Prevalence Pattern of Key Polymorphisms in the Vitamin D Receptor gene among Patients of Type 2 Diabetes Mellitus in Northeast India

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Abstract

Aims: To investigate the association between Vitamin D receptor gene polymorphisms (BsmI, TaqI and FokI) and type 2 diabetes mellitus in patients in north eastern India. **Settings and Design:** This was a case control study with 40 cases of type 2 diabetes and 20 controls. **Materials and Methods:** Genomic DNA was extracted from blood and genotyped for the single nucleotide polymorphism (SNPs) of BsmI [rs1544410], TaqI [rs731236] and FokI [rs2228570] by polymerase chain reaction and gene sequencing. Genotype distribution and allelic frequencies were compared between patients and controls. Data was expressed as mean ±standard deviation. Chi square test and t test were used to compare groups. Statistical analysis was done using SAS version 9.3 software. P value of <0.05 was considered significant. **Results:** Body weight and BMI were significantly associated with VDR polymorphisms BsmI and TaqI while BsmI was significantly associated with HbA1C. Vitamin D deficiency was significantly greater in cases than controls. The frequency of the heterozygous genotype of the BsmI polymorphism was significantly greater in type 2 diabetics than in controls. **Conclusions:** Vitamin D receptor polymorphisms are associated with type 2 diabetes in our population and require larger scale studies to be considered as possible risk factors or type 2 diabetes mellitus.

Keywords: Gene polymorphisms, type 2 diabetes, Vitamin D receptor gene

Introduction

Type 2 diabetes mellitus (T2DM) is a progressive chronic disease which is marked by the inability of tissues such as liver and skeletal muscles to respond to insulin. Diabetes mellitus is well on its way to becoming a potential epidemic in India with more than 65 million diabetic individuals currently diagnosed with the disease.[1] It is predicted that the prevalence of diabetes will double globally from 171 million in 2000 to 366 million in 2030, with a maximum increase in India.[2]

Vitamin D deficiency appears to be related to the development of T2DM and the metabolic syndrome. Recent studies have reported that high Vitamin D status provides protection against type 2 diabetes.[3,4] Vitamin D deficiency results in reduced insulin secretion in humans and rats, and its supplementation improves glucose tolerance.[5]

It is well known that Vitamin D, especially its activated metabolite 1,25- dihydroxyvitamin D3 are involved in controlling some functions of the endocrine pancreas, particularly insulin secretion; and the action of Vitamin D is mediated through binding to its nuclear receptor Vitamin D receptor (VDR).[6] The VDR primarily acts as a transcription factor. The proteins produced by the DNA that the VDR targets are responsible for various cell processes ranging from inflammation to lipid storage and calcium regulation, and deficiencies in Vitamin D are associated with many diseases, particularly type 2 diabetes.

A number of studies have been carried out in various parts of the world which have shown that polymorphisms in the VDR gene are implicated in susceptibility to T2DM. Some of the VDR polymorphisms which have been investigated for their possible associations are rs731236 (TaqI), rs1544410 (BsmI), and rs10735810 (Fok1), and these were identified using the

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Access this article online	
Quick Response Code:	**Website:** www.ijem.in
	DOI: 10.4103/ijem.IJEM_213_17

TaqI, BsmI, and FokI restriction enzymes, respectively. The results have been inconsistent and inconclusive, with wide variation in different regions and races. A study in North India explored associations between VDR polymorphisms and genetic susceptibility to type 2 diabetes and found that the genotype distribution, allele and haplotype frequencies of VDR polymorphism did not differ significantly between patients and controls.[7] However, to date, no such study exploring genetic polymorphisms in the VDR to evaluate for the risk of type 2 diabetes has been undertaken in the northeastern part of India, which was why this study was undertaken.

MATERIALS AND METHODS

The present study was a case–control study carried out in a tertiary care center from August 2015 to December 2016. The study was undertaken after obtaining approval from the Institutional Ethics Committee and written informed consent was obtained from all the participants. A total of 40 patients with T2DM diagnosed as per the American Diabetes Association (ADA) (2015) criteria for diabetes were enrolled as cases. Healthy age- and sex-matched controls with no history of T2DM and normal oral glucose tolerance test as per ADA criteria of diabetes (2015) and without any family history of diabetes mellitus in first-degree relatives were enrolled as controls. Exclusion criteria for the study were people on Vitamin D supplementation within the past 3 months, patients on drugs altering Vitamin D levels (anticonvulsants, estrogen, cholestyramine, and orlistat), chronic liver disease and CKD stages 3-5.

Study design

Demographic details were taken, and clinical examination was performed for both cases and controls. Height and weight were measured and recorded to nearest 0.5 cm and 0.1 g precision, respectively, using standard measuring tape and weighing scale. Body mass index (BMI), in kg/m^2) was calculated. Overweight was defined as BMI 25–29.9 kg/m^2 and obesity was defined as BMI $\geq$ 30 kg/m^2 for both genders. Waist circumference was measured at the midpoint between the lower margin of the least palpable rib and the top of the iliac crest, using a stretch resistant tape providing a constant 100 g tension. Hip circumference was measured around the widest portion of the buttocks, with the tape parallel to the floor. Systolic and diastolic blood pressures (SBP and DBP) was measured as the mean of three consecutive measurements using a standard mercury sphygmomanometer from the right arm of the participant, seated for 5 min before the measurement. Hypertension was defined as SBP $\geq$140 mm Hg and/or DBP $\geq$90 mm Hg, or the use of antihypertensive drug treatment or a combination of these.

Biochemical tests

Peripheral venous blood samples were collected in the morning after an 8 h fast. All controls were screened with a standard 75 g oral glucose tolerance test to exclude the presence of diabetes mellitus. Fasting and postprandial plasma glucose was measured by the glucose oxidase enzymatic assay. Glycated hemoglobin (HbA1C), serum triglycerides, serum high-density lipoprotein cholesterol (HDL-C), and serum total cholesterol (TC) was measured using an automated analyzer (VITROS® 5600 Integrated System, Ortho Clinical Diagnostics, Inc.). Low-density lipoprotein cholesterol (LDL-C) was estimated from the Friedewald formula: LDL-C (mg/dL) = TC (mg/dL) - HDL-C (mg/dL) - TG (mg/dL)/5. Dyslipidemia was defined as LDL-C >100 mg/dL, TG >150 mg/dL, TC >200 mg/dL and/or HDL-C <40 mg/dL in males or <50 mg/dL in females or with the patient on pharmacological treatment for dyslipidemia.

Serum 25-hydroxyvitamin D (25(OH)) Vitamin D was assayed by an electrochemiluminescence binding assay (Elecsys® Vitamin D total assay, Roche Diagnostics). Vitamin D deficiency was defined as per Endocrine Society guidelines[8] as a 25(OH) D below 20 ng/ml, and Vitamin D insufficiency as a 25(OH) D of 21-29 ng/ml.

Molecular analysis
Isolation of nucleic acids
DNA from fresh blood samples (of patients and healthy controls) were extracted using HiPurA™ Blood Genomic DNA Miniprep Purification Kit (Himedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India).

Qualitative polymerase chain reaction
Analysis of good quality DNA samples of cases and controls using polymerase chain reaction (PCR) sequencing for Fok1, Bsm1, and Taq1 polymorphism were performed using previously published primers.[9] After successful PCR amplification PCR products were analyzed through agarose gel electrophoresis.

Sequence analysis
50 µl of samples along with primers used for PCR were sent to a private genomics laboratory for sequencing. The interpretations of the chromatograms generated [Figure 2] were manually double-checked using bioinformatics software BioEdit. Then, these sequences were multiple aligned using software, GeneDoc and NCBI-BLAST.

Statistical analysis
For statistical analysis, data were expressed as mean ± standard deviation for normally distributed variables. Chi-square and *t*-test were used to compare groups as appropriate. *P* <0.05 was considered to be statistically significant. Statistical analysis was performed using SAS version 9.3 software (SAS institute, north carolina, US).

RESULTS

Baseline characteristics of 40 patients with T2DM and 20 healthy controls are summarized in Table 1. As shown in Table 1, the mean weight of cases (62.3 kg) was significantly greater than that of controls (57.5%, *P* = 0.01). The mean BMI of cases was 24.5 kg/m^2 (±3.19), while in controls, it was

Table 1: Baseline parameters in the two groups

Parameter	Mean±SD		P
	Cases (n=40)	Controls (n=20)	
Age (years)	49.65±9.78	48.45±8.73	0.64
Height (cm)	159.58±6.51	160.7±8.61	0.57
Weight (kg)	62.3±7.5	57.5±5.41	0.013
BMI (kg/m²)	24.51±3.19	22.26±1.32	0.0038
Waist circumference (cm)	90.05±7.35	87±4.12	0.091
Hip circumference (cm)	96.33±6.86	93.45±3.49	0.084
Waist-hip ratio	0.94±0.04	0.93±0.04	0.73
Systolic BP (mmHg)	138.35±11.55	127.8±7.81	0.0005
Diastolic BP (mmHg)	89.55±6.73	84.2±5.31	0.003
HbA1C	10.09±2.24	5.25±0.26	<0.0001
Total cholesterol (mg/dL)	140.68±39.06	135±25.39	0.64
LDL-cholesterol (mg/dL)	75±33.46	70.57±17.1	0.58
HDL-cholesterol (mg/dL)	35.07±10.74	41.75±9.79	0.03
Triglycerides (mg/dL)	149.8±39.23	116±38.68	0.0025
25 (OH) D (ng/mL)	13.91±8.5	21.98±10.15	0.0019

BP: Blood pressure, HbA1C: Glycated haemoglobin, LDL: Low-density lipoprotein, HDL: High-density lipoprotein, BMI: Body mass index, 25 (OH) D: 25-hydroxyvitamin D, SD: Standard deviation

Figure 1: Serum 25-hydroxyvitamin D levels in cases and controls; *P* < 0.05 was considered significant

22.26 (±1.32) which was statistically significant (*P* = 0.0038). Other anthropometric parameters (waist circumference and waist-hip ratio) were greater in cases than controls, but the difference was not statistically significant. Both SBP and DBP were significantly greater in cases relative to controls. Among biochemical parameters, HbA1C was significantly greater in cases (*P* < 0.01). Among lipid profile parameters HDL-C was significantly lower in cases relative to controls (*P* = 0.0269). TG was significantly higher in cases than controls. TC and LDL-C were higher in cases than controls, but the difference did not achieve statistical significance. Vitamin D levels were significantly lower in cases compared to controls (13.9 ng/ml vs. 21.9 ng/ml, *P* = 0.0019).

Overall, Vitamin D deficiency and insufficiency were found to be present in the majority of study participants, as shown in Figure 1. Vitamin D deficiency was significantly more in cases compared to controls (85% vs. 45%, *P* = 0.0001). Vitamin D insufficiency was present in more controls than cases while only a minority of cases and controls were found to have sufficient levels of Vitamin D.

Analysis of the various clinical and biochemical parameters as per the three genotypes of the VDR gene [Table 2] showed that there was a significant association of the body weight and BMI with the combined mutant and heterozygous BsmI genotypes (AA + GA) compared to the wild type(GG) genotype. The body weight and BMI were also significantly correlated with the combined mutant and heterozygous TaqI genotypes (CC + TC) compared to the wild type TaqI genotype (TT). The HbA1C levels were also significantly correlated with the combined mutant and heterozygous BsmI genotypes relative to the levels in subjects harboring the wild type BsmI genotype. However, no other association
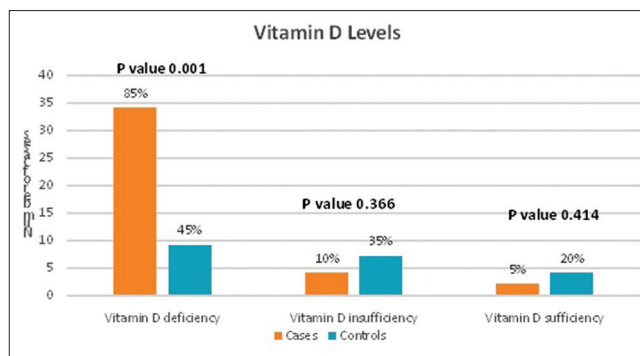
was noted with any of the other clinical and biochemical parameters with BsmI polymorphisms. Analysis of the other two polymorphisms (TaqI and FokI) also failed to demonstrate any other difference between cases and controls when the wild type genotypes (TT) were compared to the mutant and heterozygous genotypes (TC + CC).

The distribution of the BsmI polymorphism [Table 3] showed that the frequency of the heterozygous GA genotype was significantly higher in cases of type 2 diabetes compared to controls (78.57% vs. 21.43%, Chi-square value: 4.037, *P* = 0.04), with no significant difference found in the frequency of the wild type and the mutant genotypes.

On analysis of the allelic frequencies of the BsmI polymorphism [Table 4], no significant difference was found in the mutant allele (A) between cases and controls.

On analysis of the distribution of the TaqI genotypes [Table 3], the homozygous TT genotype was the most frequent variant in cases (55%) as well as in controls (70%). The mutant CC genotype was more frequent in cases (25%) compared to controls (10%). However, there was no statistically significant difference found in any of the genotypes of TaqI between cases and controls.

On comparing allelic frequencies of the TaqI polymorphism [Table 4], there was no significant difference in the frequency of the mutant C allele between cases and controls.

On analysis of the genotypes of the FokI polymorphism [Table 3], the wild type TT variant was the most common genotype in both cases (80%) and controls (75%), with the difference not being statistically significant. The heterozygous genotype (TC) was not found in the cases analyzed. The mutant CC genotype was more frequent in cases (20%) compared to controls (10%) but the difference was not statistically significant.

Comparing the allelic frequencies of the T and C alleles of the FokI polymorphism [Table 4], no significant difference was found between the mutant C allele between cases and controls.

Table 2: Anthropometric and metabolic parameters according to genotypes of vitamin D receptor polymorphisms in type 2 diabetes mellitus cases

	VDR rs1544410 (BsmI)		
Parameter	GG (wild)	GA + AA (heterozygous + homozygous mutant)	P
	Mean	Mean	
Age	50.17±10.54	49.43±9.63	0.83
Height	157.17±4.95	160.61±6.89	0.13
Weight	55.92±9.99	59.64±6.61	0.17
BMI	22.62±3.26	23.31±2.33	0.45
Waist	89.33±7.56	90.36±7.37	0.69
Hip	96.5±7.59	96.25±6.68	0.92
WHR	0.93±0.03	0.94±0.04	0.36
Systolic BP	140±10.41	137.64±12.11	0.56
Diastolic BP	91.83±7.004	88.57±6.48	0.16
HbA1C	9.22±2.09	10.46±2.23	0.11
TC	157.83±40.99	139.04±50.28	0.26
LDL-C	83.92±33.31	71.18±33.38	0.28
HDL-C	39.67±10.81	41.79±29.66	0.81
TG	126.67±45.09	134.21±61.2	0.7
Vitamin D	13.03±5.38	14.29±9.59	0.67
	VDR rs731236 (TaqI)		
Parameter	TT (Wild)	TC + CC (heterozygous + homozygous mutant)	P
	Mean	Mean	
Age	48.73±8.04	50.78±11.72	0.52
Height	159.82±6.17	159.28±7.07	0.8
Weight	58.64±9.01	58.39±6.37	0.92
BMI	23.16±2.94	23.04±2.25	0.89
Waist	90.32±7.19	89.72±7.73	0.8
Hip	97.59±6.64	94.78±7.00	0.2
WHR	0.93±0.03	0.95±0.05	0.09
Systolic BP	139.45±10.44	137.00±12.95	0.51
Diastolic BP	89.73±7.05	89.33±6.51	0.86
HbA1C	9.75±2.11	10.49±2.38	0.31
TC	142.41±43.18	147.44±54.39	0.75
LDL-C	73.95±32.51	76.28±35.48	0.83
HDL-C	38.45±14.37	44.44±34.63	0.46
TG	126.00±55.29	139.22±58.43	0.47
Vitamin D	13.33±8.65	14.62±8.50	0.64
	VDR rs10735810 (FokI)		
Parameter	TT (Wild)	TC + CC (heterozygous + homozygous mutant)	P
	Mean	Mean	
Age	49.91±10.22	48.63±8.28	0.75
Height	159.31±6.64	160.63±6.25	0.62
Weight	58.78±8.11	57.50±7.03	0.68
BMI	23.31±2.70	22.28±2.22	0.32
Waist	90.19±7.52	89.50±7.05	0.82
Hip	96.97±6.77	93.75±7.09	0.24
WHR	0.93±0.04	0.96±0.05	0.11
Systolic BP	138.19±10.40	139.00±16.21	0.86
Diastolic BP	89.75±6.64	88.75±7.48	0.71
HbA1C	9.76±2.03	11.39±2.68	0.06
TC	150.63±46.75	120.88±48.21	0.12
LDL-C	77.47±31.57	65.13±41.02	0.36

Contd...

Table 2: Contd...

	VDR rs10735810 (FokI)		
Parameter	**GG (wild)**	**GA + AA (heterozygous + homozygous mutant)**	**P**
	Mean	**Mean**	
HDL-C	43.28±27.71	32.63±8.78	0.29
TG	136.09±57.57	115.38±51.37	0.36
Vitamin D	14.16±9.07	12.89±6.00	0.71

TC: Total cholesterol, TG: Triglycerides, VDR: Vitamin D receptor gene, HbA1C: Glycated haemoglobin, LDL: Low-density lipoprotein, HDL: High-density lipoprotein, BMI: Body mass index, WH: Waist-hip ratio, BP: Blood pressure

Table 3: Distribution of BsmI, TaqI and FokI polymorphisms in type 2 diabetes mellitus cases and controls

Genotype	Cases (%)	Controls (%)	χ^2	P
BsmI				
GG (wild)	12 (30)	10 (50)	2.296	0.129
GA (heterozygous)	23 (57.5)	6 (30)	4.037	0.044
AA (mutant)	5 (12.5)	4 (20)	0.588	0.443
TaqI				
TT (wild)	22 (55)	14 (70)	1.25	0.263
TC (heterozygote)	10 (25)	4 (20)	0.186	0.666
CC (mutant)	8 (20)	2 (10)	0.96	0.327
FokI				
TT (wild)	32 (80)	15 (75)	0.196	0.657
TC (heterozygote)	0	3 (15)		
CC (mutant)	8 (20)	2 (10)	0.96	0.327

TC: Total cholesterol

Table 4: Comparison of allelic frequencies of BsmI, TaqI and FokI in type 2 diabetes mellitus cases and controls

| Alleles	Cases (%)	Controls (%)	χ^2	P
BsmI				
G	47 (58.75)	26 (65)	0.6250	0.4292
A	33 (41.25)	14 (35)		
TaqI				
T	54 (67.5)	32 (80)	2.052	0.1520
C	26 (32.5)	8 (20)		
FokI				
T	64 (80)	33 (82.5)	0.1076	0.7429
C	16 (20)	7 (17.5)		

DISCUSSION

This study was carried out to evaluate the association between VDR gene polymorphisms and T2DM in patients in North Eastern India. Previous studies in various ethnic groups have yielded inconsistent results.

Vitamin D levels were significantly lower in cases compared to controls in our study (P = 0.0019). Our study showed that Vitamin D deficiency was significantly greater in cases compared to controls. Similar to our study, Errouagui *et al*.[10] also demonstrated significantly higher levels of Vitamin D deficiency in patients with type 2 diabetes relative to controls. Their findings were similar to the results obtained from a study

carried out in the French West Indies in type 2 diabetics where the prevalence among the study subjects (all of whom were patients of T2DM) was 42%.[11] The study by Errouagui *et al*. which also looked at Vitamin D deficiency in healthy controls found the prevalence of Vitamin D deficiency in that group to be half that of cases (20%).[10] In our study, the prevalence of Vitamin D deficiency was much higher in both cases and controls (85% and 45%, respectively), which appears to be a reflection of the general status of Vitamin D levels in our country.[12]

The study also showed that like in the study by Errouagui *et al*.[10] in Moroccan subjects, Indian type 2 diabetics also had a substantially higher prevalence of Vitamin D deficiency than healthy controls. Vitamin D has been shown to play an important role in the pathogenesis of type 2 diabetes. Several preclinical studies have demonstrated that Vitamin D plays a key regulatory role in insulin secretion, beta-cell survival, and calcium flux within beta cells. A number of studies done in rats have shown that Vitamin D deficiency impairs glucose-mediated insulin secretion in rat pancreatic beta cells.[13-17] Thus, the evidence available suggests that the high Vitamin D deficient status in type 2 diabetics could be related etiologically to the disease process.

With regard to the clinical parameters assessed, we found that the body weight and BMI were significantly more in subjects with the genotypes with the presence of the mutant allele (i.e., the combined heterozygous and mutant homozygous genotypes) of the BsmI and TaqI polymorphisms. There was no difference in the other clinical parameters, between the wild type genotypes compared to the heterozygous and mutant genotypes of the three polymorphisms which were assessed (BsmI, TaqI, and FokI). Ye *et al*. in 2001, reported that the TT homozygosity for the TaqI SNP and the bb homozygous state for BsmI polymorphism were associated with higher obesity rates and higher BMI values in French subjects.[18] Filus *et al*.,[19] found an association with the BsmI polymorphism and higher BMI values, similar to our study; however, in their study, the higher BMI values were associated with the BB allele, as opposed to our study where it was the genotypes with the mutant alleles which had the higher BMI values. In their study, Bid *et al*. found a significant association between age and waist-hip ratio with TaqI polymorphism.[7]
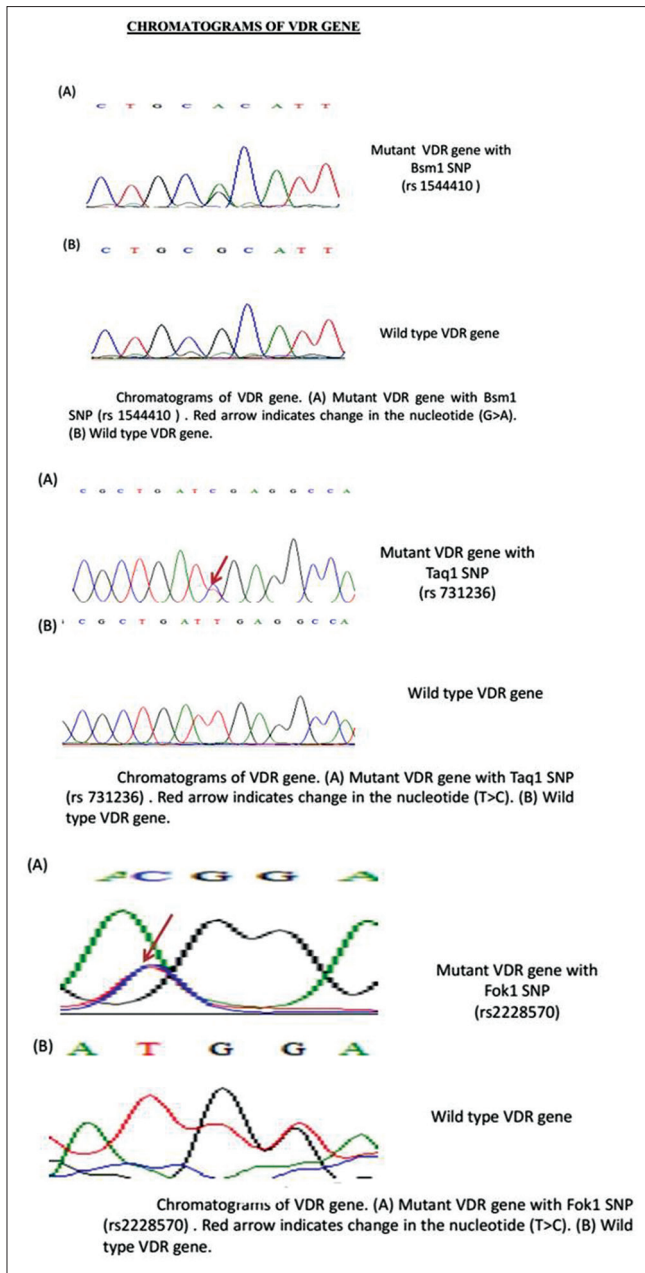
Figure 2: Sequencing chromatograms of Vitamin D receptor gene

We found that there was a significantly higher HbA1C level in cases carrying the mutant BsmI allele (AA + AG genotypes) compared to the wild type (GG genotype). Also, similar to our study, Al-Daghri *et al.*[20] showed that BsmI polymorphisms were associated with an increased risk of obesity in their study carried out in the Saudi Arabian population; however, they found lower HDL cholesterol levels also in relation to BsmI polymorphisms, which was not found in our study. On the other hand, Errouagui *et al.*[10] found that the Fok1 FF variant was significantly associated with increased levels of TC, LDL cholesterol, HDL cholesterol, and triglyceride levels in a study in Moroccan patients.

Regarding the association of T2DM and VDR polymorphisms, we found a significant difference in prevalence of the heterozygous GA genotype of the BsmI polymorphism between cases and controls. The frequency of the heterozygous GA genotype was significantly higher in cases compared to the controls. However, in case of the TaqI and FokI polymorphisms, no significant difference was found between cases and controls with respect to any of the genotypes. Similarly, Ortlepp *et al.*[21] also found that the BsmI polymorphism was significantly associated with the risk of T2DM in the German population. Errouagui *et al.*[10] showed that there was a strong association between Fok1 polymorphisms with type 2 diabetes in Moroccans.

On the other hand, Malecki *et al.*[22] found no association of Fok1, ApaI, BsmI, and TaqI polymorphisms with T2DM in Poland. Ye *et al.*[18] looked for Bsm1, TruI, Apa1 and Taq1 polymorphisms in French subjects and found no association with T2DM. Bid *et al.*[7] found that genotype distribution, allele and haplotype frequencies of BsmI, FokI, and TaqI polymorphisms did not differ significantly between patients and controls. More recently, a Tunisian study[23] looked for the association of T2DM with the FokI polymorphism only and concluded that the FokI polymorphism was not associated with T2DM. Thus, with regard to the question of VDR polymorphisms and the association with T2DM, our study threw up its own results with respect to the BsmI polymorphism, concordant with the finding of some studies while differing with others, as results have varied depending on the ethnicities of the populations where these studies have been carried out. The study was limited by the small sample size. Furthermore, the Apa1 polymorphism was not looked for in our study. While small sample sizes have been a common limitation of every study carried out in this area, the number of polymorphisms looked for has also varied from study to study. The study findings are significant as this is the first study of its kind to have been carried out in the northeastern region of India. To validate the findings of our study, however, a large-scale study design is required.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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