ORIGINAL ARTICLE

Polymorphisms in the GC Gene for Vitamin D Binding Protein and Their Association with Vitamin D and Bone Mass in Young Adults

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ABSTRACT

Objective: To identify DBP gene rs4588 and rs7041 polymorphisms and associate with participants' serum 25(OH)D and BMD levels.

Study Design: Cross-sectional descriptive study design.

Place and Duration of Study: Section of Chemical Pathology and Molecular Pathology, Department of Pathology and Laboratory Medicine, The Aga Khan University, Karachi, from July 2014 to September 2015.

Methodology: Blood samples from 98 young adults, out of 101 samples collected, were genotyped for GC rs4588 and rs7041 polymorphisms by polymerase chain reaction-based restriction fragment length polymorphism assay. Questionnaires were administered to obtain information on demographics and anthropometric characteristics, BMD was assessed with heel ultrasound and 25(OH)D was measured using a Chemiluminescence immunoassay.

Results: High prevalence of vitamin D deficiency was noted in the study population n=87 (86.1%) having median (IQR) 25(OH)D levels of 14.9 (20.9) ng/ml, in males and 12.1 (51.8) ng/ml in females. The C/C genotype of SNP rs4588 had the highest proportion n=50 (51%), whereas for rs7041 genotype G/T was most frequently observed n=53 (54%) in subjects. Highest 25(OH)D levels were observed within the homozygous genotypes C/C median 25(OH)D 14.0 (49.6) and G/G (median 25(OH)D 14.9 (37.1) ng/ml. Statistically significant relationship was noted between rs7041 genotype G/T and BMD (p 0.037).

Conclusion: Hypovitaminosis D was frequently found in young adults. Furthermore, G/T variant of rs7041 polymorphism was associated with lower 25(OH)D serum levels.

Key Words: *Vitamin D deficiency, Single nucleotide polymorphism, GC gene, BMD, 25(OH)D, Pakistani population, Vitamin D binding protein, PCR, Genotype, Allele.*

INTRODUCTION

The production of vitamin D in the skin is a two-step process. Ultraviolet B component of sunlight breaks down B ring of 7-dehydrocholesterol forming pre-vitamin D, which then isomerises to vitamin D in a thermo sensitive process. The other three main steps in vitamin D metabolism including 25 hydroxylation, 1, and 24-hydroxylation are regulated by cytochrome P450 mixed-function oxidases CYP27A1 and CYP2R1 for 25 hydroxylase, CYP27B1, and CYP24A1 for 1 alpha and 24 alpha-hydroxylase enzymes, respectively.[1]

Vitamin D binding protein (DBP) is a product of the group-specific component of DBP (GC gene), which is located on chromosome 4. It performs the transport of vitamin D to target tissues.[2] The DBP-bound vitamin is relatively unavailable to target tissues; thus, level or change in the affinity of DBP could lead to change in vitamin D bioactivity, resulting in differences in the functional status of vitamin D between individuals having same 25(OH)D concentrations. This variation has been linked to alteration in bone mineral density (BMD) in several populations. However, the precise role of DBP in vitamin D actions is still incompletely understood.[3,4] Santos *et al*. previously demonstrated the association of AA genotype of rs4588 and TT genotype of rs7041 with lower 25(OH)D levels, after an adjustment for age and season in Brazil. However, the frequencies of the polymorphism can vary between the populations.[5]

Although many genetic variations in the GC gene are described in the literature, the two most common polymorphisms reported are rs70411 and rs4588 located in the exon 11. These single nucleotide polymorphisms (SNPs) exist in complete linkage disequilibrium, so only six haplotypes are observed to any significant frequency.[5,6]

Vitamin D deficiency is common in the developing as well as developed countries alike, and all ages are equally prone to deficiency.[7,8] However, in Pakistani population significantly prominent vitamin D deficiency has been reported compared to Western communities, which has been explained by inadequate sunlight exposure, lifestyle factors, skin pigmentation and altered vitamin D metabolism.[9-12]

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Iqbal *et al*. did not find any association between vitamin D levels and VDBP genotypes surveyed in the pregnant women and neonates of urban and rural areas in Pakistan.[13] In another study, an association was found between GC 1-2 genotype of VDBP gene and risk to T2DM in a Pakistani population, while hypovitaminosis D appeared not to have any relationship with T2DM.[14] So far, no study in Pakistan has been conducted on GC; gene polymorphisms in normal healthy individuals.

This study was conducted to explore association between two non-synonymous SNPs in GC gene, specifically rs4588 and rs7041 with 25(OH)D and BMD in healthy Pakistani young adults.

METHODOLOGY

A cross-sectional descriptive study was conducted at the Chemical Pathology Section, Department of Pathology and Laboratory Medicine, The Aga Khan University (AKU), Karachi, Pakistan, between July 2014 and September 2015. The study was reviewed and approved by the Institution's Ethical Review Committee (ERC approval number: 2155-Path-ERC-12). Each participant was allotted an identifying serial number to maintain confidentiality.

Apparently 101 healthy students, enrolled at the Medical College of AKU, were invited to participate in this study and a written informed consent was obtained. Inclusion criteria included all those students who were not on any calcium and vitamin D supplements for the last six months. Those individuals who took vitamin D injection or had remarkable changes in weight or diet in the last six months or any disease / metabolic disorder that may affect their vitamin D levels, were excluded from study. Socio-demographic information (age, sex and ethnicity) and lifestyle factors were collected through interviewer administered questionnaire. Anthropometric characteristics (height, weight, and waist circumference), body mass index (BMI) and bone mass were also measured.

A 7-day camp was set up for phlebotomy and for determination of BMD by heel ultrasound. Blood samples for 25(OH)D measurements were drawn in gel separation tubes and centrifuged. Aliquot of serum were prepared, labelled with the participant's serial number and immediately stored at -20°C until analysis.

Ultrasound Bone Densitometer Sonost-3000 by Osteosys Belgium East Flanders was used for measuring BMD. This device is an ultrasound bone densitometer which has ±0.2 (T-score) precision error.

Serum 25(OH)D levels was measured by ADVIA Centaur vitamin D total assay which is a direct competitive chemiluminescence immunoassay that is standardised to the ID-LC/MS/MS 25(OH)D Reference Measurement Procedure of the Vitamin D Standardisation Programme. Each run included three controls with high, low and normal concentration of 25(OH)D provided by the manufacturer. Assay's lower and upper detection limits were 4.2 ng/ml and 150 ng/ml, respectively and its CV ranged between 4.2 and 11.9%.

Genomic DNA was extracted from WBC using Promega DNA isolation kit [Madison, WI, USA]. Purified DNA was stored at -20°C for further processing. The SNPs rs4588 and rs7041 were analysed by PCR-RFLP using a published protocol (12). PCR amplification was carried out in a total volume of 25 µL, containing 100 ng of genomic DNA, 200 µM of dNTPS, 1.5mM MgCl2 and 1 unit of *Taq* polymerase (Promega, Madison, WI, USA). PCR reactions were subjected to 40 cycles of denaturation (95°C for 30 s), annealing (60°C for 40 s) and elongation (72°C for 30 s) using a BioRad Techne 100 thermal cycler. Restriction enzymes *HaeIII* and *BtgI* (New England Biolabs, Hitchin, UK) were used for the identification of rs7041 (T/G) and rs4588(C/A) alleles, respectively. Restriction mix consisted of 15 µl PCR product, 3 µl of corresponding buffer, 0.2 µl BSA, 11.3 µl nuclease-free water and 5 units of specific enzyme, which was incubated overnight in a water bath at 37°C. Restriction products were then analysed by 3% agarose gel electrophoresis after staining with ethidium bromide. All assays were examined with appropriate controls.

Statistical analysis was done using SPSS version 21. For categorical variables, descriptive statistics such as alleles and genotypes frequencies with percentages and for quantitative data mean ±SD were performed. After testing for normality distribution using Kolmogorov-Smirnov test, data of vitamin D was found skewed and; hence, medians were reported. Vitamin D deficiency was defined as <20 ng/mL and sufficiency or optimal levels as >20 ng/mL.[14] Kruskal-Wallis and Chi-Square tests were used where applicable. Allele and genotype frequencies were calculated by direct counting. SNPStats (http://bioinfo.iconcologia.net) was applied for calculation of Hardy-Weinberg equilibrium, comparison of allele and genotype frequencies between study variables such as 25(OH)D levels and bone mass. P-value <0.05 was considered significant.

RESULTS

Table I elaborates biochemical parameters of 101 medical students enrolled in the study. Median age was 20 (7) years. Forty-two students (41.6%) and 59 (58.44%) were males and females, respectively. Eighty-seven students (86.1%) were vitamin D deficient, of which 49 (48.5%) were females while 38 (37.7%) were males. Sufficient 25(OH)D levels were present in seven (6.9%) medical students only. Notably, all those classified as having sufficient 25(OH)D levels were females. Median 25(OH)D levels of males [14.9 (20.9) ng/ml] and females [12.1 (51.8) ng/ml] were statistically different (p = 0.03).

DNAs of three students were contaminated; hence, excluded from genotype analysis. The rs4588 (p=0.81)

Table I: Demographic, anthropometric and biochemical features of study subjects (n=101).

Feature		
Median (IQR) of age in years,	20 (7)	
Sex (frequency, percentage)		
Males	42 (41.6%)	
Females	59 (58.4%)	
Median (IQR) of BMI in kg/m^2	21.9 (18.98)	
Median (IQR) of waist circumference in inches	60.3 (56.5)	
Biochemical parameters	Males	Females
Median (IQR) 25(OH)D level (ng/mL)	14.95 (20.9)	12.1 (51.8)
Median (IQR) 25(OH)D Status		
Deficient (n=87)	38 (37.6%)	49 (48.5%)
Insufficient (n=7)	4 (3.9%)	3 (2.9%)
Sufficient (n=7)	-	7 (6.9%)

The values are presented in frequency (%).

Table II: Distribution of 25(OH)D levels and bone mass according to rs4588 and rs7041 genotypes in young adults (n=98).

SNP-Genotype (n) Median (IQR) (ng/ml)	25(OH)D levels	p-value	Bone mass (g/cm^2) Median (IQR)	p-value
rs4588				
AA (7)	13.0 (16.3)	0.90	2.7 (1.2	0.88
AC (41)	14.0(49.6)		2.3 (1.5)	
CC (50)	14.0 (38)		2.3 (1.6)	
rs7041				
GG (22)	14.9 (37.1)	0.75	2.3 (1.3)	0.037*
GT (53)	13.2 (49.6)		2.2 (1.4)	
TT (23)	14.0 (21.5)		2.7 (1.5)	

**rs7041 association with bone mass was statistically significant as assessed by Kruskal-Walis test.*

and rs7041 (p=0.54) genotypes were in Hardy-Weinberg equilibrium. Within the rs4588; C/C genotype was the most frequently detected 51% (n=50), followed by A/C 42% (n=41) and A/A genotype 7% (n=7). The C allele constituted 72% (n=141) of the total allele count; whereas, the remaining 28% (n=55) were A allele. The spectrum of rs7041 genotypes consisted of heterozygous G/T 54% (n=55); the homozygous G/G and T/T were 22% (n=22) and 23% (n=23), respectively with distribution for G and T alleles of 97 (49.4%) and 99 (50.5%).

Table II shows distribution of rs4588 and rs7041 genotypes and their association with 25(OH)D levels and bone mass. Median 25(OH)D levels were deficient in all the genotypes. Comparatively higher levels were measured in C/C and G/G genotypes and lowest in A/A and G/T genotypes of rs4588 and rs7041 SNP, respectively. A statistically significant association was observed between rs7041 genotypes, and bone mass (p-value=0.037) with the lowest observed bone mass (2.2 (1.9) g/cm^3) for G/T genotype when compared to the other variants within this polymorphism. The mean bone mass observed for the rs4588 genotypes were not significantly different.

DISCUSSION

Vitamin D deficiency is a very common disease in the Pakistani population; and several studies have reported prevalence ranging from 53.5% in randomly chosen citizens to a striking 98.86% in the young adults.[13,14] This study reports prevalence of vitamin D deficiency in 85.6% young adults that correlates with earlier findings. Furthermore, the majority of those classified as deficient were females (51.8%); Iqbal *et al*. described similar findings in a previous study, carried out in the urban Pakistani population.[14]

This study was consistent with the distribution patterns of GC gene rs4,588 and rs7041 allele frequencies compared to other populations. G/T was recognised as the most common variant amongst the rs7041 polymorphisms, while the homozygous variant T/T was found in a smaller number of individuals; in case of rs4588 SNP, C/C was observed as the most common genotype, whereas A/A was rarely seen.

Several studies have shown that rs4588 variants A/A and A/C and rs7041 variants T/T and T/G are associated with lower serum vitamin D levels compared to the C/C and G/G, respectively.[5,15,16] Moreover, significantly lower levels of vitamin D were noted in individuals having rs4588A/A: rs7041 T/T haplotype compared to other combinations.[17,18] Furthermore, median serum levels of 25(OH)D have been observed to be highest for individuals with two copies of rs7041 G/G: rs4588G/G.[19] The present findings were similar to those mentioned above, lower median 25(OH)D levels were observed within the rs4588 A/A and rs7041 G/T genotypes (evaluated separately) and highest levels in rs7041 C/C and G/G genotypes; although these differences did not achieve statistical significance in our study, because of a smaller sample size. Numerically, the difference between the median serum 25(OH)D levels for C/C and A/C genotypes was less than 1 ng/mL.

The low levels of vitamin D associated with the rare alleles can be explained in part by the functionally different proteins coded by these genes, which leads to decreased synthesis of DBP by the rare genotypes, ultimately translating into lower total vitamin D metabolite levels in the serum. Interestingly, some of the variants with the higher median levels of serum 25(OH)D also showed higher frequency of Z-scores below-2 (A/C, C/C in rs4588 and G/T in rs7041) (data not shown). Indeed, it has been shown that individuals carrying the C/C variant of r4588 have a higher risk of osteoporosis despite high levels of serum 25(OH)D as underscored by Li *et al*. in a Chinese population.[20] These observations can be substantially explained by taking into consideration the structure of DBP. It is comprised of three domains; domain I is the vitamin D binding region; whereas, domains II and III are responsible for other non-binding functions, including osteoclast differentiation and activation. Thus, this protein can essentially influence bone resorption directly by functioning as a DBP-macrophage activating factor. Rats treated with DBP-

macrophage activating factor have increased osteoclastic activity and decreased bone mass.[21,22] Since these domains are independent of each other in function, the nature of the relationship between the allelic variation, vitamin D levels and osteoporotic risk can be validated.[23] Lauridsen *et al*. reported an increased fracture risk and the haplotype of DBP rs7041 G allele and rs4588 C alleles.[24]

This study showed statistically non-significant association between bone mass and the A/C and C/C genotypes, the lowest bone mass within rs7041 was observed to have a significant association with the G/T variant. Nevertheless, these findings must be interpreted in context of the genetic influence of the vitamin DBP gene on osteoporosis, and fracture risk is part of an interplay between both, environmental and other genetic risk factors in vitamin D, and hence, bone metabolism.[25]

Findings from this study improve the understanding of vitamin D homoeostasis and could assist identification of a subgroup of the Pakistani population who are at risk of hypovitaminosis D and its consequences. This is one of the few studies that addresses multiple genetic factors underlying the Pakistani population-wide vitamin D deficiency. It also highlights the need to perhaps redefine the cutoffs for Vitamin D deficiency and sufficiency for a population largely genetically predisposed to lower serum vitamin D levels than counterparts in the Western world.

CONCLUSION

Certain variants within the rs4588 and rs7041 polymorphisms, which code for the vitamin DBP are related to lower vitamin D levels, and/or osteoporosis, and this study paves the way for future studies on a larger scale to further explain and highlight these genetic associations in Pakistan.

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