**Brief Original Article**

**Relationships between low serum vitamin D levels and HBV “a” determinant mutations in chronic hepatitis B patients**

Huijuan Zhu¹, Xingxiang Liu², Yi Ding³, Hui Zhou³, Yiyong Wang¹, Zhiwei Zhou¹, Xinxin Li⁴, Zengli Zhang¹, Chen Dong¹

¹ Department of Epidemiology and Statistics, School of Public Health, Jiangsu Key Laboratory and Translational Medicine for Geriatric Disease, Medical College of Soochow University, Suzhou, China
² Hua’ian Fourth Hospital, Hua’ian, China
³ Suzhou Industrial Park Centers for Disease Control and Prevention, Suzhou, Jiangsu, China
⁴ Zhangjiagang Women and Children Health Center, Suzhou, China

**Abstract**

Introduction: Vitamin D is significantly associated with virus replication in chronic hepatitis B virus (HBV) infection. However, the relationship between low serum vitamin D levels and HBV “a” determinant mutations remains unknown.

Methodology: A total of 133 chronically HBV-infected, treatment-naive patients were randomly selected in the present study. Serum vitamin D levels were measured by using liquid chromatography-mass spectrometry. The HBV “a” determinant was amplified, sequenced, and analyzed by nested polymerase chain reaction (PCR).

Results: Among 133 patients, 36, 88, and 9 patients had vitamin D deficiency (25(OH)D < 14 ng/mL), vitamin D insufficiency (25(OH)D ≥ 14 and < 30 ng/mL), and normal vitamin D serum levels ((25(OH)D ≥ 30 ng/mL), respectively. As results showed, 36 [11 genotype B HBVs (HBV/B) and 25 genotype C HBVs (HBV/C)] were isolated from the vitamin D-deficient group, 88 (48 HBV/B and 40 HBV/C) from the vitamin D-insufficient group, and 4 HBV/C strains from the normal serum-vitamin D group. Compared to the HBV/B infected patients with vitamin D insufficiency, higher rates of amino acid mutation within “a” determinant were detected in HBV/B-infected, vitamin D-deficient patients. Moreover, the change frequency of M133 was 27.27% in HBV/B infected patients with vitamin D deficiency, which was significantly higher than those in the vitamin D-insufficient group (p = 0.040).

Conclusions: Vitamin D deficiency is significantly associated with genotype B HBV “a” determinant mutations.

**Key words:** hepatitis B virus; vitamin D; deficiency.


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**Introduction**

Hepatitis B virus (HBV) infection is the most common cause of acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) worldwide. To date, more than two billion individuals have been infected, of which 350 million are chronic carriers of the virus [1,2]. The genome of HBV is a circular, partially double-stranded DNA of approximately 3.2 kb. According to genetic heterogeneity, HBV can be classified into eight genotypes (A–H), and recently, two additional genotypes (I and J) were proposed [3]. The virus genome has four open reading frames encoding hepatitis B surface antigen (HBsAg), core antigen, X peptide, and DNA-polymerase enzyme. High genetic variability is a characteristic feature of HBV because virus polymerase lacks proofreading activity and very high virion production per day (> 1,000 viruses) [4]. In addition to natural evolutionary changes that contribute to genetic variations, increased genome mutations might also be caused by the host immune system in order for the virus to evade immune clearance [5,6].

Vitamin D is not only a secosteroid hormone with classical actions related to mineral metabolism and skeletal health, but also acts as an important modulator involved in cell proliferation and differentiation, immunomodulation, and inflammation [7-10]. According to World Health Organization (WHO) recommendations, 25(OH)D (the main circulation form of vitamin D) levels of (≥ 14 and < 30 ng/mL) and < 14 ng/mL are defined as vitamin D insufficiency and vitamin D deficiency, respectively. In clinical trials, the therapeutic value of the immunomodulatory effect of vitamin D on tuberculosis patients has been previously reported [11,12]. In a reporter assay system, the results...
showed that vitamin D could inhibit the replications of hepatitis C virus (HCV) [13]. Recently, Farnik et al. found that there is a significant association between low serum concentrations of 25(OH)D3 and higher levels of HBV replications in chronically infected patients [14]. In order to further understand the effects of vitamin D metabolism on chronic hepatitis B virus infection (CHB), we performed this study to analyze the relationships between low serum vitamin D levels and the mutations of HBV “a” determinant in CHB patients.

Methodology

Study subjects
From January 2014 to September 2014, 133 chronically HBV-infected patients were randomly selected from two hospitals of Jiangsu province, China. Because both IFN-α and some nucleoside/nucleotide analog inhibitors may have an effect on vitamin D metabolism, inclusion criteria for the present study were chronic infection with HBV, defined as a positive result of HBsAg (HBsAg+) and HBV DNA ≥ 6 months, age ≥ 18 years, and treatment-naive status. Patients were excluded if they were co-infected with HCV, human immunodeficiency virus (HIV), or hepatitis delta virus (HDV), if excessive alcohol consumption (> 40 g/day) had been reported, if they had received a liver allograft, or if a malignant disease (including HCC) or diabetes mellitus had been diagnosed. This work was approved by the ethics committee of Huai’an Fourth Hospital.

Laboratory testing
The serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured with commercial reagents. Serum 25(OH)D were measured using liquid chromatography-mass spectrometry (LC-MS) as previously described [19]. HBV titer was analyzed by real-time polymerase chain reaction (RT-PCR) with a diagnostic kit for quantification of hepatitis B virus DNA (Kehua Bio-engineering Co., Shanghai, China). The range of the quantification of HBV DNA was 500–10^5 copies/mL.

HBV DNA isolation, amplification, and sequencing
HBV DNA was extracted from 200 μL serum samples by using a viral RNA/DNA extraction kit (TaKaRa Biotechnology, Dalian, China). HBV DNA was eluted in 30 μL buffer and stored at -80°C until further analysis.

Next, 437 bp of the HBsAg gene (nt220–nt656, referred to as HBV-Chi89, AB073826) was amplified by nested PCR. The primers DZ-1, 5’-CTAGGACCCCTGCT CGTGTTAC and DZ-4, 5’-CACTGAACAAATGGC ACTAG were used for the first round of PCR, and primers DZ-2, 5’-GACAAGAACCTCTACAAATC and DZ-3, 5’-CTGAGGCCCAC TCCC CATAG were used for the nested PCR. Both first- and second-round PCR consisted of pre-denaturation at 94°C for 2 minutes, followed by 94°C for 45 seconds and 55°C for 30 seconds, extension at 72°C for 45 seconds for 35 cycles, and final extension at 72°C for 7 minutes. The PCR product was purified and sequenced using the ABI 3730 (Applied Biosystems, Foster City, USA).

Phylogenetic analysis and mutation analysis
A consensus sequence for each DNA fragment was generated on the basis of the plus and minus strand sequence using Lasergene 7.0. Due to the poor quality of some of the sequences, the sequences of the S gene were cropped to nt 236–631. A phylogenetic tree was constructed using the neighbor-joining (NJ) algorithm in MEGA version 4.1 software. The reliability of the phylogenetic tree was tested by bootstrap analysis with 1,000 replicates.

Nucleotides/amino acid variability was defined as the proportion of sequences that did not harbor the nucleotides/amino acids found in HBV reference sequences of the same genotype. For analysis, HBV “a” determinant (124–147aa) were compared with the reference wild-type sequences of the same genotype in BioEdit software.

Statistical analysis
An unpaired student’s t-test was used to test for differences between different groups of continuous variables. The prevalence of nucleotide and amino acid mutations between genotype B and C virus group were compared using the χ2 test or Fisher’s exact test. All statistical analysis was performed using SAS version 9.2 software. A two-sided p value of < 0.05 denoted statistical significance.

Nucleotide sequence accession numbers
The isolates characterized in this study were deposited in GenBank under accession numbers KP410408–KP410466 (except KP410436, KP410448, and KP410462), KP410468–KP410521 (except KP410477, KP410499, and KP410508), and KP410523–KP410546 (except KP410532, KP410541, and KP410544).
Results

Characteristics of CHB patients with lower serum vitamin D levels

A total of 133 patients were selected in the present study according to the above-described criteria. The mean serum level of 25(OH)D was 19.36 ng/mL (standard deviation: 8.18 ng/mL). The demographic characteristics are summarized in Table 1. Of the patients, 36, 88, and 9 had vitamin D deficiency (25(OH)D < 14 ng/mL), vitamin D insufficiency (25(OH)D ≥ 14 and < 30 ng/mL), and normal vitamin D serum levels (25(OH)D ≥ 30 ng/mL), respectively. There were no significant differences in age, gender, HBV quantities, ALT, and AST among these three groups.

Phylogenetic analysis

In the present study, 124 HBV sequences were isolated from 36 and 88 patients in the vitamin D-deficient group and vitamin D-insufficient group successfully. However, only 4 sequences were amplified from 9 patients with normal serum vitamin D. The phylogenetic tree constructed by 128 isolated HBV strains is shown in Figure 1. Of 128 HBV sequences isolated in this study, 59 and 69 sequences were clustered into genotype B HBV (HBV/B) and genotype C HBV (HBV/C), respectively. As the results show, 4 sequences amplified from the patients who had normal serum vitamin D belonged to genotype C, and 36 sequences from the vitamin D-deficient group comprised 11 genotype B and 25 genotype C HBV strains. Among 88 sequences isolated from the vitamin D-insufficient group, 48 sequences belonged to genotype B, and 40 sequences belonged to genotype C. Because only 4 genotype C HBVs were isolated from the patients with normal serum vitamin D levels, these sequences were not included in the further mutation analysis.

Amino acid mutations in “a” determinant

Of 11 HBV/B-infected patients in the vitamin D-deficient group, 4 patients had a total of 6 amino acid substitutions in “a” determinant. However, among 48 HBV/B-infected cases in the vitamin D-insufficient group, 6 patients (12.50%) had amino acid substitutions in “a” determinant. Of these 6 patients, 5 had a single mutation (83.33%), and 1 (16.67%) had a combination of 2 mutations, making a total of 7 mutations (Table 2). The amino acid substitution rates in the vitamin D-deficient group was significantly higher than those in the vitamin D-insufficient group (p = 0.021) (Table 2).

As shown in Table 2, (0.33%) mutated amino acids were detected in 25 HBV/C-infected cases in the vitamin D-deficient group. Similarly, there were 0.73% of amino acids substituted in “a” determinant in 40 HBV/C-infected patients in the vitamin D-insufficient group. No significant difference of amino acid substitution rates was observed in the two groups (p = 0.496). However, in the vitamin D-deficient group,

Table 1. Characteristics of chronic hepatitis B patients recruited in the present study.

<table>
<thead>
<tr>
<th>Variable</th>
<th>25(OH)D &lt; 14 ng/mL (n = 36)</th>
<th>14 ≤ 25(OH)D &lt; 30 ng/mL (n = 88)</th>
<th>25(OH)D ≥ 30 ng/mL (n = 9)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>40.36 ± 11.30</td>
<td>40.58 ± 11.75</td>
<td>42.89 ± 14.38</td>
<td>0.841</td>
</tr>
<tr>
<td>Gender (males), n (%)</td>
<td>24 (66.67%)</td>
<td>64 (72.73%)</td>
<td>9 (100%)</td>
<td>0.132</td>
</tr>
<tr>
<td>HBV DNA, log10 IU/mL</td>
<td>5.71 ± 1.67</td>
<td>5.11 ± 1.87</td>
<td>4.93 ± 1.31</td>
<td>0.308</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>61.99 ± 51.12</td>
<td>51.08 ± 66.14</td>
<td>61.33 ± 32.03</td>
<td>0.627</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>53.24 ± 51.14</td>
<td>42.66 ± 39.90</td>
<td>37.02 ± 8.60</td>
<td>0.376</td>
</tr>
</tbody>
</table>

ALT: alanine aminotransferase; AST: aspartate aminotransferase

Figure 1. Phylogenetic analysis based on nucleotide sequencing of the 396 bp (nt236–nt631) of S region.

59 strains of genotype B HBV (“●”) and 69 strains of genotype C HBV (“▲”) were isolated from Jiangsu, China. Genotype A (AB194949, AJ309369, AM180623, AJ233275, AF090838), genotype B (AB033554, D00329, EF494380, EU579441, FJ562257), genotype C (AB014381, AF330110, AJ74809, AF040627, EF494376), genotype D (AB554016, HE815465, KC875317, X65259, X85254), genotype E (AB091255, AF323631, AJ605025, AP007262, AR488645), genotype F (AB36905, AF223965, AJ179735, DQ823086, HE981185), genotype G (AB064310, AF160501, AP007264, DQ077986, EF464098), and genotype H (AB179747, AP007261, AF090454, EF157291, EU498228) were used as reference HBV strains.

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significantly higher rate of amino acid substitutions was determined in the patients with HBV/B infection compared to those with HBV/C infection (p = 0.012).

Frequency of amino acid substitution in “a” determinant

The frequency of amino acid substitution for each individual position of “a” determinant in the studied patients is shown in Table 3. In total, 22 amino acid residues were affected: T/I126 (7), P127 (3), Q129 (1), T131 (2), M133 (5), T140 (1), T/S143 (1), and G145 (2). Eight occurred in the patients in the vitamin D-deficient group, and were 14 detected in the vitamin D-insufficient group. The most frequently found substitution was T/I126, with an overall prevalence of 5.65%. Furthermore, the change frequency of M133 was 27.27% in HBV/B-infected patients with serum 25(OH)D < 14 ng/mL, which was significantly higher than that in the HBV/B-infected patients in the vitamin D-insufficient group (p = 0.040). However, no significant differences of amino acid mutations were observed between the two groups (Figure 2).

Discussion

Over the past decade, numerous studies reported that patients with chronic liver disease, especially HCV-positive and HBV-positive patients, have decreased 25(OH)D levels compared to healthy people [13-16]. By quantifying serum 25(OH)D levels in a cohort of 203 chronically HBV-infected patients, Farnik et al. reported that low 25(OH)D serum levels are strongly associated with high levels of HBV replication [14]. In addition, among the patients with chronic kidney disease, an Austrian study found that patients with vitamin D deficiency were more frequently non-responders and less frequently showed [13-16]. By quantifying serum 25(OH)D levels in a cohort of 203 chronically HBV-infected patients, Farnik et al. reported that low 25(OH)D serum levels are strongly associated with high levels of HBV replication [14]. In addition, among the patients with chronic kidney disease, an Austrian study found that patients with vitamin D deficiency were more frequently non-responders and less frequently showed

Table 2. Comparison of amino acid substitutions within “a” determinant region between vitamin D-deficient group and insufficient group.

<table>
<thead>
<tr>
<th>Amino acid substitution</th>
<th>25(OH)D &lt; 14 ng/mL</th>
<th>14 ≤ 25(OH)D &lt; 30 ng/mL</th>
<th>χ²</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>0.58</td>
<td>0.447</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substituted</td>
<td>8 (0.93%)</td>
<td>14 (0.66%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-substituted</td>
<td>856 (99.07%)</td>
<td>2,098 (99.34%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype B</td>
<td></td>
<td></td>
<td>6.55</td>
<td>0.021*</td>
</tr>
<tr>
<td>Substituted</td>
<td>6 (2.27%)</td>
<td>7 (0.61%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-substituted</td>
<td>258 (97.73%)</td>
<td>1,145 (99.39%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype C</td>
<td></td>
<td></td>
<td>1.01</td>
<td>0.496</td>
</tr>
<tr>
<td>Substituted</td>
<td>2 (0.33%)</td>
<td>7 (0.73%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-substituted</td>
<td>598 (99.67%)</td>
<td>953 (99.27%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. The frequency of amino acid substitutions in “a” determinant between vitamin D deficient group and insufficient group.

<table>
<thead>
<tr>
<th>Variable</th>
<th>25(OH)D &lt;14 ng/mL</th>
<th>14≤25(OH)D &lt;30 ng/mL</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>I/T126A/T/N/I/S</td>
<td>1(2.78%)</td>
<td>6(6.82%)</td>
<td>0.672</td>
</tr>
<tr>
<td>P127T</td>
<td>2(5.56%)</td>
<td>1(1.14%)</td>
<td>0.202</td>
</tr>
<tr>
<td>Q129H/R</td>
<td>0</td>
<td>1(1.14%)</td>
<td>1.000</td>
</tr>
<tr>
<td>T131P/N</td>
<td>1(2.78%)</td>
<td>1(1.14%)</td>
<td>0.498</td>
</tr>
<tr>
<td>M133L/T</td>
<td>3(8.33%)</td>
<td>2(2.27%)</td>
<td>0.146</td>
</tr>
<tr>
<td>T140I</td>
<td>1(2.78%)</td>
<td>0</td>
<td>0.290</td>
</tr>
<tr>
<td>S/T143M</td>
<td>0</td>
<td>1(1.14%)</td>
<td>1.000</td>
</tr>
<tr>
<td>G145R/A</td>
<td>0</td>
<td>2(2.27%)</td>
<td>1.000</td>
</tr>
</tbody>
</table>
seroconversion to hepatitis B vaccine than did patients with higher 25(OH)D levels [17]. Therefore, it is reasonable to perform a study to investigate the associations of low serum vitamin D with HBV genome mutations, especially with the mutations in different HBV genotypes. Compared to the HBV/B-infected patients with vitamin D insufficiency, higher rates of amino acid mutation within “a” determinant were detected in HBV/B-infected, vitamin D-deficient patients. Interestingly, in a more recent Chinese study, the authors reported that genotype B HBV-infected patients had a higher prevalence of vitamin D insufficiency than those infected by genotype C HBV [18]. Therefore, our results suggest that the effects of serum vitamin D deficiency on HBV infection, especially on genotype B HBV infection and disease progression, should be carefully analyzed in a long-term follow-up study.

Many special mutants within “a” determinant such as amino acid T1/I126, Q129, G130, M133, F134, C137, and T140, either alone or in combination, have been reported in association with HBsAg detection and vaccine failure and immunotherapy escape [19-21]. In the present study, 22 amino acid residues mutated within “a” determinant were determined. Furthermore, compared to the HBV/B-infected patients with vitamin D insufficiency, a higher prevalence of mutation at the positions of M133 was detected in the cases with vitamin D deficiency. In previous studies, M133 was identified as significantly related to immune escape [22,23]. Additionally, our results also indicated that among the patients with less than 14 ng/mL of serum 25(OH)D, more mutations at the positions of M133 were also determined in HBV/B-infected patients compared to those with HBV/C infection.

Conclusions
Our results report that vitamin D deficiency was significantly associated with genotype B HBV “a” determinant mutations. This should be helpful for the further understanding of the relationships between vitamin D and chronic HBV infection. In future studies, the suppression ability of vitamin D on HBV replication should be addressed because the lower seropositivity of HBV DNA in the patients with normal serum vitamin D (44.44%) was observed herein. Additionally, considering that both chronic HBV infection and vitamin D deficiency are serious public health problems in China, more studies should be performed to investigate the effects of vitamin D deficiency on the mutations of different HBV genotypes at the whole genome level.

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References


**Corresponding author**
Chen Dong
Department of Epidemiology and Statistics, School of Public Health
Jiangsu Key Laboratory and Translational Medicine for Geriatric Disease
Medical College of Soochow University
199 Renai Road, Suzhou, 215123, China
Phone and fax: 86-512-65884830
Email: cdong1974@163.com

**Conflict of interests:** No conflict of interests is declared.