**ABSTRACT**

Objective: Polymorphisms in vitamin D receptor (VDR) genes are known to be linked with different metabolic diseases including Type 2 diabetes mellitus (T2DM) also. However, the association of these polymorphisms is not much explored for the Indian population. To determine the prevalence of BsmI and TaqI polymorphism in VDR gene of T2DM patients from North India.

Methods: Blood samples were obtained from 100 well-characterized T2DM patients and 100 healthy controls. Genomic DNA was isolated from blood samples and using polymerase chain reaction/restriction fragment length polymorphism based method, the presence of these polymorphisms was investigated in these samples. The data were statistically analyzed using SPSS 21.0 software.

Results: For TaqI polymorphism, both the wild type (TT) and heterozygous (TC) genotype showed a significant difference between patients and controls (p=0.023 and p<0.001, respectively). Whereas, the frequency of GG genotype was not significantly different among these groups (p=0.506). For BsmI polymorphism also, the frequency of wild type (GG) and heterozygous (GA) genotype was significantly different in patients and controls (p=0.027 and p=0.001), respectively. However, the frequency of AA genotype was not of statistical significance in patients (p=0.071).

Conclusions: The mutant alleles of TaqI and BsmI polymorphisms are known to be associated with different metabolic diseases, including diabetes too. In our study also, there is a significant difference between the frequency of wild type and heterozygous genotype for these polymorphisms. This suggests that BsmI and TaqI polymorphisms may be associated with T2DM patients.

Keywords: Type 2 diabetes mellitus, Polymorphism, Vitamin D receptor, Patient, Control, Restriction fragment length polymorphism.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is one of the most prevalent lifestyle diseases, which is characterized by high blood glucose levels resulting from defects in insulin action. It usually initiates with insulin resistance, a disorder in which the cells do not respond to insulin properly and increases the risks of many other diseases [1]. According to the International Diabetes Federation by 2035, worldwide the number of T2DM patients is estimated to exceed more than 600 million. India is known as a capital of diabetes, shelter to more than 63 million people suffering from diabetes, expected to increase to 100 million by 2030 [2]. This increase in the T2DM patient is matter of serious concern. Being a complex disease, T2DM is known to be caused by a large number of environmental and genetic factors too. The role of vitamin D in the pathogenesis and prevention of DM has sparked widespread interest. Apart from its classical role in Ca++ homoeostasis, vitamin D also regulates insulin secretion from beta cells and its action on various target cells too [3-8]. All the vitamin D-mediated signaling is entirely dependent on its binding with the vitamin D receptors (VDR), which are present on the surface of the nuclear membrane. VDR, after binding with vitamin D, acts as a transcription factor, which controls the expression of approximately 200 genes including insulin too. Hence, variations/mutation in VDR gene is known to affect the insulin level, leading to diabetes [9-11].

Several polymorphisms in VDR gene have been reported to be associated with various metabolic diseases [12-15]. From last decade, these polymorphisms are also reported in different diabetic populations [16]. It has been hypothesized that VDR polymorphisms may influence both the risk of occurrence of diabetes and its prognosis too [17]. Out of all the reported VDR polymorphisms which are known to be associated with diabetes, four allele variants have been commonly identified and described in great detail. These polymorphisms are rs731236 (TaqI), rs1544410 (BsmI), rs7975232 (Apol), and rs10735810 (FokI). These four single nucleotide polymorphisms were identified using TaqI, BsmI, Apol, and FokI restriction enzymes, respectively. The TaqI polymorphism is located at exon 9 (T65058C bp), codon 352. Although this is a synonymous change, it is reported to be associated with lower circulating levels of active vitamin D3 [18-20]. BsmI is present in intron 8 (639980 bp) at the 3’ end, and being an intronic polymorphism, do not change the amino acid sequence of the encoded protein, but they may affect the expression of VDR gene by regulating the stability of mRNA [21]. Different research groups observed that both these polymorphisms, either independently or together, are associated with insulin resistance and increase in the fasting glucose levels [22,23]. In contrary, Ye et al., Malecki et al., and Ciganek et al. examined that there was no significant association of these polymorphisms with T2DM [24-26]. The status of association of these polymorphisms is also not very clear in context to the Indian population as there are very few reports available which highlight their association with diabetic phenotype in India [27]. Hence, the present work aims at investigating the association of the TaqI and BsmI polymorphism of VDR gene in T2DM patients from North India.

METHODS

Study population

A total of 100 T2DM patients and respective 100 controls were selected for the study. All the participants were recruited from All India Institute of Medical Sciences (AIIMS), New Delhi, India. These participants were between the ages of 35 and 55 years, non-smokers and belonged to the same ethnic group. T2DM patients were diagnosed on the basis of ADA Guidelines (2012). Individuals with fasting glucose ≥126 mg/dl and postprandial glucose ≥200 mg/dl were classified as diabetic. However, healthy controls with no metabolic disease were considered as controls. The present study was approved by the ethical committee of AIIMS.
New Delhi, India. The blood samples and clinical details were collected after taking the informed consent from all the participants.

Genomic DNA extraction
About 2 ml of the blood sample was collected in ethylenediamine tetra acetate acid coated BD Vacutainer and extraction of total genomic DNA was performed using the standard protocol of salting out method [28]. DNA extracted was stored at –80°C for further experiments.

Genotyping/polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP)
To do genotyping by RFLP, a region of 745 and 155 bp carrying the restriction sites of TaqI and BsmI, was amplified by PCR. The specific PCR products were digested with TaqI and BsmI restriction enzyme. For amplification of VDR gene segments containing TaqI and BsmI restriction sites, the published primers were used (Table 1).

PCR amplification was carried out using the Biorad DNA Engine Thermal Cycler (PTC200). The reaction mix was prepared using 50 ng of DNA, 10 pmol of both the forward (F) and reverse (R) primers (Sigma-Aldrich), 2 mM of dNTP’s (Sigma-Aldrich), 0.5U Taq polymerase (Sigma-Aldrich), and nuclease-free water was used to make volume to 20 µl. PCR conditions were initial denaturation at 95°C for 5 minutes; 30 cycles of denaturation at 95°C for 1 minute; annealing at 68°C/63°C (TaqI/BsmI) for 1 minute; extension at 72°C for 1 minute followed by final extension at 72°C for 5 minutes. The PCR product of 745 and 155 bp was digested with 10 unit of each TaqI (65°C for 4 hrs, Thermo Scientific) and BsmI (37°C for 4 hrs, Thermo Scientific) restriction enzyme, respectively. These digested products were then resolved in 4% agarose gel stained with ethidium bromide using gel electrophoresis system at 100 V for 1-2 hrs. The gel was visualized under ultraviolet light using BioRad gel doc system.

As per the different genotypes for each polymorphism, the expected size of fragments obtained after digestion with TaqI and BsmI are mentioned in Table 1.

Statistical analysis
The statistical analysis was performed using a commercially available software program (SPSS 21.0, SPSS Inc., Chicago, Illinois, USA). The difference in allelic and genotypic frequencies was compared, using the Chi-square (χ²) tests. Results were analyzed by calculating the p value and 95% confidence intervals. p values with <0.05 were considered to be statistically significant.

RESULTS
General characteristics of study population
The mean age of T2DM patients was 46.8±11.33 years, and it was found to be 39.3±11.12 years for the control group. Among all the T2DM patients, the average fasting glucose level was found to be 158.25±48.85 mg/dl, whereas postprandial plasma glucose level was 235.57±86.0 mg/dl.

Distribution of TaqI and BsmI polymorphisms in VDR gene
For TaqI polymorphism, genotype frequencies were TT (36%), TC (54%), and CC (10%) in patients and TT (50%), TC (37%), and CC (13%) in controls. It indicated that out of 3 genotypes, the heterozygous genotype was the most common in patients. On the other hand, the wild type genotype was the most common type in control. In both the groups, the mutant profile was less prevalent (Table 2).

As a result of statistical analysis, the frequency of TT and TC genotype of TaqI polymorphism showed a significant difference between patients and controls (p=0.023 and p<0.001, respectively). Whereas the frequency of mutated profile (CC) was not significantly different among these two groups, with χ²=0.442, p=0.506 (Table 2). After the comparison of the frequency of mutant allele (C), there was no significant difference observed between patients and controls (χ²=1.100, p=0.294) (Table 3).

Analysis of different genotypes of BsmI revealed that frequency of genotypes was GG (20%), GA (56%), and AA (24%) in patients and GG (9%), GA (77%), and AA (14%) for controls. Here again, the heterozygous genotype was most common in both the groups, but its frequency was higher in controls. Unlike TaqI, the least common genotype in both groups was found to be wild type genotype (Table 4).

As similar to TaqI polymorphism, the frequency of wild type (GG) and heterozygous genotype (GA) of BsmI was also found to be significantly different in patients and controls (χ²=4.48, p=0.027 and χ²=1.0945, p=0.001). However, the frequency of mutated profile (AA) was not different among these two groups of individuals (χ²=3.249, p=0.071) (Table 4). Moreover, allelic frequency of mutant allele (A) was also found to be same in patients and controls (χ²=0.20, p=0.687) (Table 5).

DISCUSSION
The importance of studying polymorphisms in the VDR gene initially emerges due to ethnic differences among different populations [18-20,31,32]. However, later it was also found to be associated with different metabolic diseases including diabetes [12-14,21-26]. As VDR gene is expressed in different types of tissues including pancreatic β-cells and adipocytes [33,34], which supports the idea that VDR polymorphisms possibly affects the insulin secretion and glucose metabolism [35,36].

In recent years, the relevance of VDR polymorphism has been investigated by a large number of studies in T2DM patients from different populations. The observations of different studies were not same. Most of the VDR gene polymorphism-related studies suggested a significant association between heterozygous and/or mutant genotype with different clinical parameters of diabetes such as glucose levels, obesity, and insulin level. For example, the study of BsmI polymorphism in T2DM patients done by Ortlepp et al. suggests a significant association of BsmI with fasting glucose levels of 1539 T2DM patients (p=0.018) [22]. Similarly, Oh et al. investigated BsmI polymorphisms and found a significant association with insulin levels of the 1,545 T2DM patients, p=0.05 [36]. A study by Al-Daghri et al. found that TaqI and BsmI polymorphisms are significantly associated with T2DM n=627, p=0.033 [37]. On the similar lines, Ogunkolade et al. found a significant association of TaqI and insulin secretion in 143 Bangladesh Asians (p<0.001) [32]. However, on the other hand, there are studies which contradict the same. For instance, the study by Bid et al., 2009

| SNP   | Position (bp) | SNP ID | Base change | Primer sequence | Annealing | Amplicon length (bp) | Genotype/RFLP product |
|-------|---------------|-------|-------------|----------------|-----------|----------------------|-----------------------|
| TaqI  | Exon 9 (65058) | rs731236 | T/C         | F5'CAGACCATGAGCAGGCGACCAA-3' | 68°C | 745 | TT/494, 251 |
|       |               |        |             | RS'GAACTCTCTGATGACTGGTCTC-3' [29] |         |                       |                       |
| BsmI  | Intron 8 (63980) | rs154410 | G/A         | F-5'GTGTCGAGGGCAGTCTGCTA-3' | 63°C | 155 | GC/293, 251, 201 |
|       |               |        |             | RS'TACCTGGCCGGAAGGAA-3'[30] |         |                       |                       |
Table 2: Distribution of TaqI polymorphism in T2DM patients and respective control

| Genotype | Patients (N=100) % | Controls (N=100) % | χ² | p value |
|----------|--------------------|--------------------|----|---------|
| TT (wild) | 36 (36)            | 50 (50)            | 5.195 | 0.023*  |
| TC (heterozygous mutant) | 54 (54) | 37 (37) | 37.72 | <0.001** |
| CC (mutant) | 10 (10) | 14 (14) | 3.442 | 0.506   |

Statistical evaluation was made using the Chi-square test, *p<0.05, **p<0.01.

T2DM: Type 2 diabetes mellitus

Table 3: Comparison of allelic frequencies of TaqI in T2DM patients and control

| Allele | Patients (%) | Controls (%) | p value |
|--------|--------------|--------------|---------|
| T      | 126 (63)     | 138 (69)     | 1.100   | 0.294   |
| C      | 74 (37)      | 62 (31)      |         |         |

Statistical evaluation was made using the Chi-square test. T2DM: Type 2 diabetes mellitus

Table 4: Distribution of BsmI polymorphism in T2DM patients and respective control

| Genotype | Patients (N=100) % | Controls (N=100) % | χ² | p value |
|----------|--------------------|--------------------|----|---------|
| GG (wild) | 20 (20)            | 9 (9)              | 4.880 | 0.027*  |
| GA (heterozygous mutant) | 56 (56) | 77 (77) | 10.945 | 0.001** |
| AA (mutant) | 24 (24) | 15 (15) | 3.442 | 0.506   |

Statistical evaluation was made using the Chi-square test, *p<0.05, **p<0.01.

T2DM: Type 2 diabetes mellitus

Table 5: Comparison of allelic frequencies of BsmI in T2DM patients and control

| Allele | Patients (%) | Controls (%) | χ² | p value |
|--------|--------------|--------------|----|---------|
| G      | 96 (48)      | 92 (46)      | 0.020 | 0.887   |
| A      | 104 (52)     | 108 (54)     |       |         |

Statistical evaluation was made using the Chi-square test. T2DM: Type 2 diabetes mellitus

observed a non-significant association between any of common VDR polymorphisms (FokI, BsmI, and TaqI) and risk of T2DM [27]. Malecki et al. also studied VDR polymorphisms (FokI, Apal, BsmI, and TaqI) in Polish population and showed no association of these polymorphisms with T2DM [25]. Cyganek et al. did the same study in 267 T2DM patients of Poland, and there was no association found with the T2DM [26]. In 1998, Hitman et al. studied Apal, BsmI, and TaqI in 164 T2DM patients and observed a non-significant association of BsmI and TaqI with T2DM [17].

In context to the Indian population, there is scarcity of the data which may support either of the view for VDR polymorphisms and its association with diabetes. Thus, in the current study, we have analyzed two common VDR gene polymorphisms; TaqI and BsmI in T2DM patients from North India. TaqI polymorphism is a silent substitution thus does not account for an amino acid change in VDR protein [32]. However, the wild type TT genotype of TaqI polymorphism is known to be associated with high copy number of VDR mRNA [17,32]. Hence, it may be hypothesized that the patients lacking wild genotype may have low copies of VDR gene transcripts which may probably affect level of VDR protein, and hence, the glucose metabolism too. In our study, the patient group had significantly a low number of wild type genotype TT, and it was found in only 36% of patients as compared to 50% of controls. The heterozygous TC genotype of TaqI polymorphism also shows a significant difference between T2DM patients and controls, further highlighting the possible role of mutant allele in determining the copy number of VDR gene transcript. The mutant profile (CC) of TaqI polymorphism was equally distributed in both the groups of individuals.

BsmI is an intronic polymorphism and proposed to affect the stability of VDR transcript [30]. It has also been demonstrated that an association exists between the BsmI polymorphisms of VDR gene and low insulin secretion in T2DM patients [17]. The study conducted by Speer et al. also showed that there is a link between heterozygous genotype of BsmI polymorphisms and T2DM in the Caucasian population [39]. In the similar context, we have also observed that the frequency of heterozygous GA genotype was significantly different among patients and controls (p=0.001). As similar to TaqI, we observed the significant difference between the frequency of wild type genotype between patients and controls, but surprisingly, the wild type genotype was higher in patients and heterozygous genotype was higher in controls. In addition, the mutant profile showed no difference between two groups of individuals as similar to TaqI data.

Comparison of frequency of mutant allele for both the polymorphisms indicated that there is no significant difference between patients and controls, however, to validate the same a large scale study design is required.

CONCLUSION

Both these polymorphisms are proposed to influence the transcription and/or stability of mRNA of VDR gene. Hence, based on our study and results from published literature, it can be proposed that TaqI and BsmI VDR polymorphisms may be associated with T2DM. Moreover, VDR signaling is also known to be important for insulin secretion and its action on the target cell. Hence, these polymorphisms may be suggested to be associated with altered insulin secretion and/or action, further contributing to the development of T2DM.

Although these two polymorphisms are not the only polymorphisms of VDR gene, known to be associated with T2DM, we propose to study the role of other VDR polymorphisms in these patients.

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