

ORIGINAL ARTICLE

IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISM IN VITAMIN D DEFICIENT PAKISTANI POPULATION

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Background: Vitamin D is an essential nutrient for the human body, which plays an important role in absorption of calcium and the maintenance of its levels. A deficiency of vitamin D is wide spread in South Asian countries like Pakistan. There are many causes of this deficiency, including the genetics. The present study was designed with the aim to find the single nucleotide polymorphism (SNP) located in the GC and vitamin D receptor genes associated with vitamin D deficiency/insufficiency in the local population of Pakistan. **Methods:** A total of four hundred subjects with age from 0 to 60 years were inducted in the present study. After estimation of vitamin D levels, two groups (vitamin D sufficient and insufficient) were formed. DNA extraction and PCR were done to compare the SNP in the two groups. Two SNPs in GC and vitamin D receptor genes were studied through TETRA-ARMS PCR. The allelic and genotypic frequencies were calculated and Chi-square was applied to find risk allele association in two groups. **Results:** Any association was lacking between vitamin D deficiency and rs3847987 / rs2282679 SNPs in this part of Pakistani population. The major allele frequency in case of rs2282679 was found equal in vitamin D deficient subjects and healthy individuals. The rs3847987 showed the major allele frequency in both vitamin D deficient subjects and controls. Increased exposure to sunlight, fortification of food and supplements of vitamin D appear to be the most effective choices to combat the vitamin D deficiency (VDD). It is concluded that in this study group SNPs rs3847987 and rs2282679 were not related to VDD.

Keywords: Vitamin D deficiency, *VDR* gene, GC genes, single nucleotide polymorphism, TETRA-ARMS PCR reaction

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INTRODUCTION

Primarily Vitamin D₃ production depends upon skin exposure to sunlight. Vitamin D₂ is synthesized by fungi. Both types undergo two consecutive hydroxylation reactions in liver and kidneys, respectively, which convert them to the active form called 1,25-dihydroxy Vitamin D. Cytochrome P450 family hydroxylases are responsible for the synthesis of 1,25(OH)₂D₃.¹ The metabolism of bone turnover, insulin-like growth factor-1, growth hormones and vitamin D are mutually modulated by each other, which influence cell proliferation, maturation, mineralization and bone resorption.² Active form of vitamin D binds to vitamin D receptors (VDRs) present on the nuclei of different cells of body. Factors like vitamin D, parathyroid hormone or fibroblast growth factors are involved in the homeostasis of phosphorus and calcium.³

We found in one of our previous studies that in spite of adequate sun exposure in Pakistan, Vitamin D deficiency (VDD) is rampant in this part of the world. Analytical cross-sectional studies by other researchers conducted on the local population in Pakistan revealed that job location, interval of sun light exposure, education as well as socioeconomic situations are responsible for hypo-vitaminosis D.⁴ Pakistan Health Research Council Centres in

Islamabad, Lahore and Karachi carried out a study in which subjects were recruited who reported for vitamin D testing or bone mineral density examination. Strangely this study revealed that VDD does not have any direct effect on serum phosphorus, calcium, alkaline phosphatase or bone mineral density.⁵ Vitamin D deficiency is on the rise worldwide. According to an estimate, about 50% or more of the world population has VDD (serum levels below 30 ng/mL). Epidemiological studies show varying vitamin D levels with age and genders.^{6,7} Inadequacy is particularly found to be high in the older population, particular geographical areas, in kids, young females, in ethnic minorities/immigrants and socio-economically poor people. Whatever the cause is, suboptimal levels of vitamin D are being linked with many diseases these days. Secondary hyperparathyroidism results from VDD. This potentially can cause low serum levels of phosphorus due to less absorption through the gut and loss in urine. Later, it may result in a decrease of the calcium phosphorus products necessary for bone mineralization. Under severe VDD states, calcium and phosphorus levels go down but alkaline phosphatase levels rise. Optimal regulation of *Pi* is disturbed if individual is vitamin D deficient. Hypophosphatemia may lead to rickets and growth retardation.³ Vitamin D

inadequacy leads to inefficiency of the human physiological systems like muscle power, neuromuscular conduction, hormonal release, autoimmunity and cancers. 1,25-dihydroxy Vitamin D acts as a hormone controller (calcium/phosphorus balance) as well as an immuno-modulator. Its deficiency can contribute to the pathogenesis of infection by HIV.⁸ According to various recent studies, VDD has been implicated in raised blood pressure, diabetes mellitus, inflammatory diseases, obesity, heart and vascular disorders. Vitamin D deficiency has also been related to numerous cancers. It affects the reproductive system indirectly through extracellular calcium/phosphorus which has been seen to cause infertility in male mice.⁹ Vitamin D serum levels can definitely be raised by food fortification, and this has been proved in many countries like USA, Canada, and Finland.

It is now well established that polymorphism in genes play a role in the metabolism of vitamin D and later on in VDD. Mutations in VDR gene may be associated with diseases like obstructive sleep apnoea syndrome.¹⁰ Genome-wide analysis of VDR is required to comprehend the physiological effects of the active form of vitamin D. FokI polymorphism of VDR causes VDD resulting in increase the risk of preeclampsia.¹¹ SNPs in the VDR gene causing VDD are also related to breast cancer.¹² It has been suggested that Vitamin D (Vit D) supplements might have protective role in certain diseases. SNP Taq-1, identified in the VDR gene though associated with the acute pancreatitis yet Vit D supplement could not play a protective role in it.¹³

In the light of above facts this study was designed to find a possible genetic cause for VDD in Pakistani population. Objective was to determine whether SNPs rs3847987 and/or rs2282679 in GC and VDR genes are responsible for serum calcitriol deficiency.

MATERIAL AND METHODS

Ethical Committee approval was sought to fulfill the criteria laid down by the Declaration of Helsinki. Healthy subjects of various age groups ranging from 0 to 60 years, belonging to both genders and living in Northern Punjab were recruited. A questionnaire was prepared to enter the demographic and other data; keeping in mind the inclusion/exclusion criteria. Five milliliters of blood were drawn from 400 subjects over a period of one year (October 2017 to October 2018). The levels of vitamin D were estimated. DNA was isolated from these 400 blood samples by the phenol-chloroform organic method. The two SNPs rs3847987 (A/C allele) and rs2282679 (T/G allele) in VDR and GC genes were selected. TETRA-ARMS PCR was used to screen the SNPs. PCR primers were designed

through primer 1 software (Table-1). The PCR reaction contained 1×PCR, 2 mM MgCl₂, 200 μM dNTPs, 1:5 ratio outer and inner primers (forward and reverse), 1 unit Taq DNA Polymerase and x volume double distilled water. TETRA-ARMS PCR was optimized for each SNP. Final conditions were 95 °C for 5 min, denaturation at 95 °C for 30 seconds, annealing at 69 °C and 57.4 °C for each SNP for 80 seconds, extension at 72 °C for 40 seconds and final extension at 72 °C for 8 minutes followed by 35 cycles. Both outer and inner primers were used in different ratios such as 1:5, 1:6 and 1:8. The best outer and inner primers ratio for TETRA-ARM PCR was optimized at 1:5 for rs3847987 and 1:3 for rs2282679. The additive model was used to calculate allelic and genotypic frequency of aforementioned SNPs. These frequencies were calculated for Hardy-Weinberg equilibrium. Chi-square was applied.

RESULTS

The DNA was resolved on 0.8% agarose gel to estimate quality and quantity. Figure-1 shows the DNA isolated from patients and control subjects. The quantity of DNA ranged from 100 ng/μl to 1.5 μg/μl, while the spectrophotometer ratio at 260 nm/280 nm was 1–1.5 (Figure-1).

TETRA-ARMS PCR is a cost effective molecular technique for the study of single nucleotide polymorphism. We obtained good results in TETRA-ARMS PCR.

Table-1: Genotypic distribution of rs3847987 and rs2282679 in VDD subjects and healthy individuals

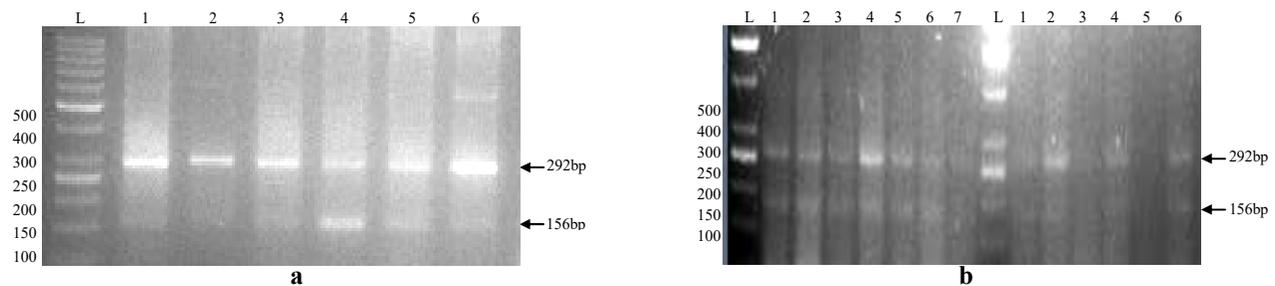
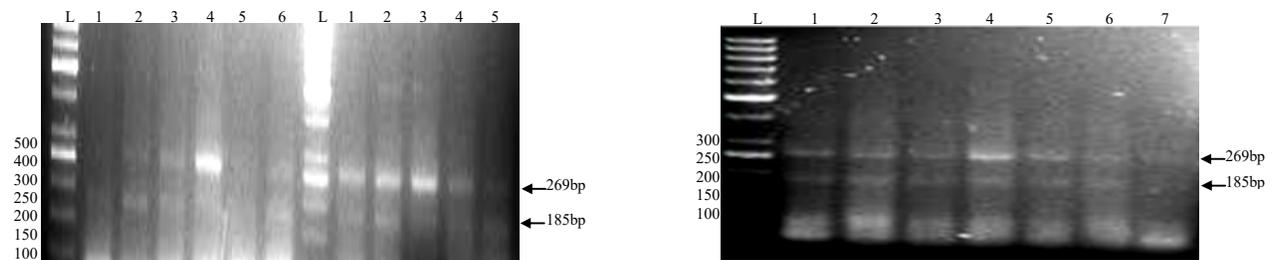
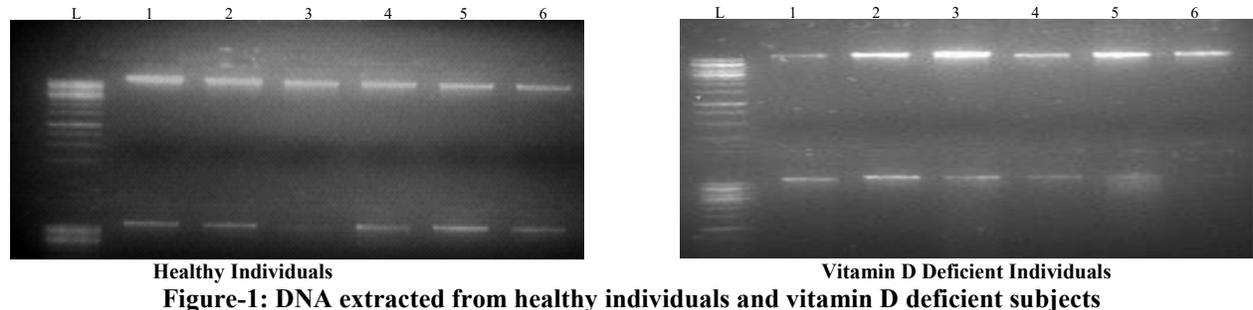
Gene/SNP	Genotype	VDD		Healthy Controls	Alleles		X ²
		Subjects [n (%)]	Alleles [n (%)]		Alleles [n (%)]		
VDR/ rs3847987	AA	300 (100%)	A=600	100 (100%)	A=200	0.6612 p=0.416	
	AC	0	(100%)	0	(100%)		
	CC	0	C=0	0	C=0		
GC/ rs2282679	GG	0	G=0		G=0	0.6612 p=0.416	
	GT	0	T=600		T=200		
	TT	300 (100%)	(100%)	100 (100%)	(100%)		

rs3847987: This SNP has alleles A>C present in the VDR gene which plays a role in VitD metabolism. The major allele A was identified in all vitamin D deficient subjects and controls. The allele C was not found in any sample (Figure-2). We obtained 269bp and 185bp PCR fragments. The 269bp fragment was amplified with outer forward and reverse primers. In a single PCR 185bp fragment was amplified with outer forward and inner reverse primers. The 185bp fragment was carrying allele A with normal distribution.

rs2282679: The major allele was T, and the minor allele was G in GC gene. The polymorphism was T>G. The major allele T was found in all vitamin D deficient subjects and controls (Figure-3). Meaning the studied SNP had no association with vitamin D

deficiency. The major allele was found with a frequency of 100% in both control and deficient subjects. The major alleles T amplified with a fragment size of 156bp. The outer primers amplified 292bp fragments. Both studied of two SNPs

statistically indicates the absence of risk association of for vitamin D deficiency. Both studied SNPs did not show an association with VDD in this group of population and were insignificant.



DISCUSSION

This is probably the first time that SNPs rs3847987 and rs2282679 correlation with VDD has been sought in Pakistan.

Serum vitamin D status was not found to be correlated with these two SNPs in this study. In this study less than 29 ng/mL vitamin D serum level was considered deficient as suggested by Endocrine Society of International Medical Organization and applied by other researchers also.¹⁴ Main aim of this study was to get insight of the genetic basis of vitamin D deficiency in Pakistan. In some other studies, no association has been found in studied SNPs and VDD. Polymorphic loci of vitamin D genes distribution may be different in Northern Punjab than rest of Pakistan. Similar variations have been found in Indian population.¹⁵ Vitamin D deficiency is common in women and children of Pakistan but GC genotypes may not be associated with

vitamin D levels in the serum.¹⁶ In another study carried out in Pakistan, VDD was present in controls as well as cases but Fok 1 and Apa 1 variants of VDR genotype were not associated with Type 1 diabetes mellitus (T1DM) cases.¹⁷ There may not be a direct relationship of VDD with bone mineral density or levels of phosphorus, calcium, and alkaline phosphatase (ALP) in Pakistani population. Probably we have adjusted to low vitamin D levels.¹⁸ Pakistani genetic determinants might be ethnicity specific as is found in the rest of the world also. There is a wide variation in the frequency of SNP and their allelic distribution in different populations. In VDR and GC genes analysis, none of the 10 SNPs studied were associated with vitamin D status in Uyghur or Kazak ethnic populations.¹⁹

Other explanation of low vitamin D levels in spite of adequate sunshine in our study group could be prolonged winter or rainy seasons in the period of study which can mitigate this beneficial effect of sunshine to

some extent. People of Northern Punjab including women have usually a lot of outdoor activities. Many women work in fields to give a helping hand to their male counter parts. A darker complexion in some cases might be a contributing factor of the deficiency. It is known that raised levels of melanin are associated with reduced skin synthesis of vitamin D.²⁰ Vegetarian diet in some families can be a contributory factor in deficiency of vitamin D. To understand vitamin D role in the human body it is necessary to correlate it with other metabolites and identify the mechanisms and pathways, specifically when quantifying them. Metabolic consumptions are influenced by physiological stimuli and tissue demands.

There were certain limitations to this study. Obese individuals are more likely to be deficient in vitamin D and have disturbed bone metabolism.²¹ Neither BMI was considered during enrolment of patients in this study nor their dietary habits were taken into account. Subjects were limited to Northern Punjab and up to 60 years of age. Therefore, these results cannot be generalized to other regions of the country or to other age groups. More research is required to look into other important SNPs also. Polymorphisms in CYP2R1-rs10766197 and DHCR7/NADSYN1 have been found to be involved in the vitamin D pathway.²²

In order to maintain optimum levels of vitamin D it is highly recommended that government should ensure proper fortification of food products. Unfortified milk has 2 IU/100 ml of vitamin D. Vitamin D supplements are also recommended to bring down the ill effects of vitamin D deficiency in affected individuals. Intestinal fractional calcium absorption (FCA) is affected by vitamin D₃ treatment. The 1 α hydroxyl calcidiol and eldcalcitol treatments increase FCA. This is because of direct stimulation of VDRs by vitamin D₃ in the gut.²³ Vitamin D supplements have a special role to ward off signs and symptoms if there are some associated conditions like early renal failure. In some diseases supplements have been found to be of no use. Severe vitamin D deficiency along with hypocalcaemia has been found to cause 'Hypocalcaemic Cardiomyopathy' but alfacalcidol at 0.5 mg/day was not able to reduce cardiovascular events.²⁴ Having said that, one has to be careful not to overdose with vitamin D supplements because musculoskeletal side effects and certain cardiac issues might crop up.

Though limited quantity of data was evaluated in available time span and resources, yet results indicate clear and helpful information. It is possible that normal values of vitamin D may be low for this part of the world as it is already seen that maximal intestinal calcium absorption can occur at lower levels of vitamin D in Pakistan.

CONCLUSION

This study indicates absence of association of SNPs rs3847987 and rs2282679 with VDD in Pakistani Population. More work needs to be done to study other reported SNPs as well as genetic mutations. Genetic sequencing can help to identify population specific SNPs. Fortification of dietary products need to be ensured in Pakistan at the government level.

REFERENCES

1. Kagi L, Bettoni C, Pastor-Arroyo EM, Schnitzbauer U, Hernando N, Wagner CA. Regulation of vitamin D metabolizing enzymes in murine renal and extrarenal tissues by dietary phosphate, FGF23, and 1,25(OH)2D3. *PLoS One* 2018;13:e0195427.
2. Witkowska-Sedek E, Stelmaszczyk-Emmel A, Majcher A, Demkow U, Pyrzak B. The relationship between alkaline phosphatase and bone alkaline phosphatase activity and the growth hormone/insulin-like growth factor-1 axis and vitamin D status in children with growth hormone deficiency. *Acta Biochim Pol* 2018;65:269–75.
3. Jacquillet G, Unwin RJ. Physiological regulation of phosphate by vitamin D, parathyroid hormone (PTH) and phosphate (Pi). *Pflugers Arch* 2019;471:83–98.
4. Roomi MA, Farooq A, Ullah E, Lone KP. Hypovitaminosis D and its association with lifestyle factors. *Pak J Med Sci* 2015;31:1236–40.
5. Nadeem S, Munim TF, Hussain HF, Hussain DF. Determinants of Vitamin D deficiency in asymptomatic healthy young medical students. *Pak J Med Sci* 2018;34:1248–52.
6. Hagenau T, Vest R, Gissel TN, Poulsen CS, Erlandsen M, Mosekilde L, *et al.* Global vitamin D levels in relation to age, gender, skin pigmentation and latitude: an ecologic meta-regression analysis. *Osteoporos Int* 2009;20:133–40.
7. Arabi A, El Rassi R, El-Hajj Fuleihan G. Hypovitaminosis D in developing countries-prevalence, risk factors and outcomes. *Nat Rev Endocrinol* 2010;6:550–61.
8. Jimenez-Sousa MA, Martinez I, Medrano LM, Fernandez-Rodriguez A, Resino S. Vitamin D in Human Immunodeficiency Virus Infection: Influence on Immunity and Disease. *Front Immunol* 2018;9:458.
9. Sun W, Chen L, Zhang W, Wang R, Goltzman D, Miao D. Active vitamin D deficiency mediated by extracellular calcium and phosphorus results in male infertility in young mice. *Am J Physiol Endocrinol Metab* 2015;308:E51–62.
10. Kirac D, Yagcioglu Yassa O, Gezmis H, Mayda Domac SF, Altunok EC, Genc E. Different VDR, VDBP genotypes and vitamin D levels may effect obstructive sleep apnea syndrome. *Cell Mol Biol (Noisy-le-grand)* 2019;65:46–51.
11. Rezavand N, Tabarok S, Rahimi Z, Vaisi-Raygani A, Mohammadi E, Rahimi Z. The effect of VDR gene polymorphisms and vitamin D level on blood pressure, risk of preeclampsia, gestational age, and body mass index. *J Cell Biochem* 2019;120:6441–8.
12. O'Brien KM, Sandler DP, Kinyamu HK, Taylor JA, Weinberg CR. Single-Nucleotide Polymorphisms in Vitamin D-Related Genes May Modify Vitamin D-Breast Cancer Associations. *Cancer Epidemiol Biomarkers Prev* 2017;26:1761–71.
13. Cieslinska A, Kostyra E, Fiedorowicz E, Snarska J, Kordulewska N, Kiper K, *et al.* Single nucleotide polymorphisms in the vitamin D receptor gene (VDR) may have an impact on acute pancreatitis (AP) development: A prospective study in populations of AP patients and alcohol-abuse controls. *Int J Mol Sci* 2018;19. doi: 10.3390/ijms19071919.
14. Nadeem S, Munim TF, Hussain HF, Hussain DF. Determinants of Vitamin D deficiency in asymptomatic healthy young medical students. *Pak J Med Sci* 2018;34:1248–52.
15. Bhanushali AA, Lajpal N, Kulkarni SS, Chavan SS, Bagadi SS, Das BR. Frequency of fokI and taqI polymorphism of vitamin D

- receptor gene in Indian population and its association with 25-hydroxyvitamin D levels. *Indian J Hum Genet* 2009;15:108–13.
16. Anwar S, Iqbal MP, Azam I, Habib A, Bhutta S, Soofi SB, Bhutta ZA. Urban and rural comparison of vitamin D status in Pakistani pregnant women and neonates. *J Obstet Gynaecol* 2016;36:318–23.
 17. Nasreen M, Lone KP, Khaliq S, Khaliq S. Serum vitamin D levels and gene polymorphisms (FokI and ApaI) in children with type I diabetes and healthy controls. *J Pak Med Assoc* 2016;66:1215–20.
 18. Nadeem Saqib MA, Rafique I, Hayder I, Irshad R, Bashir S, Ullah R, *et al.* Comparison of vitamin D levels with bone density, calcium, phosphate and alkaline phosphatase—an insight from major cities of Pakistan. *J Pak Med Assoc* 2018;68:543–7.
 19. Xu X, Mao J, Zhang M, Liu H, Li H, Lei H, *et al.* Vitamin D deficiency in Uyghurs and Kazaks is associated with polymorphisms in CYP2R1 and DHCR7/NADSYN1 genes. *Med Sci Monit* 2015;21:1960–8.
 20. Bonilla C, Ness AR, Wills AK, Lawlor DA, Lewis SJ, Davey Smith G. Skin pigmentation, sun exposure and vitamin D levels in children of the Avon Longitudinal Study of Parents and Children. *BMC Public Health* 2014;14:597.
 21. Azzam EZ, Ata MN, Younan DN, Salem TM, Abdul-Aziz AA. Obesity: Relationship between vitamin D deficiency, obesity and sclerostin as a novel biomarker of bone metabolism. *J Clin Transl Endocrinol* 2019;17:100197.
 22. Slater NA, Rager ML, Havrda DE, Harralson AF. Genetic variation in CYP2R1 and GC genes associated with vitamin D deficiency status. *J Pharm Pract* 2017;30:31–6.
 23. Uenishi K, Tokiwa M, Kato S, Shiraki M. Stimulation of intestinal calcium absorption by orally administered vitamin D3 compounds: a prospective open-label randomized trial in osteoporosis. *Osteoporos Int* 2018;29:723–32.
 24. Hamano T, Yonemoto S. [The association between vitamin D/calcium deficiency and cardiovascular events.]. *Clin Calcium*. 2019;29:185–91. (Article in Japanese)

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