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Association Between Vitamin D Levels and Vitamin D Receptor (VDR) Gene Polymorphisms in Nepalese Population

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ABSTRACT

Background: Vitamin D deficiency is a global public health concern. Small variations in the proteins involved in vitamin D metabolism affect levels and physiological roles of vitamin D. However, there is a paucity of data regarding such factors in our population. This study was designed to assess the distribution of polymorphisms of vitamin D Receptor (VDR) gene (rs2238136 and rs731236) in vitamin-D deficient Nepalese population.

Methods: A total of 400 apparently healthy individuals visiting our center were enrolled. Five ml of blood was collected in gel vial and EDTA vial. The 25(OH)D levels were estimated in ABBOTT Architect autoanalyzer. After extracting genomic DNA, the VDR gene polymorphism (rs2238136 and rs731236) was analyzed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The amplified products, 135 bp and 716 bp were digested using restriction enzymes *Bpu*10IA and *Taq*I for rs2238136 and rs731236, respectively, electrophoresed on 2% agarose gel and visualized under UV illuminator. Statistical analysis was done using SPSS version 21.0.

Results: The distribution of both rs2238136 and rs731236 followed Hardy-Weinberg equilibrium. There were 24 AA, 238 GG, and 138 AG genotypes found in rs2238136, whereas 226 TT, 34 CC, and 140 TC genotypes were found in rs731236. In contrast to rs731236, vitamin D levels varied among genotypes of rs2238136.

Conclusions: In our population, there was a higher prevalence of GG genotype (rs2238136) and TT genotype (rs731236). Increased prevalence of vitamin D deficiency in our region may be due to these genetic variations.

Keywords: Genetic polymorphism; restriction fragment length polymorphism; vitamin d; vitamin d receptor.

INTRODUCTION

Vitamin D is an important mediator of endocrine homeostasis influencing calcium and phosphorus metabolism, nerve conduction, musculoskeletal well-being, and many more. Various physiological, genetic and environmental factors including reduced sunlight exposure, genetic predisposition, altered gut microbiome and immune status, affect vitamin D levels.[1] Variations in the proteins involved in vitamin D metabolism may also cause vitamin D deficiency.[2]

The physiological effect of vitamin D ensues once its active metabolite binds the intracellular hormone receptor called as vitamin D receptor (VDR), which is encoded by *VDR* gene located at chromosome12q13.11.[2] Any deviation from its native form may alter the response of active vitamin D metabolites. Among various SNPs related to *VDR*, rs2238136 and rs731236 are well-studied.

There are no genomic studies conducted in Nepal in relation to the genes affecting vitamin D metabolism and their serum levels. This study was designed to explore the existence of functionally relevant polymorphisms (*VDR* - rs2238136 and rs731236) in Nepalese population in association with the circulating serum vitamin D levels.

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METHODS

This was a cross-sectional study conducted among apparently healthy participants visiting general health check-up clinic at our centre for a period of two years between January 2021 and December 2023.

An ethical clearance was obtained from the ethical review board of Nepal Health Research Council (NHRC Ref. No. 647/2020). Written and informed consent was taken from the participant before collecting the samples.

Individuals presenting with chronic diseases like Diabetes Mellitus, hypertension, thyroid disorders, and patients taking vitamins and calcium supplements were excluded.

The minimum sample size calculated was 376. In this study, 400 individuals were enrolled.

Using aseptic technique, three ml of blood was collected in a gel vial and two ml in Ethylene diamine tetraacetic acid (EDTA) vial after an overnight fast (8 – 12 hours).

Serum 25(OH) vitamin D levels were estimated in Abbott ARCHITECT ci4100 Integrated System Abbott IL 60064 USA. The levels of 25(OH)D below 20 ng/ml was considered as deficient.

Genomic DNA was extracted from peripheral blood leukocytes using the commercial kit according to the manufacturer's protocol (GeneDirex, Inc). For genotyping VDR rs2238136 G/A SNP, the primers used were: 5'- CAGCATGCCTGTCCTCAGC-3' and 5'-CCAGTACTGCCAGCTCCC-3',[4] yielding 135 bp products, with initial denaturation at 94°C for 10 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 66°C for 60 s, and extension at 72°C for 45 s. A final extension step was carried out at 72°C for 10 min. Then, 5 µl of the amplified products were digested, at 37 °C overnight, in a 20 µl reaction containing *Bpu*10I restriction enzyme (Thermo Fisher Scientific Baltics UAB) using manufacturer's protocol. With A allele, 135 bp product was left intact, while G allele produced two fragments of 72 and 63 bp (Table 1 and Figure 1).

For genotyping *Taq*I rs731236 (VDR) SNP, primers used were; 5'-GGG ACG CTG AGG GAT GGC AGA GC-3' and 5'-GGA AAG GGG TTA GGT TGGACAGGA-3' with initial denaturation at 94°C for 10 min, followed by 30 cycles of denaturation at 95°C for 45 s, annealing at 62°C for 60 s, and extension at 72°C for 60 s. A final extension step was carried out at 72°C for 7 min. Then, 5 µl of the amplified products were digested at 65 °C overnight, in a 20 µl reaction containing 1 µl TaqI restriction enzyme (Thermo Fisher Scientific Baltics UAB) (Table 1 and Figure 2). The wild type genotype TT produced 512 and 204 bp fragment, while mutant CC yielded 311, 201, and 204 bp fragments.[5]

Finally, the products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and viewed under ultraviolet illumination. To confirm the accuracy of genotyping, a randomly selected sub-group (15%) of the cohort was re-genotyped in duplicate and all of them were concordant. A negative control sample was prepared in each PCR run to ensure that the samples, materials used and working environment were free of any DNA contamination.

Table 1. Gene (SNP ID), Base change, PCR products, Restriction sites and RFLP fragment.

Gene (SNP ID)	Base change	PCR product (bp)	Restriction enzyme (Temp.)	Allele: RFLP fragment (bp)
VDR (rs2238136)	G/A	135	*Bpu*10I, 37ºC	A: 135 G: 72 + 63
VDR (rs731236)	T/C	716	*Taq*I, 65 ºC	T: 512 + 204 C: 311 + 201 + 204

Figure 1. rs2238136 (Bpu10I) SNP: Lane A, C, F & G (GG), Lane B & D (AG), Lane E (AA), Lane H (Ladder).

Figure 2. rs731236 (TaqI) SNP: Lane A & E (TC), Lane B & D (TT), Lane C & F (CC), Lane G (Ladder).

After entering data in Microsoft Excel 2015, statistical analysis was done using SPSS version 21.0. Kolmogorov-Smirnov test was used to test the normality of data. Descriptive statistics was used to represent the data in mean and standard deviation or median and interquartile range according to the nature of data. Hardy-Weinberg equilibrium test was employed to test the concordance between observed and expected frequency. The group-wise comparison was done using Kruskal-Wallis test. The p-value ≤ 0.05 was considered statistically significant.

RESULTS

Among 400 study participants, 78.8% (n=315) were female while 21.2% (n=85) were males. The median age group of the study population was 34 years. Young adults between the ages of 25 and 44 made up the majority of population.

rs2238136 SNP

Twenty-four people in our study were found to be homozygous wild type variant (AA genotype), 238 to be homozygous mutant (GG genotype), and 138 were heterozygous (AG genotype). This distribution was in concord with the Hardy-Weinberg equilibrium (Table 2).

Table 2. Hardy-Weinberg equilibrium test for VDR (rs2238136) polymorphism.

*Bpu*10I (rs2238136)	Observed frequency	Expected frequency	Chi-square (x^2)	p-value
AA (135)	24	22		
GG (72 + 63)	238	236	0.311	0.856
AG (135 + 72 + 63)	138	142		

There was a notable difference in the vitamin D levels found across various genotypes with AA genotype having a marginally greater vitamin D level (Table 3).

Table 3. Allelic frequency and vitamin D levels in rs2238136 polymorphism.

*Bpu*10I (rs2238136)A>G	Genotype	Frequency N(%)	Vitamin D (ng/ml)	p-value
AA (135)	Wild	24 (6.0)	14.56 ± 3.75	0.013
GG (72 + 63)	Variant	238 (59.5)	13.13 ± 3.03	
AG (135 + 72 + 63)	Variant	138 (34.5)	12.52 ± 3.37	
Total		400		
Allele		**Frequency (%)**		
A		23.25		
G		76.75		

rs731236 SNP

In this study, 226 individuals were identified as homozygous wild type variant (TT genotype), 34 as homozygous mutant (CC), while 140 as heterozygous variant (TC) (Table 4). The levels of vitamin D did not significantly differ across the genotypes (Table 5).

Table 4: Hardy-Weinberg equilibrium test for VDR (rs731236) polymorphism

*Taq*I (rs731236)	Observed frequency	Expected frequency	Chi-square (x^2)	p-value
TT (512 + 204)	226	219	3.311	0.191
TC (512 + 311 + 201 + 204)	140	154		
CC (311 + 201 + 204)	34	27		

Table 5: Allelic frequency and vitamin D levels in rs731236 polymorphism

*Taq*I (rs731236)	Genotype	Frequency N(%)	Vitamin D (ng/ml)	p-value
TT (512 + 204)	Wild	226 (56.5)	12.84 ± 3.27	0.537
TC (512 + 311 + 201 + 204)	Variant	140 (35.0)	13.16 ± 3.09	
CC (311 + 201 + 204)	Variant	34 (8.5)	13.49 ± 3.28	
Total		400		
Allele		**Frequency (%)**		
T		74.0		
C		26.0		

DISCUSSION

Nepal lies in the geographical location with adequate sunlight available throughout the year. Nonetheless, a large quotient of population has vitamin D deficiency (i.e. below 20 ng/ml) ranging from 30 - 91.2 %.[6-9] This alarming status in metabolism may be attributed to the disparities in ethnicities, socio-economic background, environmental factors and genetic composition of different study populations. This study is the first kind reporting VDR polymorphism (SNP rs2238136 and rs731236), in relation to the serum levels of 25(OH)D levels in healthy individuals in Nepal.

The -4817 G>A variant (rs2238136) is intronic and located at 5'-untranslated region of VDR gene (transcription factor binding region-GATA1).[4,10] The *Taq*I (rs731236) is located in exon 9 of VDR (base change of C>T or T>t) which causes altered mRNA stability without any apparent changes in the encoded protein with synonymously modified isoleucine in the coding sequence.[11]

In our study, individuals with AA genotypes had the least prevalence (6.0%) but their serum vitamin D levels were higher than those with other genotypes. Since this is the first study in Nepal, we could not compare our findings with the previously published results.

However, the frequency of AA genotype (rs2238136) in our study was concordant with the findings of Mahmoudi et al.[4] in Iranian population in both controls and cases of colorectal cancer. Likewise, these findings correlate well with another study in Czech population.[12] The minor allele frequency for rs2238136 in our study was similar to the findings of Kosiniak-Kamysz et al.[13]

Contrary to our findings, a Spanish study showed that among the individuals with rs2238136 TT genotype, most individuals had vitamin D above 30 ng/ml compared to other genotypes in the same SNP; however when looking at intra-genotypic variation, vitamin D levels were lower in TT genotype compared to CT or CC genotype.[2]

The frequency of rs731236 TT genotype was higher in control population in a study by Salimi et al.[5] which is consistent with our findings. On the other hand, a study done in a similar setting in Pakistan[14] showed a similar frequency of minor genotype (CC-12.4%) but a higher prevalence of rs731236 TC genotype in the control population. In addition to that, a similar genotype frequency of rs731236 was observed in another study by Akhlagi et al.[15] in Iran.

In contrast to our study, a study by Kurian et al.[16] in South India demonstrated that the heterozygous rs731236 variant was more common in healthy population compared to diseased individuals. However, the minor genotype frequency of rs731236 in their population (GG-7.1%) and ours were alike. On the other hand, our findings are consistent with the findings of Mao et al.[17] showing no statistically significant variation in serum 25(OH)D concentration among various genotypes of rs731236 in Chinese population.

Vitamin D also exerts its anti-tumor effects through the VDR, so gene variants associated VDR has expanded its quest in research.[18] Other than rs2238136 and rs731236 SNPs, a study in Nepal reports variations in VDR polymorphism of *Fok*I (rs2228570) and *Bsm*I (rs1544410) in diabetic Nepalese population.[19]

The high prevalence of vitamin D deficiency in our region may be explained by the genotypes that are most prevalent in our population having relatively lower vitamin D levels. On the other hand, the vitamin D supplementation is not sufficient to address the shortfall in a sustainable manner when the genetic buildup has its effect on deficiency. Our study could not address the impact of genetic polymorphisms on achieved vitamin D levels significantly and its association with vitamin D supplementation. It offers opportunities for further investigation into how genetic diversity affects vitamin D supplementation.

CONCLUSIONS

The genotype distribution of *VDR* gene in our population is comparable to that of other Asian countries, with a higher prevalence of GG genotype (rs2238136) and TT genotype (rs731236). The vitamin D levels were slightly higher in those with AA genotype (rs2238136) than in those with other genotypes, while there was no such distribution seen in rs731236 SNP. This genetic variation might be the reason of vitamin D deficiency seen in a large fraction of population in our region.

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CONFLICT OF INTEREST

None

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