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Association of vitamin D levels and vitamin D-related gene polymorphisms with liver fibrosis in patients with biopsy-proven nonalcoholic fatty liver disease

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ABSTRACT

Background: Vitamin D has promising anti-proliferative and anti-fibrotic properties, but its clinical utility in nonalcoholic fatty liver disease (NAFLD) is unclear.
Aims: This study aimed to clarify the association between vitamin D levels, single nucleotide polymorphisms (SNPs) in vitamin D-related genes, and the histopathological severity of disease in patients with biopsy-proven NAFLD.
Methods: SNPs in *CYP2R1*, *DHCR7*, vitamin D binding protein (*GC*), *CYP27B1*, and vitamin D receptor (*VDR*) were determined for 229 consecutive patients with biopsy-proven NAFLD.
Results: In this study, vitamin D deficiency defined as 25-hydroxyvitamin-D_3 levels of $\leq$20 ng/mL was found in 151 patients (65.9%). Multivariate analysis revealed that cold season, advanced fibrosis, and *CYP2R1* rs1993116 genotype non-AA were independent factors significantly associated with vitamin D deficiency. Old age ($p = 5.05 \times 10^{-8}$), high body mass index ($p = 2.13 \times 10^{-2}$), low total-cholesterol ($p = 1.46 \times 10^{-4}$), low serum vitamin D level ($p = 7.34 \times 10^{-3}$), and *VDR* rs1544410 genotype CC ($p = 9.15 \times 10^{-3}$) were independent factors associated with advanced liver fibrosis.
Conclusion: Serum 25-hydroxyvitamin-D_3 levels and the *VDR* gene SNP were significantly and independently associated with the severity of liver fibrosis in patients with biopsy-proven NAFLD.

1. Introduction

Vitamin D is an important group of fat-soluble secosteroids involved in calcium and bone metabolism [1]. Vitamin D is partly ingested through food, but is mostly produced in the skin under ultraviolet light irradiation. Accordingly, serum vitamin D levels vary depending on food culture, skin color, lifestyle, and latitudinal location of residential area [2,3]. Recently, deficiencies in vitamin D have been reported to be involved in the development of various diseases such as cancers of the colon, rectum, breasts, and prostate [4]; metabolic syndrome; type 2 diabetes; and cardiovascular disease [5–7]. Furthermore, vitamin D is likely to be deficient in chronic liver disease. Specifically, vitamin D deficiency is closely associated with the severity of chronic liver disease [8–10].

Nonalcoholic fatty liver disease (NAFLD) is a major chronic liver disease with a global prevalence of approximately 25% [11–13]. It is

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a multi-factorial disease that is mutually associated with metabolic syndrome [14]. There have been several reports on the association between NAFLD and vitamin D: serum vitamin D levels were lower in patients with NAFLD than in healthy subjects, and vitamin D deficiency was involved in the development and progression of NAFLD [15–17], insulin resistance [18], visceral obesity, and metabolic syndrome [19]. Regarding the association between the histopathologic manifestations of NAFLD and vitamin D, serum vitamin D levels have been reported to be closely associated with steatosis, inflammation, fibrosis [20], and hepatocyte ballooning [21].

Vitamin D-related genes, as well as serum vitamin D levels, affect the severity of liver fibrosis as well as the development and progression of hepatocellular carcinoma in patients with hepatitis B [22,23], hepatitis C [24–27], alcoholic liver disease [28], autoimmune hepatitis (AIH), and primary biliary cholangitis (PBC) [29]. Moreover, experimental studies suggest that vitamin D could exert anti-proliferative and anti-fibrotic effects through vitamin D receptors [30,31]. However, it has been reported that vitamin D-related genes and serum vitamin D levels did not affect the development and severity of NAFLD [32,33]. Therefore, the clinical significance of vitamin D and vitamin D-related genes in NAFLD is controversial and warrants further investigation.

The aim of this study was to identify factors, including vitamin D-related genes that could influence serum vitamin D levels in patients with NAFLD, and to evaluate the association of serum vitamin D levels and vitamin D-related genes with the severity of NAFLD.

2. Series and methods

2.1. Patients

Among the patients with suspected NAFLD based on unexplained alanine aminotransferase elevations and/or evidence of hepatic steatosis by ultrasound findings in Nippon Medical School Chiba Hokusoh Hospital, Nippon Medical School Hospital, Ootakanomori Hospital, and Shinmatsudo Central General Hospital between August 2013 and August 2017, 229 patients aged 18 years or older underwent histological evaluation and were diagnosed with NAFLD according to the European Association for the Study of the Liver guidelines as follows [34–36]: NAFLD was defined by the presence of steatosis in ≥5% of hepatocytes according to histological analysis. Exclusion criteria included patients with (1) daily alcohol consumption ≥30 g for men and ≥20 g for women; (2) other chronic liver diseases, such as viral hepatitis B or C, AIH, Wilson disease, and hemochromatosis; (3) secondary causes of steatosis, such as drug-induced fatty liver disease, total parenteral nutrition, and inborn errors of metabolism; and (4) pregnancy. We also excluded patients taking vitamin D supplementation, including ergocalciferol, cholecalciferol, calcitriol, and/or any multivitamin containing low-dose vitamin D; vitamin E; and thiazolidinedione (such as pioglitazone), which are agents for the treatment of NAFLD [37]. A careful interview, clinical and laboratory evaluations, and image inspection were performed at the time of the liver biopsy in all patients. The study protocol complied with the ethical guidelines established in accordance with the 2013 Declaration of Helsinki and was approved by the Ethics Committee of Nippon Medical School Chiba Hokusoh Hospital (approval number: 468). All patients provided written informed consent before the entry into this study.

2.2. Clinical and laboratory evaluation

Clinical and laboratory data were collected concurrently with liver biopsy. The body mass index (BMI) was calculated as weight (kg) divided by the square of height (m). Blood pressure was measured in a seated position twice or more at an interval of several minutes, and the mean was calculated. Hypertension was diagnosed when systolic blood pressure was 135 mmHg or higher, or diastolic blood pressure was 85 mmHg or higher, and when patients were treated with an antihypertensive drug [38]. Dyslipidemia was diagnosed when total cholesterol was 220 mg/dL or higher, high-density lipoprotein cholesterol (HDL cholesterol) was below 40 mg/dL, and/or triglyceride was 150 mg/dL or higher, as well as when patients were treated with an antihyperlipidemic drug [39]. Type 2 diabetes was diagnosed according to the 2006 World Health Organization (WHO) criteria in addition to the presence of treatment with an oral hypoglycemic agent and insulin. Laboratory evaluation included complete blood count, routine liver biochemistry (aspartate aminotransferase, alanine aminotransferase, total bilirubin, albumin, alkaline phosphatase, and gamma glutamyl transpeptidase), fasting lipids (total cholesterol, triglyceride, HDL cholesterol, and low-density lipoprotein cholesterol), fasting plasma glucose, hemoglobin A1c, and immunoreactive insulin. As an index of insulin resistance, the homeostasis model assessment-insulin resistance (HOMA-IR) was calculated using the following equation: HOMA-IR = fasting insulin (μU/mL) × plasma glucose (mg/dL)/405 [40].

2.3. Serum vitamin D level

Serum 25-hydroxyvitamin D_3 was considered to be the representative vitamin D in the serum [41], and was measured using a double-antibody radioimmunoassay kit (SRL Inc., Tokyo, Japan). Serum 25-hydroxyvitamin D_3 levels were classified as follows: vitamin D deficiency for ≤20 ng/mL; vitamin D insufficiency for 21–29 ng/mL; and vitamin D sufficiency for ≥30 ng/mL [42]. Patients in whom serum 25-hydroxyvitamin D_3 levels were measured between May and October, a period when there is longer duration of daylight, constituted the “warm season” group, and in whom the measurements were made between November and April, a period when there is shorter duration of daylight, constituted the “cold season” group.

2.4. Vitamin D-related gene polymorphisms

Single nucleotide polymorphisms (SNPs) in the genes encoding cytochrome P450 2R1 (*CYP2R1*) (rs1993116, rs10741657), 7-Dehydrocholesterol reductase (*DHCR7*) (rs7944926, rs12785878), vitamin D binding protein (*GC*) (rs2282679), cytochrome P450 27B1 (*CYP27B1*) (rs10877012), and vitamin D receptor (*VDR*) (rs2228570, rs1544410, rs7975232, rs731236) were determined by real-time detection PCR using the TaqMan SNP Genotyping Assays (Life Technologies, Carlsbad, CA). Previous studies have shown that SNP genotypes with risk alleles for *CYP2R1*, *DHCR7*, *GC*, and *CYP27B1* were significant factors associated with low serum 25-hydroxyvitamin D_3 levels in healthy subjects and patients with hepatitis C [24–26,43,44]. *VDR* SNP genotypes have been reported to be associated with the presence and severity of disease, development and progression of hepatocellular carcinoma, and therapeutic effects on other chronic liver diseases such as hepatitis B [22,23] and C [27], alcoholic liver disease [28], AIH, and PBC [29]. Therefore, patients were divided into patients with or without risk genotypes by genotyping these SNPs.

2.5. Histopathological evaluation

Histopathological evaluation was performed by experienced pathologists blinded to clinical and laboratory data of the patients. NAFLD was diagnosed when lipid droplet deposition was noted in 5% or more hepatocytes, and then steatosis, lobular inflammation, ballooning, and liver fibrosis were semi-quantitatively evaluated

Table 1
Baseline characteristics of the 229 patients.

Factors	n = 229
Age (year)	55 (18–84)
Gender (male/female)	122/107
BMI (kg/m^2)	27.8 (15.4–44.9)
Season (cold season/warm season)	103/126
Leukocytes (/mm^3)	6160 (2660–14310)
Hemoglobin (g/dL)	14.4 (7.6–19.9)
Platelets ($\times 10^4$/mm^3)	20.3 (5.0–41.1)
AST (U/L)	45 (15–182)
ALT (U/L)	61 (8–428)
γ-GTP (U/L)	63 (13–413)
Serum albumin (g/dL)	4.0 (2.2–5.2)
Prothrombin time (%)	99.1 (48.6–162.0)
Total cholesterol (mg/dL)	192 (96–285)
HDL cholesterol (mg/dL)	44 (23–115)
Triglyceride (mg/dL)	134 (40–587)
Plasma glucose (mg/dL)	101 (75–342)
Insulin (μU/mL)	11.5 (1.4–88.5)
HOMA-IR	3.06 (0.30–40.6)
Type 2 diabetes (presence/absence)	80/149
Hypertension (presence/absence)	74/155
25-hydroxyvitamin D_3 (ng/mL)	18 (7–39)
Liver steatosis (1/2/3)	127/78/24
Liver inflammation (0/1/2/3)	15/155/54/5
Liver ballooning (0/1/2)	94/110/25
Liver fibrosis stage (F0/F1/F2/F3/F4)	28/98/39/35/29

Data are presented as numbers or median (range)
BMI; body mass index, AST; aspartate aminotransferase, ALT; alanine aminotransferase, γ-GTP; gamma glutamyl transpeptidase, HDL; high-density lipoprotein, HOMA-IR; homeostasis model assessment-insulin resistance, Season of collecting sample (cold season; November to April, warm season; May to October).

according to the NASH CRN scoring system [45]: steatosis was graded 0–3 based on percent of hepatocytes on biopsy specimens (0: <5%, 1: 5–33%, 2: 33–66%, 3: >66%). Lobular inflammation was graded 0–3 based on inflammatory foci per 200 × field (0: no foci, 1: <2 foci, 2: 2–4 foci, 3: >4 foci). Ballooning was graded 0–2 based on the number of hepatocytes with this change (0: none, 1: few cells, 2: many cells/prominent ballooning). Fibrosis stage was evaluated as follows: F0 = no fibrosis, F1 = perisinusoidal or periportal fibrosis, F2 = perisinusoidal and portal/periportal fibrosis, F3 = bridging fibrosis, and F4 = cirrhosis. F3–4 was provisionally designated as advanced fibrosis.

2.6. Statistical analyses

Continuous variables were presented as medians and ranges, and categorical variables were presented as numbers and percentages. Categorical variables were compared between groups using the Pearson chi-squared test. Continuous variables with skewed distribution were compared between two groups using the Mann–Whitney test. Multiple logistic regression analysis was used to identify independent factors that were significantly associated with vitamin D deficiency and advanced fibrosis (stage F3–4). All statistical analyses were performed using IBM SPSS version 17.0 (IBM Japan, Tokyo, Japan). The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. Patients

Baseline characteristics of the 229 patients with biopsy-proven NAFLD are shown in Table 1. There were 122 males and 107 females, and the median age was 55 years (range, 18–84 years). Regarding metabolic components, the median BMI was 27.8 kg/m^2 (range, 15.4–44.9 kg/m^2); there were 80 patients with type 2 diabetes (34.9%) and 74 patients with hypertension (32.3%). Dyslipidemia was found in 138 patients (60.3%): 48 (21.0%), 65 (28.4%), and 89 (38.9%) patients had hypercholesterolemia, hypo-HDL-cholesterolemia, and hypertriglyceridemia, respectively. Upon pathological examination of the 229 liver biopsy specimens, the fibrosis stage was determined to be F0 for 28 (12.2%) patients, F1 for 98 (42.8%) patients, F2 for 39 (17.0%) patients, F3 for 35 (15.3%) patients, and F4 for 29 (12.7%) patients, indicating that advanced fibrosis (F3–4) was found in 64 (27.9%) patients. The median serum 25-hydroxyvitamin D_3 level was 18 ng/mL (range, 7–39 ng/mL). The distribution of serum 25-hydroxyvitamin D_3 levels is shown in Fig. 1. Vitamin D deficiency, vitamin D insufficiency, and vitamin D sufficiency were found in 151 (65.9%), 61 (26.6%), and 17 patients (7.4%), respectively. Table 2 shows the distribution of vitamin D-related gene polymorphisms with or without risk genotypes.

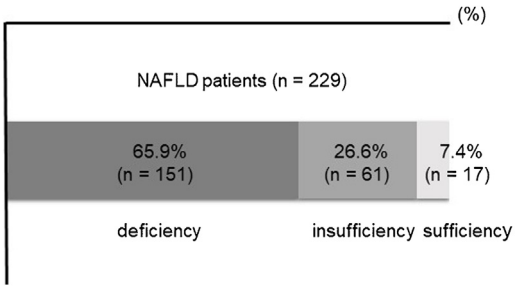

Fig. 1. Proportion of groups classified according to serum 25-hydroxyvitamin D_3 levels in patients with biopsy-proven nonalcoholic fatty liver disease (NAFLD). Serum 25-hydroxyvitamin D_3 levels of 20 ng/mL or less were defined as vitamin D deficiency, those of 21–29 ng/mL were defined as vitamin D insufficiency, and those with serum 25-hydroxyvitamin D_3 levels of 30 ng/mL or more were defined as vitamin D sufficiency.

Table 2
Distribution of vitamin D-related gene polymorphisms with or without risk genotypes in 229 patients with biopsy-proven non-alcoholic fatty liver disease.

Gene	SNPs	Risk genotype ±
CYP2R1	rs1993116 (non-AA/AA)	193 (84.3%)/ 36 (15.7%)
	rs10741657 (non-AA/AA)	189 (82.5%)/ 40 (17.5%)
DHCR7	rs7944926 (GG/non-GG)	27 (11.8%)/ 202 (88.2%)
	rs12785878 (GG/non-GG)	114 (49.8%)/ 115 (50.2%)
GC	rs2282679 (non-TT/TT)	111 (48.5%)/ 118 (51.5%)
CYP27B1	rs10877012 (non-TT/TT)	88 (38.4%)/ 141 (61.6%)
VDR	rs2228570 (GG/non-GG)	80 (34.9%)/ 149 (65.1%)
	rs1544410 (CC/non-CC)	169 (73.8%)/ 60 (26.2%)
	rs7975232 (GG/non-GG)	109 (47.6%)/ 120 (52.4%)
	rs731236 (AA/non-AA)	188 (82.1%)/ 41 (17.9%)

Data are expressed as numbers (percentages).
CYP2R1; cytochrome P450 2R1, *DHCR7*; 7-Dehydrocholesterol reductase, *GC*; vitamin D binding protein, *CYP27B1*; cytochrome P450 27B1, *VDR*; vitamin D receptor.

3.2. Factors associated with vitamin D deficiency

In the univariate analysis (Table 3), five variables (female, cold season, fibrosis stage, *CYP2R1* rs1993116 genotype non-AA, and *VDR* rs2228570 genotype GG) were significantly associated with vitamin D deficiency.

Among these variables, cold season ($p = 2.82 \times 10^{-2}$; odds ratio [OR] = 1.96; 95% confidence interval [CI] = 1.07–3.57), fibrosis stage ($p = 9.91 \times 10^{-3}$; OR = 1.40; 95% CI = 1.08–1.82), and *CYP2R1* rs1993116 genotype non-AA ($p = 3.98 \times 10^{-2}$; OR = 2.25; 95% CI = 1.04–4.89) were significantly independent in multiple logistic regression analysis. Fig. 2 shows the distribution of serum 25-hydroxyvitamin D_3 levels according to *CYP2R1* rs1993116 genotypes. Serum 25-hydroxyvitamin D_3 levels in the patients with *CYP2R1* rs1993116 genotype AA were significantly higher than those with the genotype non-AA (the median levels of 20 and 18 ng/mL, respectively; $p = 1.42 \times 10^{-2}$).

Table 3
Univariate and multivariate analyses of factors associated with vitamin D deficiency.

Factors	Category	Univariate			Multivariate		
		OR	95% CI	*p* Value	OR	95% CI	*p* Value
Age (years)	By 1 year down	1.00	0.98–1.02	0.832			
Gender	Female	1.81	1.03–3.16	3.84×10^{-2}			
BMI (kg/m^2)	By 1 kg/m^2 up	1.06	0.99–1.13	8.84×10^{-2}			
Season	Cold season	2.08	1.18–3.68	1.5×10^{-2}	1.96	1.07–3.57	2.82×10^{-2}
AST (U/L)	By 1 U/L up	1.00	1.00–1.01	0.746			
ALT (U/L)	By 1 U/L up	1.00	0.99–1.00	0.461			
Total-cholesterol (mg/dL)	By 1 mg/dL up	1.00	0.99–1.01	0.679			
HDL-cholesterol (mg/dL)	By 1 mg/dL down	1.01	0.98–1.03	0.637			
Triglyceride (mg/dL)	By 1 mg/dL up	1.00	1.00–1.00	0.441			
Plasma glucose (mg/dL)	By 1 mg/dL up	1.00	1.00–1.01	0.435			
Insulin (μU/mL)	By 1 μU/mL up	1.00	0.98–1.03	0.905			
HOMA-IR	By 1 up	1.00	0.95–1.06	0.895			
Diabetes	Presence	1.45	0.81–2.61	0.215			
Hypertension	Presence	1.45	0.79–2.67	0.234			
Liver steatosis	1 grade up	1.39	0.91–2.11	0.124			
Liver inflammation	1 grade up	1.31	0.81–2.12	0.266			
Liver ballooning	1 grade up	1.35	0.88–2.07	0.168			
Liver fibrosis stage	1 stage up	1.42	1.11–1.81	4.83×10^{-3}	1.40	1.08–1.82	9.91×10^{-3}
CYP2R1 rs1993116	Genotype non-AA	2.22	1.08–4.56	3.05×10^{-2}	2.25	1.04–4.89	3.98×10^{-2}
CYP2R1 rs10741657	Genotype non-AA	1.20	0.59–2.44	0.614			
DHCR7 rs7944926	Genotype GG	2.10	0.75–5.87	0.155			
DHCR7 rs12785878	Genotype non-GG	1.19	0.68–2.06	0.540			
GC rs2282679	Genotype non-TT	1.46	0.84–2.55	0.179			
CYP27B1 rs10877012	Genotype non-TT	1.00	0.57–1.76	0.996			
VDR rs2228570	Genotype GG	1.86	1.02–3.40	4.42×10^{-2}			
VDR rs1544410	Genotype CC	1.47	0.80–2.72	0.214			
VDR rs7975232	Genotype non-GG	1.07	0.62–1.86	0.804			
VDR rs731236	Genotype AA	1.14	0.57–2.32	0.707			

OR; odds ratio, CI; confidence interval, BMI; body mass index, AST; aspartate aminotransferase, ALT; alanine aminotransferase, HDL; high-density lipoprotein, HOMA-IR; homeostasis model assessment-insulin resistance, *CYP2R1*; cytochrome P450 2R1, *DHCR7*; 7-Dehydrocholesterol reductase, *GC*; vitamin D binding protein, *CYP27B1*; cytochrome P450 27B1, *VDR*; vitamin D receptor, Cold season; November to April.

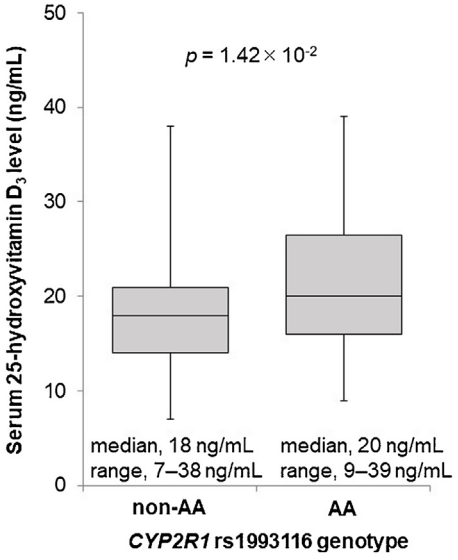

Fig. 2. Serum 25-hydroxyvitamin D_3 levels in patients with biopsy-proven non-alcoholic fatty liver disease according to the *CYP2R1* gene rs1993116 (non-AA *vs* AA).

3.3. Factors associated with advanced fibrosis (F3–4)

In the univariate analysis (Table 4), 12 variables (old age, female, high BMI, low ALT, low total-cholesterol, low triglyceride, high plasma glucose, high insulin, presence of diabetes, presence of hypertension, low serum 25-hydroxyvitamin D_3, and *VDR* rs1544410 genotype CC) were significantly associated with advanced fibrosis (stages F3–4).

Multiple logistic regression analysis showed that the following five variables were independently linked to advanced fibrosis (Table 4): old age ($p = 5.05 \times 10^{-8}$; OR = 1.10; 95% CI = 1.06–1.14), high BMI ($p = 2.13 \times 10^{-2}$; OR = 1.11; 95% CI = 1.02–1.22), low total-cholesterol ($p = 1.46 \times 10^{-4}$; OR = 1.03; 95% CI = 1.01–1.04), low serum 25-hydroxyvitamin D_3 ($p = 7.34 \times 10^{-3}$; OR = 1.09; 95% CI = 1.02–1.16), and *VDR* rs1544410 genotype CC ($p = 9.15 \times 10^{-3}$; OR, 4.04; 95% CI, 1.41–11.53).

Fig. 3(A) shows the prevalence of advanced fibrosis according to *VDR* rs1544410 genotypes: the rate (32.0%) in patients with genotype CC was significantly higher than that (16.7%) in patients with genotype non-CC ($p = 2.34 \times 10^{-2}$). Furthermore, Fig. 3 (B) shows advanced fibrosis rates according to serum 25-hydroxyvitamin D_3 levels and *VDR* rs1544410 genotypes. Only 8.0% of the patients with serum 25-hydroxyvitamin D_3 >20 ng/mL and *VDR* rs1544410 genotype non-CC genotype had advanced fibrosis. Advanced fibrosis occurred more frequently in patients who had either or both of the risk factors than in those without the risk factors ($p = 9.12 \times 10^{-3}$).

4. Discussion

Patients with chronic liver disease have been reported to have a high prevalence of vitamin D deficiency [8,9]. This study showed similar results even in patients with biopsy-proven NAFLD in Japan: 65.9% (151/229) of patients were found to have vitamin D deficiency. The reason for low serum vitamin D levels in chronic liver disease can be explained by various mechanisms [8,9]. Firstly, the exposure time to sunlight may be short because patients with chronic liver disease might go out less frequently than healthy subjects. Secondly, malabsorption of vitamin D from the intestine due to portal hypertension-induced intestinal edema and reduction of bile acid production and secretion may reduce serum vitamin D levels. Thirdly, vitamin D is biologically activated via enzymatic conversion (hydroxylation) in the liver [46], and vitamin D bind-

Table 4
Univariate and multivariate analyses of factors associated with advanced fibrosis (stages F3–4).

Factors	Category	Univariate			Multivariate		
		OR	95% CI	*p* Value	OR	95% CI	*p* Value
Age (years)	By 1 year up	1.08	1.05–1.11	5.66×10^{-9}	1.10	1.06–1.14	5.05×10^{-8}
Gender	Female	2.67	1.47–4.86	1.27×10^{-3}			
BMI (kg/m^2)	By 1 kg/m^2 up	1.07	1.00–1.14	3.72×10^{-2}	1.11	1.02–1.22	2.13×10^{-2}
AST (U/L)	By 1 U/L up	1.00	1.00–1.01	0.162			
ALT (U/L)	By 1 U/L down	1.01	1.00–1.01	3.44×10^{-2}			
Total-cholesterol (mg/dL)	By 1 mg/dL down	1.03	1.02–1.04	1.71×10^{-7}	1.03	1.01–1.04	1.46×10^{-4}
HDL-cholesterol (mg/dL)	By 1 mg/dL down	1.00	0.98–1.02	0.922			
Triglyceride (mg/dL)	By 1 mg/dL down	1.01	1.00–1.01	8.41×10^{-3}			
Plasma glucose (mg/dL)	By 1 mg/dL up	1.01	1.00–1.02	6.39×10^{-3}			
Insulin (μU/mL)	By 1 μU/mL up	1.03	1.00–1.06	4.38×10^{-2}			
HOMA-IR	By 1 up	1.09	1.00–1.19	5.35×10^{-2}			
Diabetes	Presence	2.44	1.35–4.42	3.29×10^{-3}			
Hypertension	Presence	3.11	1.62–5.99	6.72×10^{-4}			
25-hydroxyvitamin D_3 (ng/mL)	By 1 ng/mL down	1.09	1.04–1.15	1.05×10^{-3}	1.09	1.02–1.16	7.34×10^{-3}
CYP2R1 rs1993116	Genotype AA	1.23	0.56–2.68	0.612			
CYP2R1 rs10741657	Genotype AA	1.13	0.54–2.40	0.741			
DHCR7 rs7944926	Genotype GG	1.64	0.68–3.98	0.270			
DHCR7 rs12785878	Genotype GG	1.01	0.56–1.80	0.986			
GC rs2282679	Genotype TT	1.08	0.62–1.98	0.743			
CYP27B1 rs10877012	Genotype non-TT	1.04	0.58–1.89	0.887			
VDR rs2228570	Genotype GG	1.40	0.77–2.57	0.269			
VDR rs1544410	Genotype CC	2.28	1.07–4.84	3.24×10^{-2}	4.04	1.41–11.53	9.15×10^{-3}
VDR rs7975232	Genotype non-GG	1.15	0.64–2.06	0.645			
VDR rs731236	Genotype AA	1.24	0.57–2.72	0.583			

OR; odds ratio, CI; confidence interval, BMI; body mass index, AST; aspartate aminotransferase, ALT; alanine aminotransferase, HDL; high-density lipoprotein, HOMA-IR; homeostasis model assessment-insulin resistance, *CYP2R1*; cytochrome P450 2R1, *DHCR7*; 7-Dehydrocholesterol reductase, *GC*; vitamin D binding protein, *CYP27B1*; cytochrome P450 27B1, *VDR*; vitamin D receptor.

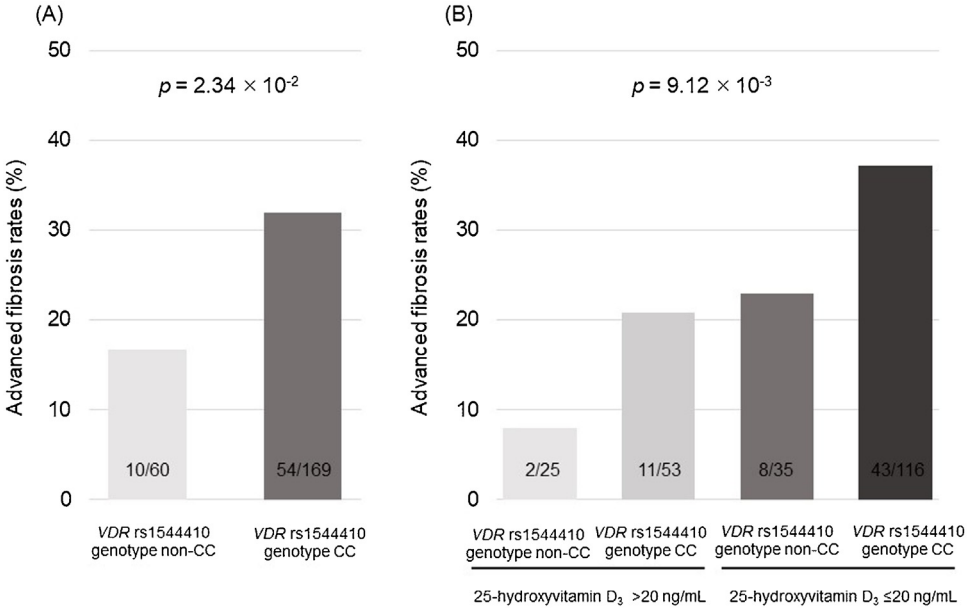

Fig. 3. (A) Prevalence of advanced fibrosis according to the vitamin D receptor (*VDR*) gene rs1544410 in 229 patients with nonalcoholic fatty liver disease. (B) Comparison of advanced fibrosis rates according to a combination of serum 25-hydroxyvitamin D_3 levels and the *VDR* gene rs1544410.

ing protein is synthesized in the liver and plays a major role in the transport and bioavailability of vitamin D. The activation of vitamin D and the level of vitamin D binding protein may be reduced with the progression of the liver disease stage [9], leading to low serum vitamin D levels.

We identified factors associated with serum 25-hydroxyvitamin D_3 levels in patients with NAFLD. Serum 25-hydroxyvitamin D_3 levels in female patients were lower than that in male patients. This was possibly related to an unbalanced diet or excessive avoidance of ultraviolet light. Actually, women covering themselves with clothes in the Middle East inhibit vitamin D synthesis in the skin; therefore, they have a higher incidence of vitamin D deficiency [47]. In addition, sex hormones such as estrogen may account for differences between male and female patient serum vitamin D levels. Serum estrogen levels reportedly have a positive association with serum vitamin D levels; declining serum estrogen levels in postmenopausal females lead to vitamin D deficiency [48,49]. This study involved a high percentage of postmenopausal female patients (74.8%, 80/107); therefore, it is one possible reason for the differences between male and female patient serum vitamin D levels. Serum 25-hydroxyvitamin D_3 levels in patients with NAFLD were also low in the cold season when the duration of sunshine is shorter.

In a cohort study involving 619 patients with chronic hepatitis C, serum 25-hydroxyvitamin D_3 levels were lower in female patients and in the cold season [50]. This study reconfirmed similar findings in patients with biopsy-proven NAFLD. We further demonstrated that the *CYP2R1* rs1993116 genotype non-AA was significantly and independently associated with vitamin D deficiency in patients with NAFLD. This is the first report to identify factors including genetic variations associated with serum 25-hydroxyvitamin D_3 levels in patients with biopsy-proven NAFLD. The association between *CYP2R1* SNPs and serum 25-hydroxyvitamin D_3 levels has also been reported from two GWAS studies conducted on the European population [43,44]. Unlike in those studies, however, the findings of our study did not indicate an association of *DHCR7* or *GC* SNPs with serum 25-hydroxyvitamin D_3 levels. This discrepancy may be due to the different ethnic and clinical backgrounds of the patient population and the limited number of patients in our study.

In the present study, serum vitamin D levels were low in most of the patients with biopsy-proven NAFLD, and the vitamin D levels were likely to decrease, creating deficiency with the progression of liver fibrosis. *In vitro* and *in vivo* experiments using a hepatic fibrosis mouse model showed the anti-proliferative and anti-fibrotic effects of vitamin D [32,33]: vitamin D inhibits the activation and proliferation of stellate cells that play a major role in fibrogenesis, reduces accumulation of extracellular matrix, activates matrix metalloproteinase that degrades extracellular collagen, and suppresses the expression of tissue inhibitors of metalloproteinases. In patients with advanced liver fibrosis, serum vitamin D levels were decreased through some hypothesized mechanisms as described above, and the reduced levels may further promote liver fibrosis. These findings encouraged us to prevent and improve liver fibrosis by supplementation of vitamin D, because no radical therapeutic drug resolving liver fibrosis is currently available, and vitamin D is inexpensive and causes very few adverse events. A prospective, randomized, controlled clinical trial is required to clarify whether supplementation of vitamin D prevents and improves liver fibrosis in patients with NAFLD.

We also demonstrated for the first time that, similar to other risk factors such as serum 25-hydroxyvitamin D_3 level, age, high BMI, and low total-cholesterol, *VDR* rs1544410 genotype CC was significantly and independently associated with advanced fibrosis in patients with biopsy-proven NAFLD. Age and BMI are established predictors of liver fibrosis and are included in the NAFLD fibrosis score formula [51]. A meta-analysis showed that serum total-cholesterol levels were significantly lower in NASH versus simple steatosis patients [52]. In general, serum cholesterol levels decrease as liver fibrosis progresses because cholesterol is synthesized in the liver. Vitamin D is involved in various physiological functions, such as calcium metabolism, bone formation, mineralization, cell proliferation, and immune function, which are mediated by the VDR in the cell nucleus, a ligand-activated transcriptional factor that regulates target gene expression [53]. In addition, vitamin D and the *VDR* gene are involved in the onset and progression of various cancers, such as skin, breast, prostate, colorectal, and lung cancer, as well as various diseases such as metabolic syndrome, type 2 diabetes, and cardiovascular disease [54]. Intriguingly, our findings demonstrated that the combination of the two factors – the *VDR* gene SNP and serum 25-hydroxyvitamin D_3 levels – were closely linked to liver fibrosis in patients with biopsy-proven NAFLD.

There were some limitations in this study. First, the number of patients in this study, especially those with cirrhosis (stage F4), was relatively small. Second, we did not validate the above results in another confirmatory cohort. Therefore, a large-scale study is needed to reach a decisive conclusion. Third, serum vitamin D levels should have been measured in the same season to generalize the clinical utility of vitamin D. This study might have overestimated or underestimated serum vitamin D levels.

In conclusion, cold season, advanced fibrosis, and the *CYP2R1* gene SNP were independently associated with vitamin D deficiency in patients with biopsy-proven NAFLD in Japan. Serum 25-hydroxyvitamin D_3 levels and the *VDR* gene SNP were independently associated with advanced fibrosis in patients with biopsy-proven NAFLD in Japan.

Conflict of interest

None declared.

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