Association of vitamin D receptor gene polymorphisms with response to peginterferon plus ribavirin in Asian patients with chronic hepatitis C


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Background/Purpose: Recent studies have shown that serum vitamin D deficiency is a negative predictor of response to peginterferon plus ribavirin therapy for Caucasian patients with chronic hepatitis C (CHC). Whether vitamin D receptor (VDR) gene polymorphisms associate with antiviral response in Asian CHC patients remains unclear.

Methods: We recruited 139 Asian patients with CHC genotype-1 who achieved 80/80/80 adherence of response-guided peginterferon plus ribavirin therapy. BsmI rs1544410, ApaI rs7975232, and TaqI rs731236 were genotyped and related to clinical and virological features and to treatment outcome.

Results: Patients carrying bAt [CCA] haplotype (p = 0.033), Apal CC genotype (p = 0.033), and TaqI AA genotype (p = 0.037) had a higher HCV load as compared to those with other haplotypes, Apal CA/AA genotype and TaqI AG genotype, respectively. A sustained virological response (SVR) was achieved in 74 (53%) of the patients. Polymorphisms in VDR gene did not correlate with rapid virological response and SVR achievement. Stepwise logistic regression analysis showed that rs12979860 CC type [odds ratio (OR): 5.56, p = 0.007], platelet counts ≥ 15 x 10^11/L (OR: 4.80, p = 0.001), and rapid virological response achievement (OR: 8.36, p < 0.001) were independent factors of SVR.

Conclusion: Despite their associations with high hepatitis C virus load, VDR gene polymorphisms are not related to the response to peginterferon plus ribavirin therapy in Asian CHC patients.

Conflicts of interest: The authors have no conflicts of interest relevant to this article.

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**Introduction**

Hepatitis C virus (HCV) is one of the most common causes in chronic infection resulting in liver cirrhosis and hepatocellular carcinoma (HCC). The associated complications, mortality, and need for liver transplantation are global problems. Currently, the adjunction of new direct antiviral agents is changing the therapeutic approach in chronic HCV infection. However, a significant number of patients are treated with the classic combination therapy of peginterferon and ribavirin where these new therapies cannot be afforded. Consequently, to identify patients who are or are not good candidates for peginterferon plus ribavirin therapy is clinically important to avoid unnecessary treatments and minimize adverse effects. Several factors probably affecting response to peginterferon plus ribavirin therapy have been reported, including age, interleukin (IL)28B polymorphisms, liver fibrosis, insulin resistance, and viral factors, such as HCV load and HCV variations.

Vitamin D is involved in the metabolism of skeleton as a systemic hormone but also has important roles in the regulation of host immune responses, fibrogenesis, and development of cancer through vitamin D receptor (VDR). Interestingly, a recent study showed that lower serum 25-hydroxylation (OH)D level was an independent negative risk factor for sustained virological response (SVR) to peginterferon plus ribavirin therapy for Caucasian patients with chronic hepatitis C. Other studies have also reported that vitamin D supplementation may enhance the antiviral response of peginterferon plus ribavirin therapy, but controversy still exists. In addition, VDR gene polymorphisms have been investigated in the context of some chronic liver diseases, such as chronic hepatitis B, primary biliary cirrhosis and autoimmune hepatitis. Another study has demonstrated that bioactive 1,25-di-(OH)D3 enhanced the inhibitory effect of interferon-α on HCV replication, and has identified the VDR as a novel suppressor of interferon-α-induced signaling through the Jak–STAT pathway.

So far, there are limited data on the association between VDR polymorphisms and the antiviral response in chronic hepatitis C. In this study, we conducted a cohort of chronic hepatitis C patients in Asia receiving response-guided therapy with peginterferon plus ribavirin to clarify this issue.

**Methods**

**Patients**

From August 2011 to July 2013, a total of 151 naive patients with chronic HCV genotype-1 infection who received peginterferon plus ribavirin therapy in single medical center in Taiwan were enrolled. All patients were seropositive for HCV antibody and HCV RNA, and all had exhibited elevated alanine aminotransferase levels. Patients were excluded if they were positive for serum hepatitis B surface antigen or anti-human immunodeficiency virus antibody, or exhibited other causes of hepatocellular injury (e.g., any history of alcoholism, autoimmune hepatitis, primary biliary cirrhosis, or treatment with hepatotoxic drugs). Clinical diagnosis of cirrhosis was based on repeated ultrasound findings suggestive of cirrhosis at least twice 3 months apart, supplemented with clinical criteria or other signs of portal hypertension. In these 151 patients, we excluded 12 patients who did not fit the 80/80/80 adherence rule (< 80% of total peginterferon or ribavirin doses or < 80% of the total duration of therapy), with a final case number of 139.

Patients were treated according to the on-treatment response as follows: 24 weeks for patients achieving a rapid virological response (RVR, seronegativity of HCV RNA at 4 weeks of therapy); 48 weeks for those with an early virological response (EVR, at least a 2 – log10 decrease from baseline of serum HCV RNA at 12 weeks of treatment); and early termination (< 16 weeks) in those without an EVR. This protocol has been recommended by the National Health Insurance Bureau in Taiwan since November 2009. All patients received either peginterferon α-2a (180 μg/week) or peginterferon α-2b (1.5 μg/kg/wk) subcutaneously plus weight-based ribavirin (1000 mg/d for weight < 75 kg and 1200 mg/d for weight > 75 kg). The endpoint of the study was achievement of a SVR, defined as seronegativity of HCV RNA throughout 24 weeks of post-treatment follow-up period. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board of the hospital.

**Qualitative and quantitative assay of HCV-RNA and HCV genotyping**

Serum was prepared in a laminar flow bench and frozen at −70°C until use. Before treatment, qualitative detection of HCV RNA was performed by a standardized qualitative reverse transcription–polymerase chain reaction (PCR) assay (AmpliSpec; Roche Diagnostics, Branchburg, NJ, USA), using biotinylated primers for the 5’ noncoding region. The lowest detection limit of this assay was 90 IU/mL. Serum HCV RNA levels were determined by COBAS TaqMan HCV Test (TaqMan HCV; Roche Molecular Systems Inc., Branchburg, NJ, USA; lower limit of detection: 15 IU/mL). Genotyping of HCV was performed by reverse hybridization assay (Inno-LiPA HCV II; Innogenetics N.V., Gent, Belgium) using the HCV-Amplicor products.

**Detection of VDR and IL28B polymorphisms**

The DNA was extracted from peripheral blood leukocytes using the Qiagen DNA isolation kit (Qiagen, Hilden, Germany). The VDR genotype was determined by PCR amplification and restriction length fragment polymorphisms as previously described. For the detection of Bsml polymorphisms, a forward primer in exon 7 (5’-CAACCAA-GACTCAAGTACCCGTCGTA-3’) and a reverse primer in intron 8 (5’-AACCAGCGAAAGTGCAACGCA-3’) were used. For the detection of Apal and TaqI polymorphisms, a forward primer in exon 8 (5’-CAGACGCTGACGGGAGCCAA-3’) and a reverse primer in exon 9 (5’-GCAAATCTCATGGCTGAGGAT-3’) were used. The PCR products for Bsml polymorphisms were 820 base pairs (bp), and for Apal/TaqI polymorphisms they were 745 bp. The PCR mix contained
annealing step for 45 seconds at optimum temperature for 2 minutes was added after the last PCR cycle. A total of 40 cycles of PCR were performed, consisting of a denaturation step for 45 seconds at 94°C for 2 minutes at 72°C for 1 minute. The fragments were separated on gels of 2% agarose. The presence of BsmI restriction site resulted in two fragments (645 bp and 177 bp). Digestion with Apal produced two fragments of 531 bp and 214 bp when the restriction site was present. Digestion with TaqI resulted in three fragments of approximately 205 bp, 290 bp, and 245 bp in the presence of TaqI polymorphic site, and in fragments of 245 bp and 495 bp in its absence.

IL28B variant rs12979860C>T was diagnosed from stored samples using direct sequencing (AmpliTaq gold DNA polymerase and BigDye terminator v1.1 cycle sequencing kit; Applied Biosystems, Warrington, Cheshire, UK). The PCR products were separated on an ABI3130 sequencer, and analyzed with SEQSCAPE 2.6 (Applied Biosystems). Primers for amplification were: forward primer: 5’-ATTCTCTG-GACGTGGATGGGTACT-3’, reverse primer: 5’-biotin-GGAGCGCCGGAGTGCAATT-3’. The sequencing primer required for the detection of a short DNA sequence around the SNP of interest was 5’-AGCTCCCCCGAGGCG-3’.

5 µL of each primer (10 pmol), 5 µL buffer, 1.5 µL MgCl2 (50 mM), 5 µL template DNA (50–100 ng), 5 µL dNTPs (2 mM), Taq polymerase (MBI Fermentas, St. Leon-Rot, Germany) 2 µL, H2O 26.5 µL. The DNA template was denatured at 95°C for 2 minutes. A total of 40 cycles of PCR were performed, consisting of a denaturation step for 45 seconds at 94°C, an annealing step for 45 seconds at optimum temperature (67°C for Apal/TaqI and 60°C for BsmI), and an extension reaction for 1 minute at 72°C. A final extension step at 72°C for 2 minutes was added after the last PCR cycle.

After amplification, the PCR products were digested with BsmI, Apal, and TaqI endonucleases. Following restriction endonuclease digestion, genotyping was determined by ethidium bromide-UVB illumination of the fragments separated on gels of 2% agarose. The presence of BsmI, Apal, or TaqI restriction sites was defined as the lower-case “b”, “a”, or “t”, respectively, and the absence of the sites defined as the upper-case “B”, “A”, or “T”.

### Statistical analysis

Continuous data are expressed as mean ± standard deviation, and the categorical data are expressed as number (percentage). Comparisons of differences in categorical data between groups were performed using the Chi-square test. Distributions of continuous variables were analyzed by the Student t test or Mann–Whitney U test for the two groups where appropriate. Independent factors possibly affecting response to peginterferon plus ribavirin therapy were determined by stepwise multiple logistic regression analysis. A p value < 0.05 was considered statistically significant.

### Results

#### Baseline characteristics

The basic demographic, virologic, and clinical features of the patients are shown in Table 1. There were 71 men and 68 women, age 20–78 years (mean, 56 years). Fourteen percent (n = 19) of patients had cirrhosis. Of these patients, 43 (31%) patients achieved a RVR, 87 (62.6%) achieved an EVR without RVR and nine (6.4%) had no EVR. The rates of SVR were 84% (n = 36) of RVR patients, 44% (n = 38) of EVR patients and 0% of non-EVR patients, respectively.

#### Comparison between bAt [CCA]-haplotype and other haplotypes

Of these patients, 76 (55%) carried bAt [CCA] haplotype. As shown in Table 2, univariate analysis revealed that only HCV viral load was significantly different between these two groups. Figure 1 shows the association of VDR genotype with HCV load. We found that the carriage of Apal CC genotype and TaqI AA genotype had significant higher viral load as compared to those with Apal CA/AA type and TaqI AG type, respectively.
Factors associated with sustained virological response to peginterferon plus ribavirin in a response-guided therapy

As shown in Figure 2, patients carrying the bAt [CCA] haplotype, ApaI CC genotype, and TaqI AA genotype had a trend in lower rate of achieving RVR and SVR. However, there were no significant associations of VDR gene polymorphisms with RVR and SVR achievement. By univariate analysis, the carriage of rs12979860 CC genotype, cirrhosis, higher platelet counts, and RVR were significantly associated with SVR. Stepwise logistic regression analysis revealed that rs12979860 CC genotype, higher platelet counts and RVR were independent predictors of SVR among these patients (Table 3).

Discussion

Experimental evidence suggests the potential ability of vitamin D, through interaction with VDR, to regulate the host immune responses, fibrogenesis, and carcinogenesis. One of the common genetic variations of VDR gene is the bAt haplotype consisting of three adjacent restriction polymorphic sites, BsmI, ApaI, and TaqI. These genetic variations have been described as important modulators of several chronic liver diseases such as primary biliary cirrhosis and autoimmune hepatitis. In addition, our previous data showed that chronic hepatitis C patients with HCC had a higher frequency of ApaI CC genotype and bAt [CCA] haplotype than controls, and ApaI C polymorphism might be used as a molecular marker to predict the risk of HCC. In this
study, we found significant associations of bAt [CCA] haplotype, Apal CC genotype, and TaqI AA genotype with higher HCV load. Although the detailed mechanisms remain to be clarified, these observations could be explained by the immunomodulatory effects of vitamin D, and thus polymorphisms in VDR could affect the host response against HCV.

Two recent studies have reported that the VDR bAt [CCA] haplotype impairs antiviral response to peginterferon plus ribavirin therapy in Caucasian patients with chronic hepatitis C. By contrast, we failed to find a significant association between VDR gene polymorphisms and treatment response, although there was a trend in lower rate of SVR among patients carrying bAt [CCA] haplotype, ApaI CC genotype, and TaqI AA genotype. These discordant results might be associated with the following facts. First, the C allele of the IL-28B rs12979860 C/T polymorphism is much more prevalent in Asians than in Caucasians, and patients with IL28B favorable genotype attain a high SVR rate up to 70–80% in genotype-1 chronic hepatitis C patients. Therefore, it is possible that the high prevalence of the C allele results in blunting of the effect by vitamin D. This possibility is supported by the finding that a group of Caucasian patients with IL28B favorable genotype who had higher 25(OH)D concentrations had an SVR rate of 85.7%, whereas those with IL28B unfavorable genotype who also had higher 25(OH)D concentrations had an SVR rate of only 36.8%. Second, it is well established that there are racial differences in the relations between the 25(OH)D concentrations, parathyroid hormone concentration, and calcium homeostasis. Racial differences in vitamin D physiology or race-specific factors that modify the effects of vitamin D may affect the immune response to HCV. Further studies with an increased number of cases are necessary to reach a firm conclusion.

A number of studies have evaluated an association between baseline 25(OH)D level and SVR to peginterferon plus ribavirin therapy in chronic HCV infection. However, studies have yielded inconsistent results because of variation in the 25(OH)D assay used, and the different ethnicities and geographic latitude of populations studied. In our study, the retrospective design precluded us to determine serum vitamin D and hence to analyze the possible influence of the studied polymorphisms on serum concentrations of vitamin D. However, this could be justified since VDR gene variants modulate biological effects of vitamin D without influencing serum vitamin D levels. In addition, serum 25(OH)D levels strongly fluctuate during seasons, with age, and as a consequence of numerous other conditions.

In conclusion, the present study suggests a significant association of VDR bAt [CCA]-haplotype, Apal CC genotype, and TaqI AA genotype with higher viral load in Asian patients with chronic HCV infection. However, VDR gene polymorphisms are not related to the response to peginterferon plus ribavirin therapy in our study population. The detailed molecular mechanisms deserve further investigation.

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References


