RESEARCH ARTICLE

Association of rs10741657 polymorphism in CYP2R1 gene with apparently healthy vitamin-D deficient Pakistani subjects

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Abstract

Objective: To determine the association of polymorphism rs10741657 in the CYP2R1 gene with vitamin D deficiency in apparently healthy subjects.

Method: The prospective, case-control study was conducted from June 2023 to January 2024 at the CREAM Lab of Army Medical College, Rawalpindi, and comprised vitamin D-deficient cases in group A and healthy controls in group B. Genotyping of rs10741657 polymorphism in the CYP2R1 gene was performed using allele-specific polymerase chain reaction (AS-PCR). Genotypic and allelic frequencies, Hardy–Weinberg equilibrium, and genetic inheritance models were analysed using SNPStats, a web-based statistical software for genetic association studies.

Results: Of the 300 subjects with a mean age of 43.56±15.26 years, 150 (50%) were in group A, comprising 91 (61%) females and 59 (39%) males, with a mean age of 44.49 ± 15.12 years. There were 150 (50%) controls in group B, including 83 (55%) males and 67 (45%) females, with a mean age of 42.63±15.40 years. The genotypic frequencies in group A were A/A 45(30%), A/C 101(67.34%), C/C 4(2.66%). The corresponding values in group B were 39(26%), 110(73.34%) and 1(0.66%). The allele frequency of A was 191 (64%) in vitamin D-deficient cases and 188 (63%) in healthy controls (p=0.8775), while the C allele frequency was 109 (36%) in cases and 112 (37%) in controls (p=0.8401). The genotype frequencies in cases were A/A 45 (30%), A/C 101 (67%), and C/C 4 (2.67%), compared to A/A 39 (26%), A/C 110 (73%), and C/C 1 (0.67%) in controls, with no statistically significant difference observed between the groups (p>0.05). The inheritance genetic models suggested no association with vitamin D deficiency (p>0.05); codominant model – A/C odds ratio 0.70 (95% confidence interval: 0.45-1.23), C/C odds ratio 4.00 (95% confidence interval: 0.45-36.96); dominant model odds ratio 0.80 (95% confidence interval: 0.49-1.35); recessive model odds ratio 4.00 (95% confidence interval: 0.45-36.96); and over dominant model odds ratio 0.70 (95% confidence interval: 0.45-1.23).

Conclusion: The A allele was found to be the most common variant of rs10741657 in both the groups. The rs10741657 polymorphism was not a risk for the susceptibility to vitamin D deficiency. No correlation of genotype with serum vitamin D levels was noted.

Keywords: Allele-specific polymerase chain reaction, Single nucleotide polymorphism, Calcitriol, 25 Hydroxylase, Vitamin D binding protein. (JPMA 76: 1252; 2026) **DOI: https://doi.org/10.47391/JPMA.22692**

Introduction

Vitamin D is an essential micronutrient which performs diverse biochemical and physiological functions in the human body. The primary response of the active form of vitamin D metabolite 1,25(OH)2D (calcitriol), is to improve calcium absorption from the stomach. It works with calcium and phosphorus to maintain bone density to prevent rickets in children and osteomalacia in adults. Low vitamin D levels affect bone mineral density (BMD) and turnover. The long-term effects of vitamin D insufficiency include muscle weakness, osteomalacia, rickets, secondary hyperparathyroidism and bone-loss that can cause osteoporosis and fractures. Calcitriol enhances the absorption of calcium ion (Ca^{2+}) in the intestine by initially entering the cell and attaching it to a cytosolic receptor. The 1,25-diOH-D3-receptor complexes subsequently travels to the nucleus, where it interacts with response regions on the deoxyribonucleic acid (DNA) in a specific manner. As a result, increased production of the calcium-binding protein calbindin promotes Ca^{2+} uptake. The mechanism of action of 1,25-diOH-D3 is similar to that of steroid hormones.[1] The biologically active form of vitamin D3, 1,25-dihydroxycholecalciferol, is produced by the kidney. The dietary calcium and phosphorus, as well as parathyroid hormone (PTH) secretion, appear to be the key variables in the production of 1,25-dihydroxycholecalciferol from 25-hydroxycholecalciferol. Chronic renal failure patients have impaired production of 1,25-dihydroxycholecalciferol.[2]

Vitamin D deficiency (VDD) is common in South Asia, including Pakistan, despite being part of the sunshine region across the year.[3] A review-based study carried over

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10 years reported 58.17% vitamin D deficiency (VDD) and 26.65% vitamin D insufficiency in Pakistan.[4] The genetic aetiology is the major factor associated with VDD besides malnutrition, physical activities and chronic diseases. The A allele of rs2189480 and G allele of rs4328262 in vitamin D receptor gene were found associated with severity of asthma and atopy where rs10500804, rs3886163, rs12794714 in CYP2R1 and CYP24A1 were not associated with vitamin D metabolism.[5] The Korean National Health and Nutrition database explored the correlation among five single nucleotide polymorphisms (SNPs) related to the vitamin D metabolic pathway, including DHCR7 rs12785878, GC rs2282679, CYP2R1 rs12794714, CYP2R1 rs10741657 and CYP24A1 rs6013897 with serum levels of 25-hydroxyvitamin D (25[OH]D). The findings revealed the association of the genotypes of rs12785878, rs2282679 and rs12794714 with the serum levels of 25(OH)D.[6] Several genes of vitamin D metabolic pathway, like GC, NADSYN1/DHCR7, CYP2R1, CYP24A1, SEC23A and AMDHD1, have also been linked to low serum levels of vitamin D in studies.[7] The CYP2R1 gene encodes the 25-hydroxylase enzyme required for the synthesis of 25(OH)D which is the most abundant vitamin D metabolite in circulation.[8] CYP2R1 is positioned on chromosome 11p15.2 and has five exons that encode 501 amino acids of protein. The core structure of CYP2R1 protein consists of 12 alpha (α)-helices, largely arrayed beta (β)-sheets, and the heme in the middle.[9] A study reported 3.7 times higher vitamin D insufficiency in subjects carrying rs10741657 in CYP2R1 gene than rs2282679 in GC gene.[10] In Bangladesh, rs10741657 in CYP2R1 has been linked with low serum 25(OH)D levels.[11] Pakistan is located in an ideal geographical region where sunlight throughout the year is adequate, but VDD and vitamin D insufficiency are on the rise. The current study was planned to determine the association of polymorphism rs10741657 in the CYP2R1 gene with VDD in apparently healthy subjects.

Subjects and Methods

The prospective, case-control study was conducted from June 2023 to January 2024 at the CREAM Lab of Army Medical College, Rawalpindi, and comprised VDD cases in group A and healthy controls in group B. Formal approval was obtained from the ethical review committee of Army Medical College. DNA samples of all the samples were taken for the analysis of genetic variation rs10741657 in CYP2R1. The allele-specific polymerase chain reaction (ASPCR) was used to screen the SNPs (Figure). PCR primers were designed on web-based allele specific primer designing tool, and included 5´GGGGAGATACTTTAGCAGGTA3´, 5´GGGGAGATACTTTAGCAGGTC3´, and 5´CTAACACCTCTCTGTCAGCC3´. The PCR reaction

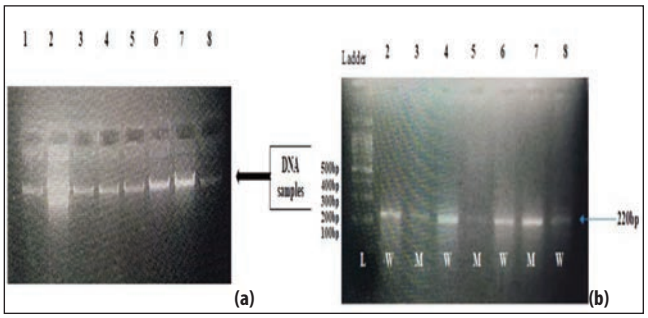

Figure-1: (a) Deoxyribonucleic acid (DNA) isolation from the samples; (b) allele-specific polymerase chain reaction (ASPCR) of the samples.

contained 1×Taq PCR Mastermix 5µl, 1 pmol of each primer, 300ng DNA and nuclease-free water. The ASPCR was performed at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 35 seconds, annealing at 57.6°C for 35 seconds, and extension at 72°C for 35 seconds. The genotypic, allelic frequencies and genetic inheritance models were analysed using biological software SNPstats.[12] The allelic and genotypic frequencies were calculated. Exact test was used to test for Hardy-Weinberg equilibrium (HWE). Also, 2xk contingency table and a chi-square test was applied to determine the association between disease and polymorphism. The odds ratio (OR) and 95% confidence interval (CI) were calculated to measure the association. The codominant model compared the homozygous recessive C/C and heterozygous A/C state of genotypes with the homozygous dominant genotype A/. The dominant model compared the homozygous recessive C/C and heterozygous A/C state of genotypes with the homozygous dominant genotype A/A. The recessive model compared the homozygous dominant A/A and heterozygous A/C state of genotypes with the homozygous recessive genotype C/C. The over dominant model compared both the homozygous dominant A/A and homozygous recessive C/C state of genotypes with the heterozygous genotype C/C. P<0.05 was considered significant.

Results

Of the 300 subjects with a mean age of 43.56±15.26 years, 150 (50%) were in group A, comprising 91 (61%) females and 59 (39%) males, with a mean age of 44.49±15.12 years. There were 150 (50%) subjects in group B, including 83 (55%) males and 67 (45%) females, with a mean age of 42.63 ± 15.40 years. The mean serum vitamin D levels were significantly lower in group A compared to group B (12.40±4.78 vs. 44.37±11.53 ng/mL; p<0.001).

The A/A and A/C genotypes were more frequent than the C/C genotype in both groups (Table 1). Among females, the A/A, A/C, and C/C genotypes were observed in 23 (25%), 67 (74%), and 1 (1%) subjects in group B, compared to 19 (32%), 44 (75%), and 4 (7%) subjects in group A. Among

Table-1: Allelic and genotypic frequencies of rs10741657 in vitamin D-deficient (VDD) cases and healthy controls.

Genetic Variation		Vitamin D deficient cases n= 150 [n (%)]	(Healthy controls) n=150 [n (%)]	*p*–value
Allele Frequencies	A	191 (64)	188 (63)	0.8775
	C	109 (36)	112 (37)	0.8401
Genotype Frequencies	A/A	45 (30)	39 (26)	0.5127
	A/C	101 (67)	110 (73)	0.5355
	C/C	4 (2.67)	1 (0.67)	0.1797

Table-2: Association of rs10741657 of CYP2R1 gene with vitamin D deficiency in the groups based on inheritance models.

Pattern of Inheritance	Genotype	Cases [n (%)]	Controls [n (%)]	OR (95% CI)	*p*-value
Codominant	A/A	45 (30%)	39 (26%)	1.00	-
	A/C	101 (67.3%)	110 (73.3%)	0.7 (0.45-1.23)	0.21
	C/C	4 (2.7%)	1 (0.7%)	4 (0.45-36.96)	0.21
Dominant	A/A	45 (30%)	39 (26%)	1.00	-
	A/C-C/C	105 (70%)	111 (74%)	0.8 (0.49-1.35)	0.41
Recessive	A/A-A/C	146 (97.3%)	149 (99.3%)	1.00	-
	C/C	4 (2.7%)	1 (0.7%)	4 (0.45-36.96)	0.21
Overdominant	A/A-C/C	49 (32.7%)	40 (26.7%)	1.00	-
	A/C	101 (67.3%)	110 (73.3%)	0.7 (0.45-1.23)	0.21

OR: Odds ratio, CI: Confidence interval; The ORs reported are unadjusted and derived from direct comparison of genotype frequencies between the groups.

males, the A/A, A/C, and C/C genotypes were found in 16 (27%), 43 (73%), and 0 (0%) subjects in group B, and 26 (29%), 57 (63%), and 0 (0%) subjects in group A, respectively.

The SNP when adjusted for to find the association with gender, the homozygous wild A/A was found in 23 healthy control females and 19 vitamin D deficient females, the heterozygous A/C was found in 67 healthy control females and 44 vitamin D deficient females, and the homozygous mutant C/C was found in 1 healthy control female and 4 vitamin D deficient females. In case of males, the homozygous wild A/A was found in 16 healthy control males and in 26 vitamin D deficient males, the heterozygous A/C was found in 43 healthy control males and in 57 vitamin D deficient males while the homozygous mutant C/C was not found in males in both groups.

The genotypic frequencies in group A were A/A 45(30%), A/C 101(67.34%), C/C 4(2.66%). The corresponding values in group B were 39(26%), 110(73.34%) and 1(0.66%). The allele frequency of A was 191 (64%) in vitamin D-deficient cases and 188 (63%) in healthy controls (p = 0.8775), while the C allele frequency was 109 (36%) in cases and 112 (37%) in controls (p=0.8401). The genotype frequencies in cases were A/A 45 (30%), A/C 101 (67%), and C/C 4 (2.67%), compared to A/A 39 (26%), A/C 110 (73%), and C/C 1 (0.67%) in controls, with no statistically significant difference observed between the groups (p>0.05). The inheritance genetic models suggested no association with vitamin D deficiency (p>0.05); codominant model – A/C odds ratio 0.70 (95% confidence interval: 0.45-1.23), C/C odds ratio 4.00 (95% confidence interval: 0.45-36.96); dominant model odds ratio 0.80 (95% confidence interval: 0.49-1.35); recessive model odds ratio 4.00 (95% confidence interval: 0.45-36.96); and over dominant model odds ratio 0.70 (95% confidence interval: 0.45-1.23) Based on the genetic patterns of inheritance, the rs10741657 polymorphism was not a risk for VDD susceptibility (Table 2).

Discussion

The primary objective of the current study was to determine the underlying genetic cause of VDD in apparently healthy Pakistani population by assessing the association of rs10741657. The study included a novel SNP, not previously reported in the seemingly healthy VDD Pakistani population. Genetic factors have been implicated in the regulation of vitamin D levels, and SNP rs10741657 in CYP2R1 gene has been a subject of interest in this context.[13]

This SNP was previously reported in Bangladeshi population and significant association was found with low serum vitamin D levels.[11] However, the current study did not find any significant association of the studied SNP with VDD. Moreover, there was no correlation of individual genotype found with decreased serum vitamin D levels because the heterozygous genotype A/C was detected more commonly than the other genotypes. The heterozygous genotypes were found in the highest number in the current study. The heterozygousity shows more genetic variability in a population. The heterozygous pattern in the study predicted beneficial effects against VDD.

The rs10741657 polymorphism in CYP2R1 was found to be associated with VDD in Iranian population in two studies, which was contradictory to the current study.[14,15] A study in Kuwait also reported the association of CYP2R1 polymorphisms (rs10741657, rs11023374 and rs12794714) with serum vitamin D levels in adolescents of Arab ethnicity.[16] The current results suggest that polymorphism can cause a distinct phenotype across populations due to geographical location, ethnicity and lifestyle changes. The results emphasise the need of genetic data of polymorphisms either involved in VDD or acting as protective variants in Pakistani population. Additional, genetic variants may interact with rs10741657 to influence vitamin D levels, making it difficult to isolate the effects of

this specific SNP.

The mean age of the current VDD cases was 43.56 years, which was similar to a study in Kazakhstan.[17] Moreover, VDD was more common in females compared to males in the current study which was in line with an earlier study.[18] SNP rs10741657 plays a key role in the promoter region of CYP2R1 gene, which encodes the 25-hydroxylase enzyme. The variations in rs10741657 can influence the rate and activity of this enzyme. The frequency of heterozygous A/C genotypes was found to be common in both the cases and controls, while the frequencies of homozygous A.A was found to be less common in both the groups. These findings suggest the need for further molecular profiling of the respective gene and their SNPs to have a generalised inference related to the local population. The current study determined that genotypes of rs10741657 were inconsistent with the HWE ($p<0.05$). It might be due to random stratification of the studied population and heterogeneity mixture of the Pakistani population.

The current study has limitations as it did not explore the element of ethnicity due to limited resources. There is a strong possibility that SNPs other than these may be the cause of VDD in the sample. Further research should be conducted with subjects from various ethnic backgrounds/ regions to determine the association of VDD with SNPs. Haplotype analysis of the polymorphisms in CYP2R1 gene may also be performed to determine strong association on a larger sample size.

Conclusion

The A allele was found to be the most common allele of rs10741657 in both the groups. The rs10741657 polymorphism was not found to be a risk factor for VDD susceptibility, and was not a major determinant of VDD. No correlation of individual genotype with decreased serum vitamin D levels was found.

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Conflict of Interest: None.

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Author Contribution:

AR: Concept, data acquisition, analysis, interpretation, drafting and agreement to be accountable for all aspects of the work.

AM: Concept, design, revision, final approval and agreement to be accountable for all aspects of the work.