GG, which are relatively resistant to IFN therapy.

Figure 5. Decrease in serum viral load from weeks 8 to 24 in patients with the TG/GG genotypes randomized to the vitamin D and control groups. The decrease in viral load was

significantly greater in the vitamin D than in the control group.

Table 1. Baseline Characteristics of patients in the control and vitamin D groups.

Characteristic		Control Group n=42	Vitamin D group n=42	P value
Age	years	59 (36-70)	60 (30-78)	0.59
Male:Female	ratio	23:19	20:22	0.58
Previous IFN* therapy	No/yes	28 / 9	32 / 9	1
Body mass index	Kg/m^2	22.5 (18.9-31.6)	22.1 (18.1-27.7)	0.43
Stage of fibrosis	0-2/3,4/ND	19 / 17 / 5	17 / 12 / 13	0.15
IL28B rs8099917	TT/TG/GG/ND	30 / 8 / 0 / 2	29 / 8 / 2 / 3	0.89
HCV-RNA log_{10}	IU/ml	6.3 (4.7-7.3)	6.3 (4.7-7.3)	1
HCV genotype 1b	No.(%)	41(100%)	42(100%)	
Platelet count	no./μl	18.3 (8.3-36.2)	15.2 (7.8-27.2)	0.01
Hemoglobin	g/dl	14.5 (10.9-16.4)	13.7 (11.7-17.0)	0.15
ALT*	IU/L	60.0 (10.0-495.0)	51.0 (14.0-250.0)	0.34
AST*	IU/L	51.0 (12.0-236.0)	44.5 (16.0-167.0)	0.31
γ-GTP*	IU/L	15.5 (13.0-282.0)	30.0 (11.0-240.0)	0.10
HbA1c	%	5.1 (3.9-10.2)	5.1 (4.4-11.8)	0.47
T-Cho*	mg/dl	173.0 (125.0-242.0)	180.0 (113.0-257.0)	0.37
25(OH)vitamin D3	ng/ml	25.0 (14.0-40.0)	22.0 (12.0-29.0)	0.028

*ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyl transpeptidase; HbA1c, hemoglobin A1c; T-Cho, total cholesterol