



VDR downregulation and promoter hypermethylation as one of the causes for triggering type 2 diabetes mellitus: Clinical and molecular studies

Najeebul Tarfeen¹ · Shariq Rashid Masoodi² · Khair Ul Nisa³ · Shafat Ali¹ · Mir Bilal Ahmad⁴ · Bashir Ahmad Ganai¹

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Abstract

Background Our present study was to investigate the methylation and Gene expression of the vitamin D receptor (VDR) gene in the causing T2DM and to determine the inflammatory biomarkers in exaggerating T2DM in Kashmiri population.

Methods In this study, T2DM cases ($n = 100$) and controls ($n = 100$) of Kashmiri population were designed. Blood samples were taken from both groups, and serum vitamin D levels, inflammatory biomarkers (TNF- α , IL-6, IL-10, CRP, Leptin and adiponectin) were estimated by ELISA. By using methylation-specific PCR (MS-PCR) and RT-PCR, respectively, the levels of methylation and expression were measured after the extraction of DNA and RNA.

Results Studies using RT-PCR demonstrated that patients with diabetes had a lower degree of VDR expression than control subjects ($P > 0.05$). The T2DM was shown to be strongly correlated with hypermethylation (p -value < 0.001 , OR 2.9; 95%CI 1.6–5.54). When compared to control groups, T2DM patients' levels of vitamin D in their serum were considerably lower ($p < 0.01$). Pro-inflammatory mediators like TNF- α , CRP, IL-6, and leptin levels were discovered to be higher, and concentrations of anti-inflammatory mediators like IL-10 and adiponectin were observed to be lower in people with T2DM than in people without the condition ($p < 0.05$).

Conclusions This study suggests the hypermethylation and down expression of VDR as one of the basis for causing T2DM in kashmiri individuals, exaggerated by enhanced degree of TNF- α , CRP, IL-6 and leptin and diminished concentration of IL-10 and adiponectin in T2DM.

Keywords Diabetes mellitus · Promoter methylation · Vitamin D · VDR · Inflammatory biomarkers

Abbreviations

VDR	Vitamin D receptor
FPG	Fasting Plasma Glucose
HbA1C	Haemoglobin A1C
BMI	Basal Metabolic Index
HDL	High Density Lipoprotein
LDL	Low Density Lipoprotein
TG	Triglycerides

HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
CRP	C-reactive protein
TNF- α	Tumor necrosis factor-alpha
IL-1 β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-17	Interleukin-17
IL-37	Interleukin-37
IFN- γ	Interferon Gamma
TGF- β	Transforming growth factor beta
MCP-1	Monocyte chemoattractant protein-1
NF- κ B	Nuclear Factor Kappa B
IRS	Insulin receptor substrate
GLUT4	Glucose transporter-4
MS-PCR	Methylation specific polymerase chain reaction
RT-PCR	Real time Polymerase chain reaction

✉ Bashir Ahmad Ganai
bbcganai@gmail.com

¹ Centre of Research for Development, University of Kashmir, Srinagar 190006, Jammu and Kashmir, India

² Division of Endocrinology, Sher-i-Kashmir Institute of Medical Sciences, Srinagar, Jammu and Kashmir, India

³ Department of Environmental Science, University of Kashmir, Srinagar 190006, Jammu and Kashmir, India

⁴ Department of Biochemistry, University of Kashmir, Srinagar 190006, Jammu and Kashmir, India

Introduction

Diabetes mellitus is an assemblage of disorders defined by disturbed metabolism that resulted in hyperglycemia due to reduced insulin activity and insulin insufficiency or both [1]. DM is a major health issue at the global level. By 2025, it is reported that the population of Diabetes will be escalated to 300 million, with diabetes mellitus (type II) (T2DM) contributing to the majority of DM [2], especially in China. T2DM is a complicated life-long disease with the environmental, genetic, and epigenetic component, which remarkably affects life quality [3]. Pathogenesis of T2DM is an outcome of multiple interactions between a variety of susceptibility genes and coordination between environment and genetic components [4]. There are a multitude number of genes, including vitamin D receptor gene (VDR), studied to have their involvement in T2DM in various populations [5].

The broadly known biological activity of vitamin D is to maintain homeostasis of phosphorus (P) and calcium (Ca) [6], besides, that it is connected with numerous biological events, including apoptosis, immunological response, differentiation, and proliferation [7]. The biochemical processes of vitamin D are achieved by gene expression via the VD-VDR complex formation. The biological basis for the wide effects of VDR and its ligands is provided by the VDR gene expression in almost all of the tissues and cells in the body [8]. The gene that codes for the VDR is positioned on the chromosome 12 (12q13.11), and it has four potential promoter regions, eight introns, and nine exons (The exons 1a, 1c, and 1d of the VDR show good homology, but exons 1b, 1e, and 1f have weak homology). The robust exon1a promoter has several transcription element binding spots (AP2 and SP1) [9]. Various studies have shown the role of vitamin D receptor in causing T2DM. Polymorphisms in the VDR gene have been found to influence the VDR protein's activity. It has been hypothesised that genetic differences in the VDR affect Ca metabolism, adipose function, insulin release, and cytokine production, which all contribute to the aetiology of T2DM [10]. A study reported that the VDR gene's FokI polymorphism may increase the incidence of T2D, particularly in Asian populations [11]. T2DM patients were shown to have a significant rate of vitamin D deficiency, which was correlated with VDR gene FokI and ApaI polymorphisms in the Caribbean population [12]. Studies by Cyganek et al. (2006) reported the role of VDR polymorphism in causing T2DM in Polish population [13]. Another study by Ma et al. (2020) revealed that the variants of VDR polymorphisms (*FokI* and *TaqI*) are associated with susceptibility to T2D and its clinical manifestation [14]. Fok-I, Taq-1 and Bsm-1 polymorphism was strongly associated with T2DM, suggesting that this gene polymorphism may be a risk factor for T2DM in the Kashmiri population [15].

Various expressional studies have been done on VDR which revealed its association with various diseases. Research on some polymorphisms, such as FokI, revealed that VDR is down-expressed, in pulmonary tuberculosis [16], in Behcet's disease [17], in alopecia areata [18], and increased VDR expression in prostate cancer patients [19] (Fig. 1).

Epigenetic changes alter the transcription level of the gene without affecting the DNA sequences. Various environmental aspects (like solar exposure, diet, infections, and pollution) have impact on the VDR by changing the levels of VD. Activated form of VD, i.e., 1,25(OH)₂D₃ after linking with VDR, influences the expression of VDR by DNA methylation, DNA demethylation, and histone modification [20]. Findings revealed that increased methylation in the promoter site of VDR was connected to lower VDR mRNA and protein.

Several studies revealed a linkage between VDR hypermethylation and infectious diseases [21]. Methylation of VDR promoter has been reported to cause colorectal cancer, breast cancer, prostate cancer [22], adrenal carcinoma [23] and idiopathic infertility [24].

Various inflammatory biomarkers have been reported to take part in insulin signalling and in the advancement of insulin resistance in β -cells of pancreas. An equilibrium between the pro and anti-inflammatory biological markers is vital for the proper functioning of endocrine nature of pancreatic β -cells. Various studies reported the higher degree of TNF- α , CRP, IL-1 β , IL-6, IL-8, IL-17, IL-37, IFN- γ , MCP-1, TGF- β and leptin in T2DM [25–34] and reduced levels of anti-inflammatory biomarkers in T2DM [35, 36]. IFN- γ is known to increase the production of TNF- α and IL-6 by enhancing the phosphorylation of STAT-1 in monocytes under high glucose conditions [37]. In our study we will investigate the levels of TNF- α , CRP, IL-6, IL-10, leptin, adiponectin in T2DM. Various studies have been done so far that demonstrated that VD has a role in triggering the T2DM and few studies exist about epigenetic control of the VDR gene in T2DM. Our study is to examine the CpG island methylation and gene expression levels of VDR in subjects with T2DM in Kashmiri population.

Materials and methods

Sample collection

Subjects with T2DM ($n = 100$) and controls ($n = 100$) of age group (27–65) had participated in the study. Among cases, 52 were males and 48 were females, whereas, among controls 51 were males and 49 were females. Participants were recruited during November 2019 and June 2021 from the Department of Endocrinology at the

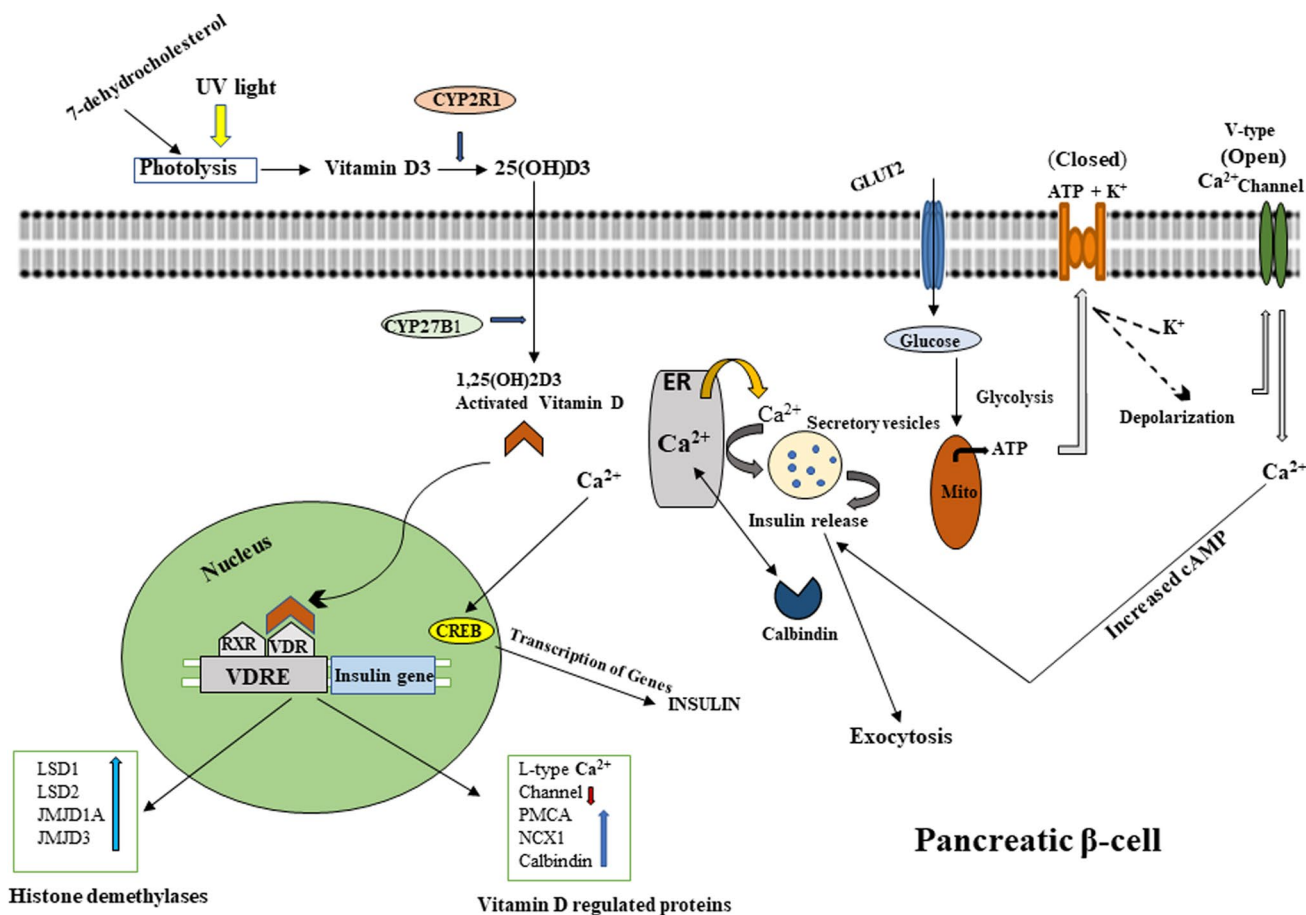


Fig. 1 Illustrates the activation mechanism of vitamin D and its role in insulin release as well as causing Diabetes; 7-dehydrocholesterol present in the skin cells after UV mediated photolysis gets converted into inactivated form of vitamin D3. The vitamin D3 enters into circulation, where first hydroxylation occurs in liver by enzyme vitamin D-25-hydroxylase (encoded by CYP2R1 gene) and gets converted into 25(OH)D3, after that, second hydroxylation occurs in target tissues such as beta cells of pancreas where 1,25(OH)2D3 is formed by enzyme 1 α -hydroxylase (encoded by CYP27B1 gene), enters into the nucleus and forms complex with VDR that gets attached to VDRE to regulate the transcription of number of genes. VDR controls insulin

release via genomic and non-genomic mechanisms, genomic mechanism is because of direct action of VDR on insulin gene and non-genomic is due to the balance of Ca²⁺ levels intracellularly as well as extracellularly. Optimum Ca²⁺ is needed for the release of insulin from secretory granules while as, increased Ca²⁺ is often associated with the onset of diabetes. Vitamin D acts to maintain the levels of Ca²⁺ by enhanced expression of NCX1 and PMCA. Vitamin D also regulates the expression of DNA demethylases like LSD1 and LSD2 as well as JMJD1A and JMJD3, that have a role in prevention of promoter hypermethylation responsible for decreasing gene transcription. Fig created by MS ppt reported the version

SKIMS, Soura, Srinagar, J&K. Patients gave their consent in writing, and recruitment began after receiving approval from the IEC ethics committee, SKIMS. American Diabetes Association (ADA) criteria were used as the basis for the cases' inclusion criteria, patients having HbA1c $\geq 6.5\%$ and FPG ≥ 126 mg/dl were taken as cases and those with HbA1c $< 5.7\%$ and FPG < 100 mg/dl were taken as controls. Patients with gestational diabetes, T1DM, metabolic syndrome, and anyone with at least one of these concomitant conditions—cancer, autoimmune illnesses, heart issues, and congenital abnormalities—were omitted from the research study. 3-4 ml of blood were drawn from diabetic and normal volunteers, and they were stored at -20°C before processing. Information related to

the lifestyle, dietary and environmental factors were collected using an organized questionnaire from each subject (Table 1).

DNA extraction from blood samples

From the blood samples, extraction of DNA was done manually by using Suguna et al. (2014) method [38]. The dissolution of DNA was carried out in the Tris-EDTA and was preserved at -20°C till further use. The DNA sample was then run on 1.5% agarose gel stained with EtBr by using electrophoretic device. The DNA concentration and purity was measured using Nanodrop (Thermofisher) measuring A₂₆₀nm and A₂₆₀/A₂₈₀ ratio respectively.

Table 1 Represents anthropometric measurements, dietary habits, lifestyle and environmental factors

Characteristics	Type 2 Diabetes Mellitus (n = 100)	Controls (n = 100)	P-value ($p \leq 0.05$)
Age (Years)	42.2 ± 7.2	45.7 ± 9	$p < 0.05$
Weight (kg)	65.3 ± 1	60.4 ± 0.9	$p < 0.05$
Gender (M/F)	52/48	51/49	$p < 0.05$
BMI (kg/m ²)	26.8 ± 1.2	21.92 ± 5.2	$p < 0.05$
Pollutant exposure	62(%)	53(%)	$p < 0.05$
Vegeterian	42(%)	51(%)	$p < 0.05$
Non-vegetarian	58(%)	49(%)	$p < 0.05$
Overweight	38(%)	21(%)	$p < 0.05$
Normal	62(%)	79(%)	$p < 0.05$
Physically active	48(%)	58(%)	$p < 0.05$
Sedendary	52(%)	42(%)	$p < 0.05$
Smoking	34(%)	25(%)	$p < 0.05$
No smoking	66(%)	75(%)	$p < 0.05$

Modification of DNA by Bisulfite treatment and MS-PCR

CpG islands are a stretch of GC content DNA positioned in the promoter site of the genes and are the sites where methylation occurs. Methylation in DNA can be determined by sodium bisulfite treatment subsequent to MS-PCR (23). The genomic DNA (~20 µL) with a concentration of 80 ng/µL and pure DNA as indicated by A_{260}/A_{280} ratio as 1.79, was treated with bisulfite using a (Promega, Madison, USA) DNA methylation kit. This treatment converted unmethylated cytosines into uracil, but it does not affect methylated cytosines. During the PCR step (MS-PCR), unmethylated cytosines get converted into thymidines, while the methylated cytosines remain unaffected displaying a difference between unmethylated and methylated DNA. The VDR promoter of the converted DNA was then targeted by VDR primers using MS-PCR. The methylation included both methylated and unmethylated primers for the promoter site of VDR; VDR- FP methylated 5' TTCGTAGTAGGTTGGGTAGAATTAC 3' VDR-RP methylated 3' ACCCTCTATAAAACAAAATTATCGAT 5'; VDR-FP unmethylated 5' TGTAGTAGGTTGGGTAGAATTATGG 3', VDR-RP unmethylated 3' ACCCTCTATAAAAACAAAATTATCA AT 5'.

For PCR amplification, the total volume of reaction mixture was taken to be 25 µL which contains Mastermix (Promega, Madison, USA) of 12.5 µL (mixture of dNTPs, MgCl₂ buffer, Taq DNA polymerase), 2 µL of DNA, and 10.5 µL of PCR grade water. Amplification of DNA was achieved in thermal cycler (Applied Biosystems), and PCR conditions were; denaturation step (95°C-3 min) continued by 40 cycles, second denaturation (95°C-50 s), annealing at (52°C-35 s), extension at (72°C-1 min) and final extension for 10 min at 70°C. The amplification yield after MS-PCR was 118 and

116 bps for methylated and unmethylated products respectively. The products were resolved by using 1.5% agarose gel that contains 4 µL of EtBr and were visualized under UV light (Fig. 2A).

RNA isolation from Blood samples

From blood samples, PBMC were separated by Ficoll-Hypaque density gradient centrifugation (Lymphodex, Inno-Train, Germany) and homogenization of cells were done in Trizol (Invitrogen, CA), and RNA was obtained from monocytes according to the protocol. Dissolution of obtained RNA was done in 30 µL DEPC-treated water, measured with Nanodrop (spectrophotometry), and the RNA integrity was assessed by 2% agarose gel electrophoresis. DNA contamination was removed using a DNase kit (Promega, Madison, USA), Synthesis of cDNA was achieved by cDNA synthesis kit (first strand) (Thermo Fisher Scientific), and a RT- polymerase chain reaction technique was used for expression studies.

Quantitative Real-Time PCR reactions

In an RT-PCR study, transcripts specific to the target gene, in this case VDR, and GAPDH (internal reference), are amplified. The research was conducted on the basis of a semi-quantitative investigation of the degree of relative mRNA expression. Requirements were optimized for PCR; the total reaction volume was taken to be 20 µL, which contains 10 µL Master Mix (MAXIMA SYBR Green; Thermo Fisher Scientific), 1 µL cDNA, 1 µL each of reverse and forward primers (0.5 µM), and 7 µL of DNA/ RNA free water was employed to perform real-time PCR experiments. The VDR gene FP (5'- TTGCCATACTGCTGGACGC -3') and VDR-RP (5'- GGCTCCCTCCACCATCATT -3') as well as

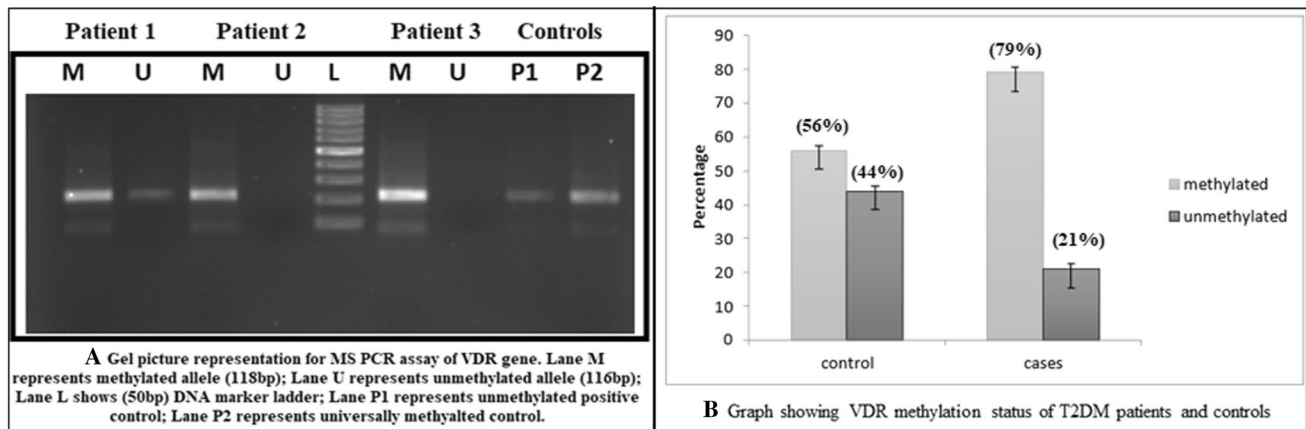


Fig. 2 A Gel picture representation for MS PCR assay of VDR gene. Lane M represents methylated allele (118 bp); Lane U represents unmethylated allele (116 bp); Lane L shows (50 bp) DNA marker ladder; Lane P1 represents unmethylated positive control; Lane P2 represents universally methylated control.

the GAPDH gene GAPDH-FP (5'- TTTGGCTACAGCAAC AGGGT -3') and GAPDH-RP (5'- TCCTCTTGCTTGCTGG -3') were employed in the RT-PCR tests. For the process of amplification of cDNA through the cycling platform, the Realtime PCR Detection System was used. Employing CFX96 cyclor (Bio-Rad Laboratories, CA), the denaturation stage was carried out at (95 °C-7 min), subsequent to a denaturation second phase (95 °C-35 s), annealing at (57 °C-35 s), and extension for (72 °C-10 min) (40 cycles). One identical product was amplified for every single sample, according to melting curve studies, and dissociation curve plots were performed to evaluate the purity of the PCR products.

Vitamin D and immunoassay analysis

Serum 25(OH)D3 levels were estimated by ELISA (Abbot Laboratories Illinois, USA) and the measuring range of serum vitamin D are as ($\geq 30\text{ng/ml}$) as vitamin D sufficient; and ($< 20\text{ng/ml}$) as vitamin D deficient and in between (20-30ng/dl) was taken as vitamin D insufficient. Serum inflammatory mediators (IL-6, IL-10, C-reactive protein, leptin, adiponectin and TNF- α) were assayed by ELISA using commercial kits and according to manufacturer's guidelines (Diaclone, France; DRG international, USA; Calbiotech, CA, USA).

Statistical analysis

SPSS (SPSS, Chicago, IL) and GraphPad InStat software were used to conduct the statistical analysis. The parameters for the "cycle threshold" (Ct) were determined using 7500 software version 2.3 amplification charts. The $2^{-\Delta\Delta\text{Ct}}$ technique was used to analyse the differential expression quantities of VDR using GAPDH as the reference gene, and the results as fold change down/upregulation were produced. The normal distributions expression data (Ct) were

examined using the Kolmogorov–Smirnov test with a Lilliefors correction. Standard deviation (SD) and mean were used to report the quantitative variables. The student T-test and Chi square test were employed to evaluate the relationships between categorical data. Utilising independent samples t-tests and Mann Whitney U tests, the RNA expression levels and VDR methylation pattern were compared between diabetic and control participants. Student's T-test was carried out for inflammation, immunoassay, and biochemical indicators. The Pearson correlation coefficient (r) was found for the purposes of correlational analysis. The findings were determined at a statistical significance threshold of $p \leq 0.05$.

Results

Dietary, lifestyle and environmental factors

In the study, we divided dietary preferences into vegetarian and non-vegetarian categories. Of the patients, 42% prefer vegetarian diets, while 58% choose non-vegetarian diets. 48% of people with diabetes engage in physical activity, while 52% prefer a sedentary lifestyle. In my study, there were 34% diabetes smokers with percentage more than healthy controls (25%). Pollutant exposure is higher for participants with diabetes (62%) than for healthy controls (53%).

Biochemical and immunological assay

Biochemical analysis The biochemical parameters of the research study were summarized in (Table 2) and revealed that the levels of FPG, HbA1c, BMI, LDL, TG and Cholesterol were observed to be higher in T2DM and control groups and statistically significant outcome was discovered

Table 2 Represents the clinical parameters of patients (T2DM) and controls

Parameters	T2DM (n=100)	Control (n=100)	P-value
FPG (mg/dl)	175.34 ± 33	85.2 ± 21	0.0001
HbA1c (%)	7.9 ± 1.7	5.1 ± 0.47	0.0001
BMI (kg/m ²)	26.8 ± 3.01	21.92 ± 5.2	0.0001
HDL-C (mg/dl)	38.85 ± 6.2	42.6 ± 16.4	0.034
LDL-C (mg/dl)	111.9 ± 31	99.3 ± 23	0.0001
Cholesterol (mg/dl)	178.9 ± 21	164.89 ± 31	0.0003
Calcium (mg/dl)	6.01 ± 1.2	8.42 ± 1.1	0.0001
Phosphorus (mg/dl)	3.86 ± 1.3	2.8 ± 1.2	0.79
Urea (mg/dl)	30.12 ± 1.1	35.12 ± 1.2	0.59
Uric acid (mg/dl)	4.12 ± 2.1	3.65 ± 2	0.75
HOMA-IR	13.49 ± 4.1	1.5 ± 1	0.00
Insulin (μU/ml)	31.95 ± 3.5	7.9 ± 1.2	0.00
Vitamin D (ng/mL)	14.73 ± 6.1	18.88 ± 8.1	0.007
Albumin (g/dl)	4.34 ± 1.02	4.31 ± 0.34	0.1
Triglycerides (mg/dl)	256.79 ± 31	201.7 ± 39	0.04

($p < 0.05$). The immunoassay analysis of the study revealed an increasing trend in the Insulin levels among patient group ($31.95 \pm 3.5 \mu\text{U/ml}$) in comparison to healthy group ($7.9 \pm 1.2 \mu\text{U/ml}$) and was found to be statistically significant ($p < 0.05$). The HOMA-IR index which represents the insulin sensitivity was found greater in patients than controls showing a significance statistically ($p < 0.05$).

Immunological analysis The results of the study group's immunological analysis were compiled in (Table 3) and showed that the concentrations of pro-inflammatory cytokines like TNF- α , CRP, IL-6, Leptin, and WBCs to be greater and anti-inflammatory markers like IL-10 and adiponectin were assessed as being low in T2DM compared to their healthy counterparts, attaining a degree of statistical significance ($p < 0.05$). The correlation method by Karl Pearson was utilised to examine the relationship between cytokines that promote inflammation in T2DM participants and healthy individuals. In T2DM patients, there was a significant positive association of TNF- α , with HbA1c, and FPG ($r = 0.87$; $p = 0.002$ and $r = 0.43$; $p = 0.05$) and non-significant association was

observed between diabetes related factors (HbA1c, FPG) with inflammatory mediators (both pro and inflammatory) ($p > 0.05$). Between anti-inflammatory and pro-inflammatory markers, significant negative correlation was seen between IL-10 and IL-6 levels ($r = -0.145$; $p = 0.034$). Non-significant correlation of vitamin D with TNF- α and leptin was found in T2DM ($r = 0.05$; $p = 0.6$, $r = 0.03$; $p = 0.4$).

Methylation study of VDR gene

Hypermethylation was observed in promoter of VDR gene in T2DM patients in comparison to controls (OR = 2.9; 95% CI = 1.6–5.54) and p-value was analysed to be $p = 0.0005$ ($p < 0.001$) shown in (Table 4). VDR gene methylation was higher in cases with VD deficiency ($< 30 \text{ ng/ml}$) in comparison to those having adequate VD ($\geq 30 \text{ ng/ml}$), but it did not achieve statistical significance Fig. 2B. (Table 3). Inverse association was observed between vitamin D levels with DNA methylation ($p > 0.05$) and no significant association were observed between VDR gene methylation with other clinical and demographic factors ($P > 0.05$).

Quantitative RT-PCR

VDR levels of gene expression in patients have been discovered to be lower than the levels in normal subjects after real-time PCR data processing (1.07 ± 0.75 and 2.14 ± 1.16 respectively, $P\text{-value} > 0.05$). We also analyzed the link between expression levels of VDR and clinical and demographic features of patient groups. Results revealed the non-significant difference between male and female VDR gene expression levels ($P = 0.03$). With regard to other clinical and demographic characteristics of the participants, there was no significant difference in the expression of the VDR gene ($P > 0.05$) (Fig. 3).

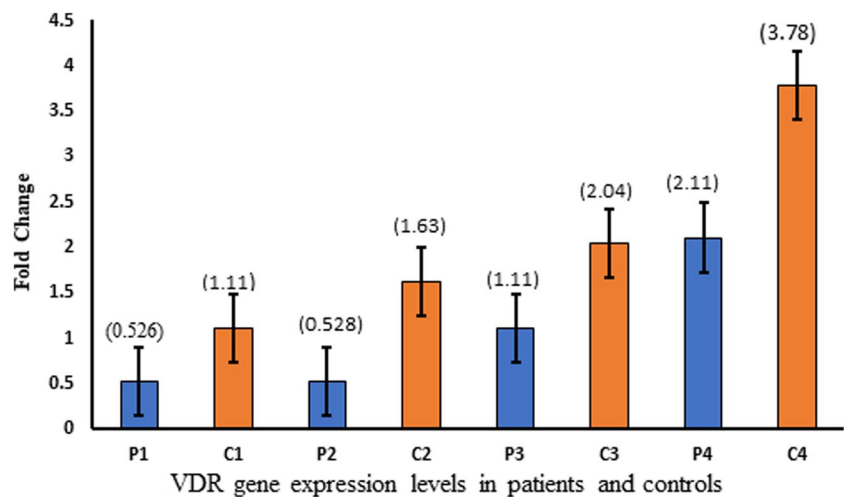
Table 4 Promoter hypermethylation in T2DM

Methylation Status	Case, (%)	Control, (%)	OR (95% CI)	P value
Unmethylated	21	44	Referent	
Methylated	79	56	2.9(1.6–5.54)	0.0005

OR (odds ratio), CI (confidence interval)

Table 3 Represents the inflammatory biomarkers in T2DM and control groups

Inflammatory Biomarkers	T2DM (n=100)	Controls (n=100)	P value (<0.05)
Leptin (ng/mL)	15.5 ± 6.1	8.3 ± 5.6	<0.001
IL-6 (pg/dl)	18.92 ± 7.1	2.9 ± 1.5	<0.001
TNF- α (pg/mL)	33.95 ± 8.81	11.97 ± 3.41	<0.001
CRP (mg/dl)	4.32 ± 1.2	1.5 ± 1	<0.001
TLC (thousands)	8456 ± 1789	7345 ± 1589	<0.001
IL-10 (pg/ml)	6.41 ± 1.89	9.41 ± 2.9	<0.001
Adiponectin (ng/ml)	12.91 ± 2.13	16.23 ± 3.43	<0.001

Fig. 3 VDR gene expression levels in patients and controls

Assessment of vitamin D in T2DM In compared to normal individuals (mean = 18.88 8.1 ng/ml), vitamin D concentrations in serum were considerably lower in patients (mean = 14.73 7.6 ng/ml), ($p < 0.05$). The correlation between VD levels and T2DM was positive, although it failed to achieve statistical significance. The relationship between 25(OH)D3 with VDR expression or with gene methylation was not statistically significant ($p > 0.05$).

Discussion

Our study focused on the genetic and epigenetic contributions of vitamin D to the progression of type 2 diabetes in the Kashmiri community. We saw a rise in diabetic cases in developing nations, which is impacted by environmental, hormonal, and hereditary variables. Numerous parameters in this investigation were shown to be greater in T2DM than in healthy controls. While addressing to the basic parameters, the diabetic category had more smokers (34%) than normal controls (25%) ($p < 0.05$). Smokers are 30%–40% more susceptible to acquire T2DM, as reported in the US Department of Health and Human Services (USDHHS) publication on Diabetes [39]. Smoking may disrupt the way that insulin is regulated because nicotine can make insulin less effective and make smokers demand more insulin to keep up their glucose levels. Oxidative strain and inflammation are also exacerbated by smoking, which negatively affects β -cell activity [35]. According to the majority of research conducted throughout the world that link sedentary lifestyle and low physical activity with the onset and severity of T2DM, over 52% of enrolled patients had sedentary lifestyles indicating that the persons who are engaged in rigorous exercise have a decreased risk of developing diabetes than those who live sedentary lifestyles. This

study's findings that TG, TC, LDL were considerably greater in T2DM cases than in control groups raise the possibility that dyslipidemia [35], which is triggered by an enhanced level of lipid parameters, may influence the onset and progression of T2DM in Kashmiri population.

Inflammatory substances such as TNF- α , CRP, IL-6, and leptin are known to be present in greater levels in people with T2DM, and this enhanced concentration is correlated with the emergence of insulin resistance [27, 40]. Our study revealed the enhanced levels of TNF- α , CRP, IL-6 and leptin T2DM than healthy controls. Anti-inflammatory cytokines like IL-10 and adiponectin are observed to be lower in our patient study group than controls. In T2DM patients, there was a non-significant correlation between diabetes-related parameters (HbA1c, FPG) and inflammatory mediators (both pro and inflammatory) ($p > 0.05$), but a substantial positive association between TNF- α with HbA1c, and FPG ($r = 0.87$; $p = 0.002$ and $r = 0.43$; $p = 0.05$). In our study we observed that patients with vitamin D insufficiency were having higher levels of pro-inflammatory cytokines though the results seems to be insignificant. Vitamin D, regulates a multiple number of genes involved in modulating the expression of inflammatory proteins [41]. Deficiency of vitamin D promotes the production of pro-inflammatory substances while lowering the expression of anti-inflammatory mediators. According to this study, people who have impaired vitamin D levels had greater concentrations of pro-inflammatory mediators and a reduced level of anti-inflammatory ones. Thus, a vitamin D shortage affects the expression of the genes that result in T2DM either directly or indirectly [42].

DNA methylation, an epigenetic modulation imparts an essential function in gene expression. DNA methylation on CpG dinucleotides is present on CpG islands (CGI) are often present in the promoter regions. CpG islands contributes to 1–2% of genome, consists of 200 bp to several

kb in length [43]. Most of the CpG's that lies outside the CGI region are methylated, suggesting its role in genome maintenance levels [23, 44]. The analysis revealed that the VDR promoter is rich in CpG islands and contains many regulatory elements required for transcription and that DNA methylation can impair the transcription process, resulting in the VDR gene silencing. Methylation may be the main mechanism leading to loss-of-function, As, somatic mutations proved to be a very rare occurrence in the VDR gene. Our research showed that diabetic individuals had a higher degree of methylation in the VDR promoter region and a lower level of VDR gene expression than healthy controls. Serum vitamin D concentration in the T2DM patient population was observed to be low in comparison to control groups. We also examined the connection between low levels of expression and hypermethylation of the VDR promoter with other clinicopathological and demographic variables. Non-significant relation was detected between 25(OH)D3 concentrations and VDR expression or with the methylation profile of VDR. These findings additionally demonstrated that 25(OH)D3 concentrations might not be the only factor influencing the degree of VDR expression. The serum 25(OH)D3 levels and the VDR's methylation state did not significantly correlate with one another. This study also presented that VDR gene promoter could be influenced by methylation of DNA which could impact on expression levels increasing the risk of T2DM. More studies need to be conducted that include a large population size to confirm the present results of our work and be performed in many regions to determine the impact of vitamin D and VDR in T2DM among diverse population groups.

Conclusions

This study suggests the hypermethylation and down expression of VDR as one of the basis for causing T2DM in kashmiri individuals, exaggerated by raised concentrations of TNF- α , CRP, IL-6 and leptin and diminished concentration of IL-10 and adiponectin in T2DM.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s40200-023-01266-6>.

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Data availability The data supporting the findings of this study is available in supplementary material.

Declarations

Ethical approval Following permission from the IEC (ethical committee) of the SKIMS, Srinagar, India, all patients completed their informed consent forms in full, and patient recruitment began. Reference number: SIMS 1 131/IEC-SKIMS/2019–513.

Conflict of interest According to the authors, there are not any conflicts of interest in this research.

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