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Genetic Variation in *CYP2R1* and *GC* Genes Associated With Vitamin D Deficiency Status

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Abstract

This cross-sectional study enrolled 180 patients at a private family practice in Virginia. Total serum vitamin D concentrations were obtained weekly from January 30, 2013, through March 30, 2013, in consecutive patients regularly scheduled for laboratory work at the practice. Patients were categorized into 2 groups and analyzed for variant alleles in vitamin D receptor (*VDR*; rs2228570), cytochrome P450 2R1 (*CYP2R1*; rs10741657), 7-dehydrocholesterol reductase (*DHCR7*; rs12785878), and group-specific component (*GC*; rs2282679) to determine whether variants of those alleles influenced total serum 25(OH)D concentrations. One-hundred and eighty patients were enrolled, with 40 (22%) being sufficient, 25-hydroxy vitamin D level 25(OH)D $\geq$ 30 ng/mL, and 140 (78%) being insufficient, 25(OH)D < 30 ng/mL. Of the 4 genes, 2 genes, *CYP2R1* (rs10741657) and *GC* (rs2282679), demonstrated a significant association related to vitamin D status. Subjects with 1 or more variant alleles at rs10741657 were almost 3.7 (odds ratio [OR] 3.67; 95% confidence interval [CI]: 1.35-9.99) times more likely be insufficient in vitamin D and subjects with 1 or more variant alleles at rs2282679 were about half (OR 0.42; 95% CI: 0.18-0.93) as likely to be insufficient in vitamin D. Allelic variations in *CYP2R1* (rs10741657) and *GC* (rs2282679) affect vitamin D levels, but variant alleles on *VDR* (rs2228570) and *DHCR7* (rs12785878) were not correlated with vitamin D deficiency, 25(OH)D < 30 ng/mL.

Keywords

genetics, vitamin D, deficiency, rs2228570, rs10741657, rs12785878, rs2282679

Background

Vitamin D, a fat-soluble steroid hormone, helps maintain adequate levels of both calcium and phosphate needed to support normal bone health and prevent diseases such as rickets and osteoporosis as well as decrease fracture risk.[1] Vitamin D receptors (VDRs) are found on a variety of cells and have been shown to regulate cell growth, promote neuromuscular and immune functions, and reduce inflammation.[1,2] Over the past several years, a number of studies have established an association of vitamin D deficiency with increased risk of cancers, particularly colon, prostate, breast, and pancreatic.[3-6] Autoimmune diseases, such as type 1 diabetes mellitus, rheumatoid arthritis, Crohn's disease, and multiple sclerosis as well as cardiovascular disease, hypertension, and myocardial infarction (MI), have also been linked to vitamin D deficiency.[3,7-12] Total serum 25-hydroxy vitamin D level 25(OH)D is an important biomarker for vitamin D status and has been found to vary by season, with the use of vitamin D supplementation, and altered by diet factors.[1] The Endocrine Society and the National Osteoporosis foundation agree that levels of total serum 25(OH)D above 30 ng/mL are considered adequate to maintain bone health and other important bodily functions.[3,13,14]

Several genes involved with vitamin D synthesis, metabolism, and transport have been reported to be associated with vitamin D deficiency in previous studies.[15,16] *Cytochrome P450 2R1* (*CYP2R1*) encodes for vitamin D 25-hydroxylase, an enzyme that converts vitamin D into 25-hydroxyvitamin D (calcidiol), the major circulating form of vitamin D. Polymorphisms of the CYP2R1 enzyme, which have been shown to play a role in the development of type 1 diabetes in children, have been linked to vitamin D deficiency.[15,17-19] The 7-dehydrocholesterol reductase (DHCR7) gene encodes for the enzyme that transforms 7-dehydrocholesterol in the skin

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to precholecalciferol that is rapidly turned into cholecalciferol (D_3). Variants of this gene have been associated with a decrease in vitamin D levels.[15,20] The group-specific component (GC) gene, also known as the vitamin D-binding protein (DBP), encodes a protein that belongs to the albumin gene family that binds to vitamin D and transports it to target tissues. Genetic mutations of these genes have been associated with vitamin D deficiency in numerous studies as well.[19,21,22] Three alleles of the genes *CYP2R1*, *DHCR7*, and *GC* were found to be associated with lower vitamin D levels in a large genome-wide association study by Wang et al.[23] The investigators found that *CYP2R1* was linked to deficiency in people with the rs10741657 variation, the *DHCR7* gene was linked to deficiency in people with the s12785878 variation, and the *GC* gene was linked to deficiency in people with the rs2282679 variation. In this study, subjects with multiple variant alleles were at a greater risk of vitamin D deficiency.[23]

In addition to CYP2R1, *DHCR7*, and *GC*, genetic mutations of the *VDR* gene have been linked to vitamin D deficiency and clinical outcome events including hip fractures and MI.[10] *VDR* encodes for the receptor that binds to activated vitamin D_3 and regulates many biological activities including calcium and phosphorous homeostasis, apoptosis, and cell differentiation. The *VDR* gene has also been associated with an increased risk of cancers including ovarian and breast and has been correlated with a decreased survival rate in those with oral carcinomas.[24-26] Engelman et al investigated the relationship between several alleles on the *VDR* gene and vitamin D levels in African Americans and Hispanics. The *VDR* allele investigated in that study, rs2228570, previously numbered rs10735810, was not found to affect vitamin D levels in the African American or Hispanic populations prompting further investigation.[16]

To further investigate the association between vitamin D-associated gene variants including *VDR* (rs2228570), *CYP2R1* (rs10741657), *DHCR7* (rs12785878), and *GC* (rs2282679) and vitamin D deficiency, this study sought to determine whether there was an increased frequency of those variants in patients with vitamin D deficiency.

Study Methods

This cross-sectional study was conducted at a private family practice clinic in northern Virginia. Patients scheduled to have routine laboratory blood work completed at the practice site were eligible to participate as long as they were over the age of 18, not pregnant, and had the capacity to give informed consent. Consecutive patients scheduled for routine blood work on Monday and Wednesday mornings between 7 AM and 11 AM from January 30, 2013, through March 30, 2013, were asked to participate. Those who were willing to participate were given an explanation of the purpose of the study and any possible adverse events associated with participation and were asked to sign the informed consent documents.

Two blood samples, one for a total serum 25(OH)D level and one for genetic testing, were obtained from each patient. The first blood sample was sent to a local laboratory (Piedmont Medical Laboratories, Winchester, Virginia) for evaluation of total serum 25(OH)D. The results were returned to the practice site and evaluated by the patient's physician to determine the need for treatment. The second blood sample was placed in a BD Vacutainer ETDA anticoagulated test tube and stored in a refrigerated space at the practice site until the time of DNA extraction. Each test tube was labeled with a unique identification number that correlated with the patient's individual consent form to keep patient information confidential throughout the data collection process. Genomic DNA was isolated from 200 μL of the blood sample using a Mini DNA Blood Kit and a Qiacube workstation (Qiagen Inc, Chatsworth, California) following the manufacturer's protocol and then frozen at −20°C until the time of genotyping.

Genotyping was performed by allelic discrimination using *Taq*Man real-time polymerase chain reaction (RT-PCR) 5′ nuclease assays. The assay mix contained nonlabeled primers and proprietary fluorescent *Taq*Man Minor Groove Binder VIC- and FAM-labeled oligonucleotide probes, one for the wild-type allele and one for the specific variant allele. RT-PCR was performed with a total reaction volume of 10 μL, which included 4.75 μL of *Taq*Man Universal PCR Master Mix, 0.5 μL of 20× assay mix, 3.75 μL of diethylpyrocarbonate H_2O, and 1 μL of gDNA. The PCR cycling conditions were as follows: 1 cycle of 50°C for 2 minutes, followed by 1 cycle of 95°C for 10 minutes, and then 50 cycles of 92°C for 15 seconds and 60°C for 90 seconds. Appropriate negative controls were performed for each PCR run. Allelic discrimination was carried out by measuring endpoint fluorescence intensity with a 7300 RT-PCR System (Applied Biosystems, Foster city, California). The results of the measurements and subsequent genotype were evaluated with SDS software Version 1.3 (Applied Biosystems). Allelic variation was characterized as present if the gene was heterozygous or homozygous variant and absent if homozygous wild type. Data collected included gender, race, and age, height, weight, body mass index (BMI), estimated glomerular filtration rate (eGFR), and prior vitamin D levels. Comorbid disease states, including kidney disease, severe liver disease, Crohn's disease, ulcerative colitis, celiac disease, and cystic fibrosis, and concomitant medications were also recorded. The use of vitamin D supplementation was determined by reviewing the patients most current medication list at the time of enrollment and patients were categorized as taking a supplement or not taking a supplement. For comparison, the subjects were separated into 2 groups based on the vitamin D level obtained at the time of enrollment and defined as either sufficient, total 25(OH)D $\geq$ 30 ng/mL, or insufficient, 25(OH)D < 30 ng/mL.

Based on the results of previous trials, a sample size calculation was carried out that assumed at least a 15% difference in allele frequencies between the sufficient and insufficient groups, power of 80%, and a 2-sided level of significance of 0.05. Based on this calculation, a total sample size of 180 would be needed, with 90 patients in each group.

Means and standard deviations were calculated for continuous variables. Based on the sample distribution, continuous variables were evaluated by the independent-samples *t* test, the

Table 1. Demographics.

Variable	Total (n = 180)	Sufficient (n = 40)	Insufficient (n = 140)	*P* Value
Mean age, years (SD)	62.3 (±11.2)	67.3 (±12)	60.9 (±10.6)	.001
Male, No. (%)	98 (54.4)	16 (16.3)	82 (83.4)	.048
Mean BMI, kg/m^2 (SD)	31.6 (±6.32)	30.7 (±6.4)	31.8 (±6.3)	.349
Mean eGFR, mL/min/1.73 m^2 (SD)	66.8 (±15.8)	63.6 (±13.3)	67.7 (±16.4)	.140
Mean vitamin D level, ng/mL (SD)	23.1 (±9.7)	36.7 (±6.6)	19.2 (±6.4)	.000
Race (No.)				
White	172	29	131	
Asian	2		2	
Hispanic	6		6	
Use of vitamin D supplementation, No. (%)				
Not taking	122 (67.8)	13 (32.5)	109 (77.8)	<.005
Taking	58 (32.2)	27 (67.5	31 (22.1)	

Abbreviations: BMI, body mass index; eGFR, estimated glomerular filtration rate; SD, standard deviation.

Mann-Whitney *U* test, and Kruskal-Wallace test as appropriate. Frequencies and percentages were determined for categorical variables. Categorical variables were compared using the chi-square or Fisher exact test. Binary logistic regression analysis was used to evaluate whether the allelic variants and other potentially confounding factors were associated with the patient's vitamin D status. The confounding factors included age, gender, height, weight, BMI, eGFR, disease states, concomitant medications, and use of vitamin D supplementation.

The initial model included the allelic variation of the 4 genes and all of the potentially confounding variables. Confounding variables that were not significant at 0.25 level were eliminated from the model. Categorical variables with frequencies of less than 10 were also excluded which resulted in the elimination of disease states and concomitant medications from the model. The forward likelihood-ratio statistic was used to eliminate final variables that did not reach significance.

A *P* value of <.05 was considered statistically significant. All statistical analyses were completed using IBM SPSS for Macintosh version 22 software (SPSS, Armonk, New York). The Shenandoah University Institutional Review Board approved the study protocol.

Results

One hundred and eighty-three patients were asked to be in the study. Three patients declined enrollment, leaving a total of 180 patients to participate with 40 (22%) in the sufficient group and 140 (78%) in the insufficient group. The sufficient group was more likely to be older, male, have a lower eGFR, and a slightly lower BMI than those who were insufficient. Demographic information can be found in Table 1. The average vitamin D level of sufficient patients was 36.7 ± 6.6 ng/mL while patients who were insufficient had an average vitamin D level of 19.2 ± 6.4 ng/mL. Supplements were used by 53% of the patients in the sufficient group and by 47% of patients in the insufficient group ($P = .005$). Furthermore, those who were taking a vitamin D supplement at the time of enrollment were roughly 4 times more likely (odds ratio [OR] 3.992; 95% confidence interval [CI]: 2.090-7.628) to be sufficient in vitamin D than those who were not taking a supplement (Table 2).

Table 2. Mean Allele Frequencies.

Gene	SNP	Alleles	MAF	*P* Value
VDR	rs2228570	A>G	0.36	.803
CYP2R1	rs10741657	A>G	0.42	.783
DHCR7	rs12785878	G>T	0.27	.948
GC	rs2282679	G>T	0.33	.376

Abbreviations: ; CYP2R1, cytochrome P450 2R1; DHCR7, 7-dehydrocholesterol reductase; GC, group-specific component; MAF, mean allele frequency; SNP, single-nucleotide polymorphism; VDR, vitamin D receptor.

It is interesting to note that although you would expect patients receiving vitamin D supplementation more likely to be in the sufficient group, when this variable is controlled for with logistic regression analysis, the role of the genetic variants is still significant. Based on the Nagelkerke R^2, about 18% of the variation in the outcome variable is explained by vitamin D supplementation alone in the logistic regression model. However, when the allelic variation is also included in the model over 30% of the variation can be accounted for, indicating it may be as important as supplementation in its effect on vitamin D sufficiency.

Of the 4 genotyped alleles, 2 genes, rs10741657 and rs2282679 in genes *CYP2R1* and *GC* respectively, demonstrated a significant association with vitamin D status. Patients who had 1 or more variant alleles on *CYP2R1* (rs10741657) were over 4 times more likely to have insufficient vitamin D levels. Additionally, participants who had 1 or more variant alleles in the *GC* gene (rs2282679) were less than half as likely to have low vitamin D levels when compared to those that were homozygous wild type (Table 3). No significant differences were found for the *VDR* variant allele (rs2228570) or the *DHCR7* variant allele (rs12785878).

No influence was found for vitamin D sufficiency based on gender or BMI but age and eGFR were statistically significant based on univariate analysis. The mean age for the sufficient

Table 3. Significance of Having a Variant Allele in Relation to Vitamin D Status.

Gene	Allele Variant	Odds Ratio (95% CI)	*P* Value
VDR	rs2228570	1.33 (0.62-2.86)	.459
CYP2R1	rs10741657	3.67 (1.35-9.99)	.011
DHCR7	rs12785878	0.87 (0.42-1.81)	.706
GC	rs2282679	0.42 (0.18-0.93)	.037

Abbreviations: CI, confidence interval; CYP2R1, cytochrome P450 2R1; DHCR7, 7-dehydrocholesterol reductase; GC, group-specific component; VDR, vitamin D receptor.

Table 4. Variables Used in the Final Regression Model.

Variable	*P* Value	OR (95% CI)
rs2228570	.230	1.722 (0.709-4.186)
rs10741657	.008	4.040 (1.429-11.420)
rs12785878	.908	0.952 (0.412-2.196)
rs2282697	.019	0.346 (0.143-0.838)
Age group	.013	1.726 (1.120-2.661)
Gender	.330	0.647 (0.269-1.554)
BMI	.916	0.996 (0.932-1.066)
Using a vit D supplement	.000	3.992 (2.090-7.628)

Abbreviations: BMI, body mass index; CI, confidence interval; OR, odds ratio; vit, vitamin.

group and the insufficient group was 67.3 $\pm$ 12.0 and 60.9 $\pm$ 10.6 years, respectively ($P = .001$). When dividing patients into age categories, the mean vitamin D level (27.7 $\pm$ 11.3 ng/mL) was highest in the older age-group, which was statistically significant when compared to the middle-aged group, who had a mean vitamin D level of 21.9 $\pm$ 8.5 ng/mL ($P = .004$). Older patients (66.92 $\pm$ 9.2 years) were also more likely to be taking vitamin D supplementation prior to enrollment when compared to younger patients (60.5 $\pm$ 11.5 years). Therefore, older patients were roughly 2 times more likely to be sufficient in vitamin D (OR 1.726; 95% CI 1.120-2.661). Patients with a lower eGFR (62.1 $\pm$ 14.2 mL/min/1.73 m^2) were also more likely to be sufficient in vitamin D than those with a higher eGFR (67.7 $\pm$ 16.0 mL/min/1.73 m^2, $P = .035$). Additionally, patients with a lower eGFR (65.3 $\pm$ 14.6 mL/min/1.73 m^2) were more likely to be taking a vitamin D supplement than those with a higher eGFR (67.4 $\pm$ 16.3 mL/min/1.73 m^2), but this was not found to be significant ($P = .342$; Table 4).

Discussion

This study found a significant association with vitamin D levels for patients with variant alleles on genes *CYP2R1* (rs10741657) and *GC* (rs2282679) but did not find a significant association with vitamin D deficiency on genes *VDR* (rs2228570) or *DHCR7* (rs12785878). Vitamin D deficiency has been associated with certain vitamin D pathway genes in a number of studies, and this study is consistent for the association with the *CYP2R1* and *GC* genes but not for the *VDR* or *DHCR7* genes.[8,10,15,16,19,27]

In 2010, the Study of Underlying Genetic Determinants of Vitamin D and Highly Related Traits (SUNLIGHT) Consortium, published by Wang et al, identified 4 different gene variants in the *GC*, *DHCR7*, *CYP2R1*, and *CYP24A1* genes, which corresponded with vitamin D deficiency. Wang et al analyzed vitamin D levels in patients with a level of less than 30 ng/mL, less than 20 ng/mL, and severe deficiency in those with a level less than 10 ng/mL. Eighteen alleles were chosen for investigation. Three of the variant alleles in *GC* (rs2282679), *DHCR7* (rs12785878), and *CYP2R1* (rs10741657) genes were significant on a genome-wide basis in 15 cohorts with over 30 000 subjects of European decent. The more variants the subject had, the greater their vitamin D deficiency.[23] This study also examined the *GC* (rs2282679) and *CYP2R1* (rs10741657) alleles and found a significant effect on vitamin D levels, but no significant difference was found for *DHCR7* (rs12785878). This study included primarily caucasian patients so the role of ethnic variation could not be evaluated. This study also evaluated a possible relationship with older age and lower eGFR on vitamin D levels that warrants further evaluation, whereas Wang et al did not examine that relationship.

Englemen and colleagues examined 30 different alleles on 3 different genes, DBP, VDR, and CYP27B1, in African American and Hispanic populations from California, Colorado, and Texas. The primary objective was to examine the relationship between the genes and plasma levels of 25(OH)D and 1,25-dihydroxy vitamin D. The investigators evaluated 30 alleles on 3 genes but found only 2 significant alleles (rs4588 and rs7041) in the *DBP* gene in all 3 populations that was associated with lower 25(OH)D levels and 1 allele (rs4588) in the *DBP* gene was associated with lower levels of $1,25(OH)_2D$.[16] Because our study did not evaluate all of the same genes and alleles as the study by Englemen and colleagues, the results are not directly comparable. Our findings with respect to the *VDR* allele rs2228570, previously known as rs10735810, in caucasians are consistent with Engleman's findings in African Americans and Hispanics.

A recent study in the *Journal of Nutrition* examined 29 different alleles on 4 genes including *GC*, *CYP2R1*, *DHCR7*, and *CYP24A1*. Six alleles on 2 genes, *GC* and *CYP2R1*, were associated with a significantly lower vitamin D level based on the season of the blood draw. Those who had blood drawn in the winter/spring months were more likely to be deficient in vitamin D than those in the summer/fall. The study population consisted of only postmenopausal women, between the ages of 50 and 79, who were initially enrolled in one of the Women's Health Initiative observational studies. Other variables such as age, BMI, and supplement use were evaluated in the analysis as well. No significance was found correlating allelic variations to vitamin D status with regard to age. However, the results showed a significant association between BMI and lower vitamin D levels in obese patients. The use of supplementation was also found to be significantly related to vitamin D levels. Those who took more than 670 IU of vitamin D, even with multiple

risk alleles, were more likely to be sufficient in vitamin D than those who did not. Similarly, this study demonstrated that 2 of the same genes, *GC* and *CYP2R1*, significantly affected the vitamin D levels, however the specific alleles were different.[19] This study population included both men and women with varying ages between 21 and 80. The majority of patients were also overweight, but this did not appear to have a significant effect on whether vitamin D levels were sufficient. Some studies have found that overweight and obese patients are more likely to be deficient in vitamin D, although this study did not identify association with BMI. This is contrary to what is expected, given that vitamin D is fat soluble and adipose tissue serves as a reservoir for vitamin D.[28-30] This finding warrants further investigation however, given that the size of the sufficient group was much smaller than that of the insufficient group.

Furthermore, patients were not asked to verify food intake or dosing of supplements in this study to associate a specific intake amount to an individual's vitamin D level, but the results from this study did show that supplementation use in general was associated with a higher vitamin D level in older patients. Research has demonstrated that older adults are less likely to be sufficient in vitamin D.[1,31] However, older patients in this study had a higher rate of vitamin D supplementation than younger patients.

Another area for further research may be treatment response to vitamin D supplementation once genetic variations have been identified. This may provide clinical insight demonstrating that genetics may not necessarily affect this vitamin D levels, but rather a patient's response to vitamin D supplementation and treatment.

In this study, unequal distribution between sufficient and insufficient vitamin D level groups may have limited the ability to detect an association between eGFR and vitamin D supplementation. Furthermore, limited representation of comorbid disease states and concomitant medications may influence the vitamin D level, precluding an assessment in this group of patients. The demographic group included in this study consisted primarily of caucasian, middle-aged adults, and therefore these results may not be generalizable to other ethnicities or age groups. Verifying the use of vitamin D supplementation for each patient at the time of enrollment would have been a better way to categorize patients as using or not using supplementation, rather than just relying on the electronic medical record for verification.

Conclusion

In summary, this study examined the association between alleles on *VDR* (rs2228570), *CYP2R1* (rs10741657), *DHCR7* (rs12785878), and *GC* (rs2282679), vitamin D levels, and a number of potentially confounding variables. The results of this study demonstrate that allelic variations in the *CYP2R1* (rs10741657) and *GC* (rs2282679) genes affect the patient's vitamin D sufficiency status while controlling for supplementation and age effects that were also significant. Additional studies are needed to further delineate how genetic variations affect vitamin D levels in the context of other factors known to be associated with vitamin D deficiency.

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Declaration of Conflicting Interests

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