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Correlation of *CYP2R1* gene promoter methylation with circulating vitamin D levels among healthy adults

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***Background & objectives*: Despite being a tropical country, vitamin D deficiency is highly prevalent in India with studies indicating 40-99 per cent prevalence. Apart from calcium and phosphate metabolism, vitamin D is involved in cell cycle regulation, cardiovascular, hepatoprotection. The metabolism of vitamin D is regulated by vitamin D tool genes (*CYP2R1/CYP27B1/CYP24A1/VDR*). The promoter regions of some of these genes have CpG islands, making them prone to methylation induced gene silencing, which may cause a reduction in circulating vitamin D levels. Epigenetic basis of vitamin D deficiency is yet to be studied in India, and hence, this pilot study was aimed to analyze whether methylation levels of *CYP2R1* gene were correlated with the levels of 25(OH)D in healthy, adult individuals in Indian population.**

***Methods*: In this cross-sectional study, healthy adults of 18-45 yr of age with no history of malabsorption, thyroidectomy, chronic illness or therapeutic vitamin D supplementation were recruited. DNA methylation analysis was carried out by methylation specific quantitative PCR. Serum calcium, phosphate and vitamin D levels were also quantified. Statistical analysis was done by R 4.0.5 software.**

***Results*: A total of 61 apparently healthy adults were analyzed. The serum vitamin D levels did not correlate with *CYP2R1* methylation levels in our study population. Significant positive correlation was observed between age and serum vitamin D levels. Significant association of gender was found with *CYP2R1* methylation levels.**

***Interpretation & conclusions*: This study found no significant correlation between levels of *CYP2R1* methylation and circulating 25(OH)D deficiency. Further studies on the Indian population having a larger sample size including entire vitamin D tool genes, among different ethnic groups may be conducted to elucidate molecular etiology of circulating 25(OH)D deficiency. The high prevalence of normal serum calcium and phosphate levels among vitamin D deficient subjects in this study coupled with the strikingly high prevalence of the deficiency at the national level, may suggest the need to revise the cut-off criteria for vitamin D deficiency in the Indian population.**

Key words Circulating 25(OH)D - *CYP2R1* - *CYP27B1* - *CYP24A1* - epigenetics - vitamin D deficiency - *VDR* - vitamin D tool genes

Vitamin D deficiency is highly prevalent in India, with studies reporting a prevalence of 40-99 per cent, across the Indian population[1,2]. The 'sunshine vitamin' has cell cycle regulatory, immunomodulatory and anti-cancer properties[3-5]. The metabolism of vitamin D involves enzymes for 25-hydroxylation, 1α-hydroxylation and 24-hydroxylation. The 'vitamin D tool' genes (*CYP2R1*, *CYP27B1*, *CYP24A1* and *VDR)*[6,7] which regulate these enzymes, are being identified as possible targets of mutations or epigenetic modifications leading to deficiency in circulating levels of active vitamin D.

The *CYP2R1,* located on chromosome 11p15.2, is one of the key genes in vitamin D metabolism through 25-hydroxylation of vitamin D[8-12]. Previous studies have established the role of genetic polymorphisms and epigenetic modifications in *CYP2R1* causing deficiency in Nigerian, Lebanese, Caucasian and African-American cohorts[9-13]. The promoter region of the *CYP2R1* gene has CpG islands predisposing it to epigenetic modification, *i.e.* methylation induced gene silencing[6]. At present, the molecular basis of vitamin D deficiency is yet to be established in the Indian population.

Due to the non-availability of previous DNA methylation studies of *CYP2R1* on vitamin D deficiency in the Indian subcontinent, the present study was conducted as a pilot study with an aim to analyze methylation levels of the *CYP2R1* gene and its correlation with levels of total 25-hydroxy vitamin D [25(OH)D] in healthy adults. Total 25(OH)D was measured since it includes both 25-OH and 1, 25-OH_2 vitamin D. As the role of vitamin D in calcium and phosphate metabolism is well established, both these markers were also analyzed.

Material & Methods

This cross-sectional study was undertaken in the department of Biochemistry, Armed Forces Medical College (AFMC), Pune, a tertiary care centre in western Maharashtra, between September 2020 and January 2021. The study was approved by the Institutional Ethics Committee and a written informed consent from all the participants.

A total of 94 consecutive adults in the age group of 19-41 yr were screened. Participants were students and staff at AFMC, Pune. All participants had recently undergone an annual medical examination and were found healthy based on physical, biochemical and radiological parameters. Sixty one participants fulfilling the above mentioned inclusion criteria and the following exclusion criteria: (*i*) history of consuming prescribed vitamin D supplements within the past six months, (*ii*) history of thyroidectomy, malabsorption syndromes, inflammatory bowel disease or any other chronic illness were selected for the study.

Blood samples (5 ml each) were collected from all participants in vacutainer tubes with K_2-EDTA (Potassium-ethylenediaminetetraacetic acid) or without additives, respectively, for molecular and biochemical studies.

DNA isolation & methylation specific PCR: DNA extraction was performed using the silica column-based Qiagen kit (Qiagen, Valencia, CA, USA). Isolated DNA was checked for purity (1.7-1.9) and quantity by Nanodrop ND2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA (500 ng) was modified using Qiagen EpiTect© Bisulfite Kit (Qiagen) followed by quantitative methylation-specific PCR using SYBR Green (Promega Corp., Madison, WI, USA) using specific primers designed for *CYP2R1*, using an Applied Biosystem QuantStudio 5 real-time PCR System (Applied Biosystems, Foster City, CA, USA). Universal methylated DNA (Qiagen) was included as a positive control in addition to no template control. The primers were designed specifically for *CYP2R1* promoter methylation using MethPrimer software[14]: forward 5'-TTTAGGGTTGTTGTGGAGTTC-3' and reverse 5'-AACTAACGAACCCCTAACGC-3'; and for *beta-actin* (housekeeping/reference gene) forward 5'-TGGAGGTTGTAGTTAGTTGAGATT-3'and reverse 5'-AACTAAATAAAACTAAACTCATCCAA-3'. Cycle threshold (Ct) values were collected for *CYP2R1* and *beta-actin*. The level of *CYP2R1* promoter methylation was determined by 2^(−ΔCt) method[15].

Biochemical analysis: Briefly, blood samples (5 ml each) were incubated for 30 min at room temperature followed by serum isolation by centrifugation at 1000 g for 10 min. The levels of circulating 25(OH)D, calcium and phosphate were detected in the serum sample using the Siemens automated biochemistry analyser Dimension EXL200 (Siemens LOCI®, Munich, Germany). Total vitamin D assay was employed and ranges for vitamin deficiency, insufficiency and sufficiency were taken as <20, 20-30 and >30 ng/dl, respectively.

Statistical analysis: Statistical software package R.4.0.5 (R development Core Team, Vienna, Austria) was

used for the statistical analysis. Categorical data were described using percentages and numbers; numerical variables were described using median. The normality of data was checked using the Shapiro-Wilk test. Correlation was analyzed using Pearson's correlation test and the association was analyzed using Kruskal-Wallis test. $P< 0.05$ were considered significant.

Results

Out of 61 participants, 37 (60.7%) were male and 24 (39.3%) were female. The age range of participants was 19-41 yr (mean 24.4±5.0 yr).

The serum vitamin D levels did not correlate with *CYP2R1* methylation levels in our cohort (r=−0.056, P=0.670). Vitamin D or *CYP2R1* methylation levels were not significantly correlated with calcium and/or phosphates levels, based on Kruskal-Wallis test. It is worth mentioning that the calcium and phosphate levels were within the normal range in 45 (80.4%) and 49 (87.5%) of 56 (54 deficient and 2 insufficient) individuals having vitamin D deficiency. Of these, seven (12.5%) of them had elevated phosphate levels. A significant positive correlation was observed between age and serum vitamin D levels (Figure and Table). No significant effect of the categorical independent variables (deficiency status of serum vitamin D, calcium and phosphate) on the continuous dependent variables (age, levels of *CYP2R1* methylation, serum calcium, phosphate and vitamin D) was elicited. However, gender was found to have a significant association with *CYP2R1* methylation levels (median methylation level in females being 0.042, compared to 0.002 in males, P=0.022).

Discussion

The present study aimed to correlate the levels of circulating 25(OH)D with the promoter DNA methylation of the *CYP2R1* gene in healthy individuals, however, results of our study failed to establish the correlation. This is in corroboration with the previous studies that reported a weak correlation between these two variables[9,10,16]. Possibly, the methylation of the specific CpG sites chosen by us may not have contributed towards silencing of *CYP2R1* to the extent required for manifestation as vitamin D deficiency. This does not rule out the possibility of a correlation existing with respect to other CpG sites. Our study showed a positive correlation between age and serum 25(OH)D levels. However, other studies have shown a reduction in these levels with age[17,18]. This could possibly be explained by the fact that our study population comprised of adolescents and middle-aged individuals. A systematic review[17] has reported higher serum levels of vitamin D in middle-aged individuals, as seen in our study.

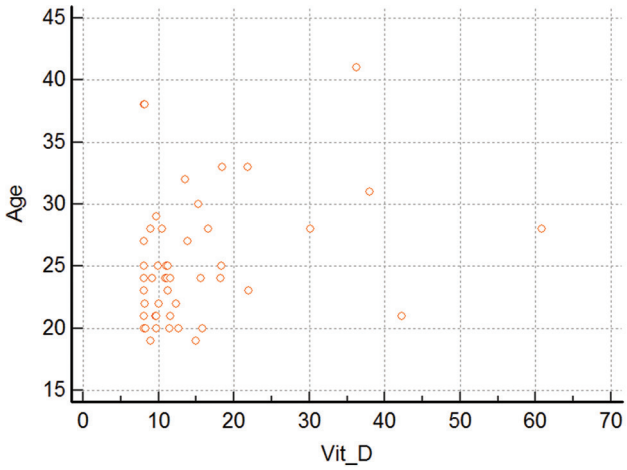

Figure. Correlation of serum vitamin D with age.

Our study also showed a positive correlation between gender and *CYP2R1* methylation levels, with the methylation being significantly more in females than males. However, the median vitamin D levels were also higher in females than males. This is contrary to the expectation that higher levels of methylation lead to a greater decrease in circulating vitamin D. Taking the lead from this, more elaborate studies involving a larger sample size can be planned to investigate this association.

Despite being a pilot study, a sample size of 61 participants could be obtained, which is higher than previous studies[9,10]. The selection of apparently healthy individuals helped to exclude a disease-induced reduction in circulating vitamin D levels, which may act as a confounder. There were some limitations in our study. The proportion of vitamin D deficient/insufficient subjects far outnumbered the vitamin D sufficient participants and the *CYP2R1* gene was the only gene analyzed from vitamin D tool genes in this study.

Further studies on the Indian population, having a larger sample size including all vitamin D tool genes, among different ethnic groups, may be conducted to elucidate the molecular aetiology of circulating 25(OH)D deficiency. A high prevalence of normal serum calcium and phosphate levels among vitamin D deficient participants was seen in

Table. Correlation of different pairs of variables: There is a significant correlation between age and vitamin D levels

Variable	Variable (n=61)	Correlation coefficient (r)	Square of correlation coefficient (r^2)	SE	P
Vitamin D	Ca2	0.155	0.024	0.129	0.234
Vitamin D	PO4	−0.089	0.008	0.130	0.495
Vitamin D	*CYP2R1*	−0.056	0.003	0.130	0.670
Ca2	PO4	0.146	0.021	0.129	0.261
Ca2	*CYP2R1*	0.222	0.049	0.127	0.085
PO4	*CYP2R1*	−0.665	0.442	0.097	0.610
Age	Vitamin D	0.349	0.122	0.122	0.006
Age	Ca2	0.193	0.037	0.128	0.136
Age	PO4	−0.144	0.021	0.129	0.268
Age	*CYP2R1*	−0.100	0.010	0.130	0.445

SE, standard error

our study. Considering this, and the strikingly high prevalence of the deficiency at the national level, a need to revise cut-off criteria for deficiency may be assessed through nationwide studies having larger sample sizes.

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***Conflicts of Interest*:** None.

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