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A genome-wide methylation study of severe vitamin D deficiency in African American adolescents

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Abstract

Objectives—To test the hypothesis that changes in DNA methylation are involved in vitamin D deficiency–related immune cell regulation using an unbiased genome-wide approach combined with a genomic and epigenomic integrative approach.

Study design—We performed a genome-wide methylation scan using the Illumina HumanMethylation 27 BeadChip on leukocytes DNAs of 11 cases of vitamin D deficiency (serum 25(OH)D≤ 25 nmol/L) and 11 age-matched controls (serum 25(OH)D>75 nmol/L); the subjects were African American normal-weight (BMI<85th percentile) males aged 14-19 years. The Limma package was used to analyze each CpG site for differential methylation between cases and controls. To correct for multiple testing, the set of raw *p* values were converted to false discovery rates (FDR). We also compared our findings with the recent data from GWAS of circulating 25(OH) D levels and then performed a permutation test to examine whether the "double hit" genes were randomly enriched.

Results—A total of 79 CpG sites achieved raw p<0.001. Of the 79 CpG sites, 2 CpG sites survived multiple testing: cg16317961 (raw p=3.5 × 10^{-6}, FDR=0.078, in *MAPRE2*) and cg04623955 (raw p=5.9 × 10^{-6}, FDR=0.078, in *DIO3*). Furthermore, 3 out of the 4 genes previously identified in the two GWAS studies were also significant at the methylation level (*DHCR7*: cg07487535, p=0.015 & cg10763288, p=0.017; *CYP2R1*: cg25454890, p=0.040; *CYP24A1*: cg18956481, p=0.022), reflecting significant enrichment (p=0.0098).

Conclusion—Severe vitamin D deficiency is associated with methylation changes in leukocyte DNA. The genomic and epigenomic approach reinforce the crucial roles played by the *DHCR7*, *CYP2R1* and *CYP24A1* genes in vitamin D metabolism.

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Keywords

Vitamin D deficiency; DNA methylation; genome-wide association study; African Americans; extreme phenotypes

Vitamin D is considered to play an important role in a broad range of bodily functions beyond bone health [1-4]. The latest assay-adjusted National Health and Nutrition Examination Survey (NHANES) 2001-2006 in 5,867 adolescents aged 12-19 years demonstrates that low 25-hydroxyvitamin D [25(OH)D] is associated with several cardiometabolic risk factors such as metabolic syndrome and insulin resistance[5].

The prevalence of 25(OH)D levels of < 25 nmol/L increased from 2% during NHANES 1988-1994 to 6% during NHANES 2001-2004. The prevalence of 25(OH)D levels of < 25 nmol/L in African Americans rose from 9% to 29%[6]. We recently reported that African American adolescents living in a year-round sunny climate area like Augusta, Georgia (∼33° North latitude), circulating 25(OH)D concentrations were less than half those of Caucasian adolescents in every season[7]. In addition, 6.2% African American males and 17.2% African America females suffered from severe vitamin D deficiency (<25 nmol/L)[7].

Vitamin D has known immunomodulatory effects on a wide range of immune cells including activated CD4 and CD8 T lymphocytes, B lymphocytes, macrophages as well as dendritic cells[1,8]. Serum 25(OH)D levels are inversely associated with levels of C-reactive protein (CRP), interleukin-6 (IL-6) and tumor necrosis factor -α (TNF-α)[9]. Vitamin D supplementation has been shown to down-regulate nuclear factor-kB activity, increase anti-inflammatory IL-10 levels and decrease the pro-inflammatory cytokines IL-6, IL-12, interferon-γ and TNF-α[10-11]. Recent hierarchical and model-based clustering analysis of 200 vitamin-related proteins revealed that vitamin D-related proteins were strongly represented among network hubs, highlighting the pervasive effects of this nutrient primarily related to immune system and cancer process[12].

Epigenetic factors are at the interface between environmental stimuli and long-lasting molecular, cellular and behavioral phenotypes[13]. Epigenetics defines all meiotically and mitotically heritable changes in chromosome function/gene expression that are not coded in the DNA sequence itself. Recently, there has been a greater appreciation of the role of histone modification in vitamin D-mediated immune-regulatory and inflammation response[14]. However, whether DNA methylation is also involved in vitamin D-mediated immune regulation remains unknown.

Genome-wide association studies (GWAS) of circulating 25 (OH)D, identified 4 genes, which are involved in cholesterol synthesis, hydroxylation, and vitamin D transport[15-16]. Genetic variation at these loci identifies individuals who have substantially elevated risk of vitamin D insufficiency. Therefore, the goal of this study was to characterize DNA methylation profile in peripheral blood leukocytes in African American adolescents with and without severe vitamin D deficiency using an unbiased genome-wide approach combined with a genomic and epigenomic integrative approach comparing our methylation profiling data with the candidate genes identified from GWAS on circulating vitamin D and vitamin D insufficiency

Methods

The genome-wide methylation analysis was conducted in 11 children with vitamin D deficiency (plasma 25(OH)D≤ 25 nmol/L) and 11 age-matched controls (plasma 25(OH)D> 75 nmol/L) selected at the two extreme ends of the 25(OH)D distribution (extreme

phenotypes) from previous studies[7,17]. All the subjects from the two studies were apparently healthy and were not taking any medications or vitamin supplements. The 22 subjects aged 14-19 were selected based on the following criteria: (1) African American ancestry by self-report; (2) male; (3) apparently healthy (normotensive, non-diabetic) (4) body mass index (BMI) < 85th percentile for age and sex; (5) having leukocyte DNA available; (6) at the lowest and the highest ends of the 25(OH)D distribution. The institutional Review Board at the Georgia Health Sciences University approved the studies. Informed consent was obtained from all subjects and by parents if subjects were less than 18 years of age.

Height and weight were measured by standard methods using a wall-mounted stadiometer and a scale, respectively. BMI was calculated as weight/height^2. After participants quietly rested for 10 minutes, systolic blood pressure and diastolic blood pressure were measured in a supine position with a Dinamap monitor (Critikon, Tampa, FL). Blood samples were collected. The plasma and buffy coat were separated and stored at -80°C.

Plasma 25(OH)D measurement

Liquid chromatography tandem mass spectroscopy (LC-MS/MS) was used to measure circulating plasma levels of 25(OH)D_2 and 25(OH)D_3 as described previously[18]. LC-MS/MS procedures is one of the most sensitive methods of measuring 25 (OH)D[19]. The detection limits for 25(OH)D_2 and 25(OH)D_3 were 4.6 and 1.2 nmol/L. The detection range is 1-250 nmol/L[18]. The intra-assay (inter-assay) coefficients of variation for 25(OH)D_2 and 25(OH)D_3 were 6-9% (9-12%) and 7-11% (8-13%) respectively. Regarding the assay specificity, LC–MS/MS has the advantage of being able to measure both 25(OH)D_2 and 25(OH)D_3 independently[19]. The levels of total 25(OH)D including 25(OH)D_2 and 25(OH)D_3 were used in the selection process.

Genome-wide methylation chip

The HumanMethylation27 BeadChip from Illumina (Illumina, San Diego, CA, USA) was used. This chip can quantitatively measure 27, 000 CpG sites, covering more than 14,000 well-annotated genes at single-CpG resolution. After bisulfite treatment, 200ng of the converted DNA was whole genome amplified (WGA) and enzymatically fragmented. The bisulfite-converted WGA-DNA samples were purified and applied to the BeadChips. Image processing and intensity data extraction were performed according to Illumina's instructions (http://www.illumina.com/products/infinium_humanmethylation27_beadchip_kits.ilmn). Each methylation data point is represented by fluorescent signals from the methylated and unmethylated alleles. DNA methylation beta values are continuous variables between 0 (completely unmethylated) and 1 (completely methylated), representing the ratio of the intensity of the methylated bead type to the combined locus intensity. Initial array processing and quality control were performed with BeadStudio software.

Statistical Analyses

The general characteristics of the subjects are presented as mean ± SD. Independent *t*-tests were conducted to examine the differences of covariates between cases and controls using SPSS17.0 (SPSS Inc., Chicago, IL). For the genome-wide methylation analysis, the Limma package[20] was used to analyze each CpG site for differential methylation between cases and controls under the design matrix of a paired test. CpG sites on the X and Y chromosomes were excluded from the analysis. Each CpG site was assigned a raw p value based on a moderated t statistic. To correct for multiple testing, the set of raw p values were converted to false discovery rates (FDR) according to Benjamini and Hochberg[21]. Rather than expressing the probability of a single false-positive result among all tests, the FDR value estimates the proportion of results declared interesting that are actually false. We used a

FDR value threshold of 0.20[22], meaning that one should expect at most 20% of declared discovery to be false.

Gene Ontology (GO) analysis was conducted with the FatiGO tool [23]. FatiGO takes two lists of genes and converts them into two lists of GO terms. Then a Fisher's exact test for 2 × 2 contingency tables was used to check for significant over-presentation of GO terms in one of the sets with respect to the other one. Multiple testing correction (indexed by adjusted p-values) to account for the multiple hypotheses tested (one for each GO term) was applied to reduce false positives. Because at least two CpG sites were included for the majority of genes in this genome wide chip, for each gene we only selected the CpG sites with the lowest p value.

Additionally, we applied an integrative approach compared our methylation profiling data with the candidate SNPs identified from GWAS on circulating vitamin D and vitamin D insufficiency[15-16] to identiy the “double hit” genes. We then performed a permutation test to examine whether the “double hit” genes were randomly enriched (STATA 8, StataCorp, College station, TX).

Results

Table I displays the general characteristics of the study sample. Except for the plasma 25(OH)D concentrations, all other variables were similar for the case and control groups. The Figure is a volcano plot showing the raw p-values for all CpG sites versus mean methylation difference between the severe vitamin D deficiency group and the control group. In general, participants with severe vitamin D deficiency showed a pattern of reduced methylation compared with controls. Table II lists the top 10 most differently methylated CpG sites. Again, 9 out of the top 10 CpG sites showed reduced methylation levels compared with controls. The top two significan CpG sites survived multiple testing using an FDR of 10% (cg16317961, raw $p = 3.5 \times 10^{-6}$, FDR=0.078, in *MAPRE2*; cg04623955, raw $p= 5.92 \times 10^{-6}$, FDR=0.07, in *DIO3*).

Gene Ontology analysis was performed to test whether some common functional trends in molecular functions and biological processes were associated with the genes exhibiting differences between cases of vitamin D deficiency and controls in the genome-wide chip. We assigned those genes with a raw p <0.001 to the first list (n=79) and included all other genes in the second list (n=13,811). As expected from a study in only 22 subjects, we did not observe any GO categories that survived multiple testing. Overall, we observed enriched functional processes that are potentially relevant for regulation of: metabolic processes; cellular development; cell adhesion, motility and differentiation; cellular defense; transcriptional factor binding; sterol binding; and Wnt-protein binding. Table III (available at www.jpeds.com) lists the GO categories with raw p values < 0.05.

Furthermore, our genomic and epigenomic integration approach showed that of the 3 loci (within or near *GC*, *DHCR7/NADSYN1* and *CYP2R1* genes) independently discovered in these two recent GWAS studies[15-16], the methylation differences in the promoter CpG sites of the two genes (*DHCR7* and *CYP2R1*) were statistically significant between the severe vitamin D deficiency group and the control group (*DHCR7*: cg07487535, p=0.015 & cg10763288, p=0.017; *CYP2R1*: cg25454890, p=0.040). Moreover, a SNP near *CYP24A1* showed an overall p value of 6.0×10^{-10} in one of the GWAS studies[16]. The methylation differences in the CpG site of the *CYP24A1* gene were also statistically significant between the severe vitamin D deficiency group and the control group (cg18956481, p=0.022, Table IV).

To test whether the above finding (ie, 3 of the 4 candidates with GWA genes were significantly associated with severe vitamin D deficiency) was randomly enriched, we performed the permutation test. Of total 26,486 CpG sites, 26,460 were mapped on 13,872 genes. For each gene, we assigned the highest statistic value (i.e. smallest p value) among the multiple CpG sites mapped to the gene as the statistic value of the gene. Four genes were randomly selected from these 13,872 genes. The number of genes with p value ≤0.05 was counted. This procedure was repeated 10000 times, and a distribution of count number of significant genes (p≤0.05) out of 4 genes was obtained. In these 10000 repetitions, only 98 had a count number equal or larger than 3. In other words, these 3 genes (*DHCR7*, *CYP2R1* and *CYP24A1*) were significantly enriched with respect to severe vitamin D deficiency (p=0.0098), indicative of not a chance finding.

Discussion

In this study, we identified methylation differences in leukocyte DNA in relation to vitamin D deficiency. Moreover, the genomic and epigenomic integrative approach confirmed the *DHCR7, CYP2R1* and *CYP24A1* genes, which may play crucial roles in vitamin D metabolism.

MAPRE2 (also known as *EB2*, *RP1*) has the most significant differently methylated promoter CpG site. The methylation level was 37% lower (3.7%/10%) in the group with vitamin D deficiency compared with the controls. Moreover, the other CpG site of the *MAPRE2* gene was also significantly differentially methylated (p=0.016). The *MAPRE* gene family consists of three members (*MAPRE1, 2 and 3*) that code for three microtubule associated proteins. They are important for many cellular processes i.e. cell division, cytoplasmatic organization, maintenance of cell polarity, chromosome segregation and cell migration[24-26]. These microtubule associated proteins regulate microtubule functions and dynamics, which is fundamentally important to the way cells response to their environment[24, 27]. *MAPRE2* is expressed in T cells and lymphocyte tumor cell lines and activated by cell surface antigens and/or cytokines[28]. *MAPRE2* over expression induced nuclear accumulation of β-catenin and promoted the transcriptional activity of β-catenin/T-cell factor[29]. Because the expression is rapidly up-regulated in activated T cells, *MAPRE2* is considered a member of the immediate-early T-cell regulatory gene family[28]. The proteins encoded by the immediate-early gene can act as pleiotropic regulators of cellular activation, such as transcription factors and proteins involved in signal transduction cascades[30]. *MAPRE2* can also be found in rapidly proliferating tumor cells and its expression correlates with the cell cycle activity indicates that its protein (RP1) might be critically involved in processes controlling cell proliferation[28, 31].

DIO3, the second most significant site identified, is located in the imprinted region in chromosome 14q32 and plays a critical role in maturation and function of the thyroid axis which is also critical to the development of the human central nervous system[32]. Meta-analysis of genes modulating intracellular T3 bio-availability has revealed a possible role for *DIO3* gene in osteoarthritis[33]. Recent data have shown that this gene is hypermethylated in hematological tumors[34-35]. In the present study, the promoter CpG site methylation was 24.2% (4.6%/19%) higher in the group with vitamin D deficiency as compared with the controls.

As demonstrated by previous studies, 3-10% of the human genome is regulated directly and indirectly by the vitamin D endocrine system[36-37]. Genome-wide VDR has a few thousand chromosomal target sites that regulate several hundred 1, 25$(OH)_2$D3 target genes in a cell-specific fashion[38-39]. Methylation/demethylation reflects vitamin D-mediated transcriptional control via vitamin D response element (VDRE)[40]. VDRE can directly bind to DNA

methyltransferases 1 and 3B (DNMT1 and DNMT3B)[41]. A global transcriptomic study showed that 1, 25 (OH)2D3 significantly changed the RNA expression profile in human colon cancer cells involving many different cell functions, including transcription, cell adhesion, DNA synthesis, apoptosis and intracellular signaling[42]. Recent hierarchical and model-based clustering analysis of the protein-protein interaction network of 200 vitamin-related proteins revealed that a subgroup of 22 proteins that are highly central, most of these proteins are key immune regulators such as cytokines and transcription factors such as SMAD3 and VDR. Moreover, 17 out of the 22 central proteins in the protein-protein interaction network are vitamin D-related proteins, highlighting the pervasive effects of this nutrient primarily related to immune system and cancer process[12]. We also showed that many functional pathways are involved in severe vitamin D deficiency-related methylation changes in leukocytes including regulation of metabolic processes, cellular development, cell adhesion, motility and differentiation, cellular defense response as well as transcriptional factor binding, sterol binding, and Wnt-protein binding (Table III). The finding that vitamin D receptor deficiency enhances Wnt/β-Catenin signaling in human color cancer cells[43] parallels our finding that Wnt-protein binding was an enriched molecular function for differentially methylated genes in leukocytes of individuals with vitamin D deficiency.

Our epigenomic findings confirmed 3 genes out of the 4 genes in GWAS findings, which reinforce the crucial roles played by those 3 genes in vitamin D metabolism. *DHCR7* encodes the enzyme 7-dehydrocholesterol (7-DHC) reductase, which converts 7-DHC to cholesterol, thereby removing the substrate from the synthetic pathway of vitamin D_3, a precursor of $25OHD_3$. *DHCR7* is a novel gene for association with vitamin D levels identified in the two recent GWAS studies[15-16]. *CYP2R1* (cytochrome P450, family 2, subfamily R, polypeptide 1) encodes a hepatic microsomal enzyme 25-hydroxylase that converts vitamin D3 to an active vitamin D receptor ligand. Previous candidate[44-45] and GWAS[15-16] studies suggested that this gene is associated with vitamin D metabolism. *CYP24A1,* which encodes 24-hydroxylase, has been identified as a candidate gene for vitamin D insufficiency in one GWAS[16], not the other[15]. This mitochondrial protein initiates the degradation of 1,25-dihydroxyvitamin D3 and plays a role in calcium homeostasis and vitamin D metabolism. Our findings suggested that subjects with vitamin D deficiency are more likely to have reduced synthesis and increased metabolism of active vitamin D. Our epigenetic data provide the additional body of evidence that those 3 enzymes are the key enzymes underlying the crucial steps in vitamin D metabolism.

The observed DNA methylation differences between cases with vitamin D deficiency and controls were relatively small (<5%), which is in agreement with the two recent genome-wide methylation studies in leukocyte DNA on obesity and type 2 diabetes[46,47]. Toperoff et al demonstrated that the odd of belonging to the type 2 diabetes group increased by 6% for every 1% decrease in methylation in whole blood DNA[47]. It is possible that the methylation differences identified in leukocyte DNA could be attributed to differences in the leukocyte profile, rather than epigenetic changes induced within specific subtypes of leukocytes. However, Toperoff et al further analyzed the potential contribution of differential case-control composition of blood cells to the observed methylation differences. No significant differences in blood counts appeared between cases and controls, and adjustment for blood lineage count did not affect the association of methylation with the incidence of type 2 diabetes[47].

Our use of a hypothesis-free approach avoided the limitations imposed by candidate gene studies and allowed us to search the whole genome in an unbiased manner. Genomic and epigenomic data integration provides greater understanding of physiology and etiology of the complex traits. The use of extreme phenotypes based on plasma vitamin D values

maximized the power to make discoveries. The selection of normal weight cases and controls eliminated the confounding effect of obesity on methylation differences. The focus on adolescents prior to the development of target organ damages optimized the chances to unmask important etiologic relationships.

Several limitations are noteworthy. First, we were unable to determine whether the between group methylation differences we observed were associated with differences in transcription level between the two groups because cellular RNA samples were unavailable. Second, although we hypothesize that severe vitamin D deficiency will lead to methylation changes in leukocyte DNA, our case-control study design cannot determine whether the identified methylation differences are the cause or the consequence of severe vitamin D deficiency. Third, the methylation profiling was done in whole blood leukocytes. However, the blood lineage data was not collected at the time of sampling. Forth, the Infinium HumanMethylation27 Beadchip was used because of the availability at that time. The limited coverage of this genome-wide chip will restrict the findings to certain CpG sites within certain genes. Genome chips with more complete coverage such as the recently released 450K Infinium Methylation BeadChip from Illumina should be used in future studies. Last, the study is limited by lack of cases with severe vitamin D deficiency and matched controls for further validation; future studies are needed to replicate and validate the findings by independent methods in independent cohorts including female populations.

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Abbreviations

25(OH)D	25-hydroxyvitamin D
BMI	Body mass index
DBP	Diastolic blood pressure
FDR	False discovery rate
GO	Gene Ontology
GWAS	Genome-wide association study

IL	Interleukin
SBP	Systolic blood pressure
WGA	whole genome amplification

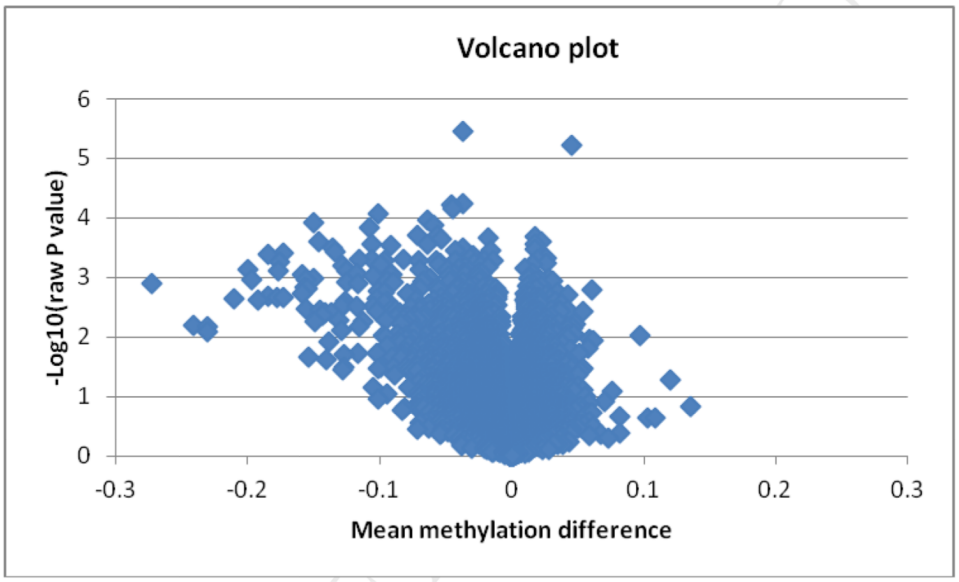

Figure 1.
Legend: Volcano plot showing raw p-values versus mean methylation differences between severe vitamin D deficient cases and matched controls.

Table 1
General characteristics of the African American male participants

	Cases 25(OH)D ≤ 25 nmol/L & BMI < 85th percentile	Controls 25(OH)D > 75 nmol/L & BMI < 85th percentile	p
N	11	11	-
Age, years	16.2 ± 1.2	16.1 ± 1.3	0.791
Age range, years	14.8-18	14.2-18.6	-
Height, cm	174.6 ± 6.1	174.3 ± 8.8	0.930
Weight, kg	63.2 ± 7.4	62.5 ± 8.5	0.841
BMI, kg/m^2	20.7 ± 2.5	20.6 ± 2.4	0.897
BMI percentile (%)*	43.6 ± 24.5	46.2 ± 25.8	0.739
Waist circumference, c	m 71.2 ± 4.7	70.9 ± 4.3	0.878
SBP, mmHg	113.0 ± 5.7	112.6 ± 4.7	0.855
DBP, mmHg*	58.5 ± 6.1	58.2 ± 4.5	0.940
25(OH)D, nmol/L	19.8 ± 3.8	92.0 ± 12.4	<0.0001

Values are mean ± SD. Tests of significance between groups were based on independent *t* test. SBP: systolic blood pressure. DBP: diastolic blood pressure.

*BMI% and DBP were log-transformed prior to the analysis.

Table 2

Top 10 differentially methylated CpG sites

Gene	Illumina ID	Distance to TSS	Methylation		P	FDR
			Case/Control (%)	Difference (%)		
MAPRE2	cg16317961	357	8.0/11.7	-3.7	0.0000035	0.078
DIO3	cg04623955	1312	21.3/16.7	4.6	0.0000059	0.078
ASTN	cg23492043	242	14.1/17.8	-3.7	0.0000588	0.317
PAWR	cg00864867	478	6.5/11.1	-4.6	0.0000592	0.317
MEPCE	cg11979382	510	9.5/14.1	-4.6	0.0000693	0.317
FLI23191	cg24646710	402	15.9/26.0	-10.1	0.0000866	0.317
ZBTB38	cg17718515	479	6.6/13.0	-6.4	0.000107	0.317
MLLT6	cg04133652	904	20.1/35.1	-15.0	0.000118	0.317
PARL	cg24355091	482	28.7/34.6	-5.9	0.000132	0.317
HRK	cg16826718	573	32.4/43.2	-10.8	0.000148	0.317

TSS, transcription starting site.

Table III

Gene-Ontology analysis

	GO. ID	Term	P value	FDR
Biological process	GO:0006139	Nucleotide and nucleic acid metabolic process	0.0081	0.936
	GO:0019222	Regulation of metabolic process	0.014	0.936
	GO:0050794	Regulation of cellular process	0.0141	0.936
	GO:0048518	Positive regulation of biological process	0.0182	0.936
	GO:0040029	Regulation of gene expression, epigenetic	0.0221	0.936
	GO:0040012	Regulation of locomotion	0.0328	0.936
	GO:0006928	Cell motility	0.036	0.936
	GO:0030154	Cell differentiation	0.036	0.936
	GO:0006968	Cellular defense response	0.0409	0.936
	GO:0050790	Regulation of catalytic activity	0.0469	0.936
	GO:0043283	Biopolymer metabolic process	0.0481	0.936
Molecular function	GO:0008134	Transcriptional factor binding	0.00058	0.11
	GO:0032934	Sterol binding	0.00079	0.11
	GO:0030020	Extracellular matrix structural constituent conferring tensile strength	0.0063	0.58
	GO:0030023	Extracellular matrix constituent conferring elasticity	0.0125	0.78
	GO:0003704	Specific RNA polymerase II transcription factor activity	0.0141	0.78
	GO:0017147	Wnt-protein binding	0.0187	0.87
	GO:0015248	Sterol transporter activity	0.0431	1

Table 4

Differentially methylated CpG sites in 3 genes identified in GWAS of vitamin D level and insufficency

Gene	Illumina ID	Distance to TSS	Methylation		P	FDR
			Case/Contrl (%)	Difference (%)		
DHCR7	cg07487535	506	11.5/10.1	1.4	0.015	0.543
	cg10763288	474	39.3/42.6	-3.3	0.017	0.554
CYP2R1	cg25454890	131	16.7/14.8	1.9	0.040	0.635
CYP24A1	cg18956481	385	8.3/9.8	-1.5	0.022	0.586

TSS, transcription starting site.