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Vitamin D Receptor Gene Polymorphism in Type 1 Diabetes Mellitus

Dr. Barkha Jain[1], Dr. Subhalakshmi Das[2], Dr. Gargi Choudhury[3], Dr. Neelakshi Bhattacharyya[4]

[1]Senior Resident, Department of Medicine, Assam Medical College and Hospital, Dibrugarh, Assam, India

[2] Professor, Department of Medicine, Assam Medical College and Hospital, Dibrugarh, Assam, India

[3]Associate Professor, Department of Microbiology, Assam Medical College and Hospital, Dibrugarh, Assam, India

[4]Scientist C, MRU, Department of Microbiology, Assam Medical College and Hospital, Dibrugarh, Assam, India

Abstract: ***Background: Polymorphisms within the VDR gene have been associated with altered gene expression or gene function. Vitamin D polymorphism is associated with the development and the severity of several autoimmune diseases such as RA, MS, SLE, IBDs, and T1DM. This study is designed to establish any potential association of VDR polymorphism in patients with type 1 DM. Methods: This study was a hospital based case control study done among 40cases and 40 healthy controls. Type1DM cases were selected based on ADA 2020 guidelines. Vitamin D receptor Polymorphism was assessed using ARMS PCR technique. Vitamin D levels were estimated using ELISA. The statistical analysis was done using SPSS 21.0, p value of <0.05 was considered significant. Results: The outcome parameter, VDR FokI genotypes (FF, Ff, ff) and BsmI genotypes (BB, Bb, bb) distribution and allele F, f; B, b frequency among cases and controls was analyzed. The distribution of FokI Genotypes (Ff, ff) was significantly different among T1DM cases and healthy controls. It was found that Ff genotype doubles the risk of T1DM when compared to other genotypes (p=0.045; OR=2.51). The distribution of BsmI genotypes (Bb and bb) was significant among cases and controls (p<0.05). It was found that bb genotype increases the risk of T1DM compared to other genotypes (p=0.003; OR=4.20). Conclusion: We found that vitamin D receptor gene (BsmI and FokI) polymorphism is associated with T1DM.***

Keywords: Type 1 Diabetes Mellitus, Vitamin D receptor (VDR), Polymorphsim, ARMS PCR

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder of great impact affecting people worldwide. It was first known to mankind dating from 1550 BC in ancient Egypt. [1, 2] DM is a group of heterogeneous metabolic diseases which is characterized by chronic hyperglycemia that results from defect in insulin secretion, action or both.3 Based on underlying pathology, majority of diabetic patients fall under 2 broad categories; Type 1 DM which is characterized by absolute insulin deficiency due to immune - mediated pancreatic beta - cell destruction and Type2 DM, characterized by peripheral insulin resistance and inadequate secretory response by pancreatic beta cells. Other remaining forms include Gestational diabetes, monogenetic form, and diabetes from secondary causes.4 Vitamin D is a major fat - soluble steroid hormone. Apart from playing an important role in bone metabolism it is also endowed with the capability of modulating inflammatory and immune function. The active form of vitamin D has important immunomodulatory properties and it regulates both innate and adaptive immunity. It inhibits activation of lymphocyte and also suppresses other elements of the immune system, like it prevents cytokine and immunoglobulin production, as well as expression of major histocompatibility complex (MHC) class II and cluster differentiation 4 (CD - 4).5, [6] Vitamin D exerts its action via the nuclear vitamin D receptor (VDR). The VDR belongs to the steroid receptor super - family and is widely expressed in many cell types, including lymphocytes, macrophages, and pancreatic cells.7 VDR gene is located on chromosome 12.8 VDR gene shows an extensive polymorphism. The most common type of genetic variation among people are single nucleotide polymorphisms, frequently called SNPs (pronounced "snips"). Each SNP represents a difference in a single DNA building block, called a nucleotide.9 Polymorphisms within the VDR gene have been associated with altered gene expression or gene function.1[0]With the help of associated restriction enzyme, five single nucleotide polymorphisms (SNP) in VDR gene have been defined historically. They are in exon 2 (FokI), intron 8 (BsmI, Tru9I, ApaI), and exon 9 (TaqI).1[1]Several studies have also been reported regarding relationship between low levels of vitamin D and the development and the severity of several autoimmune diseases such as RA, MS, SLE, IBDs, and T1DM. Association studies of VDR allelic variations and T1D has been done in many countries, including different populations however the results were inconsistent; some showed significant association while others failed to reach statistical significance. These different results are probably due to differences in ethnicity of the populations studied, genetic interactions or environmental factors involved in the pathogenesis of Type 1 DM.1[2]It is anticipated that this study may shed light on better understanding of pathogenesis of Type 1 DM and may be integrated in clinical practice to identify the at risk group and guide management decisions. This study is designed to establish any potential association of VDR polymorphism in patients with type 1 DM.

2. Methods

This was a hospital based observational case control study conducted for a period of one year from June 2020 to May 2021 which was carried out after obtaining approval from Institutional Ethics Committee. A total of 40 cases and 40 controls with age more or equal to 13 years unrelated Type 1

Diabetes Mellitus patients were selected. The subjects were pooled from those attending the Medicine Outpatient Department or the Diabetic clinic, and those admitted as inpatients at the Department of Medicine, Assam Medical College, Dibrugarh. Age and Sex matched unrelated subjects without Diabetes and without family history of diabetes were selected as control.

Criteria for the Diagnosis of Type 1 Diabetes Mellitus according to ADA 2020 guidelines of Stage 3 type 1 DM.1[3]All patients with sudden onset of serious symptoms or rapid progression to overt diabetes presenting as ketosis prone hyperglycemia and need of exogenous insulin from beginning of the disease with a fasting C - peptide level of below 5microunit/mL (0.6ng / ml).1[4] were taken for the study.

An elaborate history was taken in each patient with special reference to the points concerning age of onset of disease, age of first insulin administration, history of any autoimmune disease, complications of diabetes.

A detailed general physical examination was carried out. Systemic examination was done with weightage on every system affected by diabetes. Fundoscopy was done to gather evidence of premature cataract and retinopathy in every subject. The respiratory and gastrointestinal systems were also meticulously examined in each patient.

Technique of Laboratory Investigations:

- **Complete Blood Count:** It was performed using the Sysmex XS 800i autoanalyzer.
- **Urine routine examination**: It was done especially for sugar and albumin using UroColor 2 Urine Test Strips.
- **Renal Function Test**: Blood Urea was measured using the Modified Berthelot method. Serum Creatinine was measured by the alkaline Picrate method.
- **Estimation of Blood Glucose**: Glucose oxidase and Peroxidase Method.
- **Estimation of Glycated Hemoglobin (HbA1c):** High - performance liquid chromatography (HPLC assay) using the Variant Machine.
- **Serum Electrolytes**: Serum sodium, serum potassium estimation was done in the automated analyzer, Siemens Dimension RXL MAX auto - analyzer.
- **Fasting lipid profile**: It was measured in the Seimon's autoanalyzer.
- **Thyroid function test**: by radio - immuno essay.
- **C - peptide**: by ELISA using Robonik (Read Well) plate analyser.

Serum Vitamin D estimation:

25 (OH) D is the major circulating form of vitamin D, with a circulating half - life of 2–3 weeks while the circulating half - life of 1, 25 (OH) $_2$D is around 4 hours. It circulates at 1000 times lower concentration than 25 (OH) D, and the blood level is tightly regulated by serum levels of PTH, calcium, and phosphate. Serum 1, 25 (OH) $_2$D does not reflect vitamin D reserves, and measurement of 1, 25 (OH) $_2$D is not useful for monitoring the vitamin D status of patients. Serum 1, 25 (OH) $_2$D is frequently either normal or even elevated in those with vitamin D deficiency, due to secondary hyperparathyroidism. Thus, 1, 25 (OH) $_2$D measurement does not reflect vitamin D status. So 25 (OH) D is the best indicator to monitor for vitamin D status.

MicroO - D 25 - OH Vitamin D Sandwich ELISA kit from Affemedix Diagnostics was used for the study.

VDR Gene Polymorphism Detection

Two VDR gene polymorphism Fok I (F/f) and BsmI (B/b) was studied in all subjects using single ARMS PCR (Single Amplification Refractory Mutation System) technique. In most of the previous studies, VDR genotyping has been performed by polymerase chain reaction (PCR) - restriction fragment length polymorphism (RFLP) assays, which are cumbersome and time consuming, and their results are also sometimes difficult to interpret. One of the advances in DNA amplification technology was allele - specific PCR or the amplification refractory mutation system (ARMS).1[5] This technique is a rapid and specific test that was developed to detect known single - base substitutions or insertions.

The molecular study was done to detect VDR gene polymorphism in diabetic patients and healthy subjects. It included DNA extraction, Allele - specific PCR or the amplification refractory mutation system (ARMS) and Gel electrophoresis.

Principle of DNA Extraction: DNA is organized as chromosomes in an organelle called the nucleus. The basic principle of DNA extraction involves cell lysis using a proteolytic enzyme, DNA precipitation from cellular debris to make DNA free and its purification using alcohol.

Principle of Gel Electrophoresis: Agarose gel electrophoresis is a method used to separate DNA molecules based on their size. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field (electrophoresis). Shorter molecules move faster and migrate further than longer ones. DNA fragments are then visualized by staining with ethidium bromide. This fluorescent dye intercalates between the bases of DNA and fluoresces under UV light in Gel Documentation system.

Principle of PCR: PCR is a laboratory technique for DNA replication that allows a "target" DNA sequence to be selectively amplified. It involves the primer mediated enzymatic amplification of DNA using the ability of DNA polymerase to synthesize a new strand of DNA complementary to the offered target DNA sequence. Primer is needed because DNA polymerase can add a nucleotide only onto a preexisting 3′ - OH group to add the first nucleotide. The DNA polymerase then elongates its 3′ - end by adding more nucleotides to generate an extended region of double - stranded DNA. There are different types of PCR, commonly used are:

- Amplified fragment length polymorphism (AFLP) PCR
- Allele specific PCR or the amplification refractory mutation system (ARMS)
- Real - Time PCR (Quantitative PCR)
- Reverse Transcriptase PCR (RT - PCR)
- Variable Number of Tandem Repeats (VNTR) PCR

Principle of ARMS PCR: Allele specific polymerase chain reaction (AS - PCR) is a technique based on allele specific primers, which can be used to analyse single nucleotide polymorphism (SNP). It is also called the ARMS - PCR corresponding to the use of two different primers for two different alleles. One is the mutant set of primers which are refractory to the normal PCR, and the other is the normal set of primers, which are refractory to the mutant PCR reaction. The 3´ends of these primers are modified such that one set of the primer can amplify the normal allele while others amplify the mutant allele. This mismatch allows the primer to amplify a single allele.

Procedure of Molecular Study

Sample Collection: 2 ml of venous blood was collected in an EDTA vial for DNA extraction.

Storage of Samples: The samples were stored at the Multidisciplinary Research Unit, Department of Microbiology, Assam medical College. DNA was extracted from the samples within 24 hours of collection. DNA extracted from the samples were stored at a deep freezer at - 20° and - 80°C for short and long term storage respectively.

DNA Extraction from Whole Blood
DNA was extracted using the QIAmp DNA mini kit (QIAGEN) following manufacturer instructions. QIAamp DNA Mini Kit provides fast and easy methods for extraction of total DNA for reliable PCR. QIAamp DNA Mini Kit is designed for the rapid extraction of an average of 6 µg of total DNA from 200 µl of whole human blood. QIAamp DNA Mini Kit contains Proteinase K which is the optimal enzyme for use with the lysis buffer provided in Mini Kit. Proteinase K is completely free of DNAase and RNAase activity. The lysate buffering conditions were adjusted to allow optimal binding of the DNA to the QIAamp membrane before the sample was loaded onto the QIAamp Mini spin column. DNA was adsorbed onto the QIAamp silica membrane during brief centrifugation. Salt and pH conditions in the lysate ensured that protein and other contaminants, which could inhibit PCR and other downstream enzymatic reactions, were not retained on the QIAamp membrane.

The use of 2 different wash buffers, Buffer AW1, and Buffer AW2, significantly improved the purity of the eluted DNA. Wash conditions ensured complete removal of any residual contaminants without affecting DNA binding. Purified DNA was eluted from the QIAamp Mini spin column in a concentrated form in either Buffer AE.

DNA Yield and Purity Assessment
Absorbance Essay: The extracted DNA samples were quantified using spectrophotometry. The most common purity calculation is the ratio of the absorbance at 260nm divided by the reading at 280nm. A ratio of ~1.8 is generally accepted as pure DNA. A lower ratio indicates more contaminants are present.

Agarose Gel Electrophoresis: The quality of extracted DNA samples was checked in 0.8% agarose gel electrophoresis by comparing the band intensity. The brighter band suggests a high concentration of DNA and if the band is faint then the concentration is low.

Selection of Primer:
For detection of FokI and BsmI polymorphism, PCR primer sequences used.

ARMS - Polymerase Chain Reaction (PCR)
PCR involves the amplification of the target - specific VDR gene, using commercial Master mix and gene - specific primer.

In the single ARMS - PCR method, two complementary reactions for each polymorphism were performed; one contained a primer specific for the mutant allele and the other contained one for the wild - type allele. Both reactions used a common primer, a given number of base pairs away from the mutant/wild - type sequence. The mutant and wild type primers differed by a single nucleotide at the 3´end. Since Taq DNA polymerase lacks the 3´ to 5´ exonuclease activity, a mismatch at the 3´ terminal would reduce the efficiency of the extension. Genotyping was based on whether there was allele - specific amplification in one or both reactions. As an internal control, a pair of primers that amplified a 783 - bp constant region from the third intron of HLA - DRB1 gene was added to each reaction. The validity of amplification reactions was confirmed by size determination and sequencing of PCR products.

Procedure
- Gel tray was prepared by sealing the edges with the adhesive tape and the gel comb was placed in position.
- 2% agarose gel was prepared by adding 0.8g of agarose to 40 ml of 1X TAE buffer in a conical flask.
- The agarose was dissolved by heating in a microwave oven.
- The temperature of the solution was brought down to ~45 - 50°C.
- 3µl of Ethidium bromide was added into the solution and mixed well.
- The gel was slowly poured onto a gel casting tray and allowed to solidify.
- After solidification, the comb and the adhesive tape were carefully removed.
- The gel tray was kept inside the electrophoresis tank and 1X TAE buffer was added to a level that immerses the gel.
- 10µl of each sample was mixed with 3 µl of loading dye before loading into the wells.
- The molecular weight ladder was loaded into the first well, followed by the samples.
- The power pack was connected to gel tank and electrophoresis was carried out at 60V for 30 min.
- At the end of the run, the power supply was disconnected.
- The gel was removed from the electrophoresis tank, placed inside a gel documentation system.

Interpretation of Electrophoresis Results:
A band only in the wild - type reaction indicated a homozygote for the wild - type allele, a band only in the mutant reaction indicated a homozygote for the mutant

allele, and a band in both reactions indicated a heterozygote state. Each reaction contains a control amplicon (internal control; 783 - bp, behind the specific products), verifying successful amplification, with or without an allele - specific product. (Figure 1 and 2)

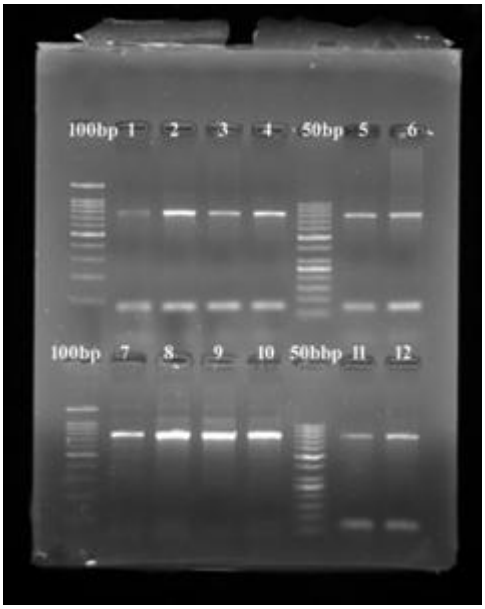

Figure 1: Agarose gel electrophoresis (2%) of ARMS - PCR technique of the amplified FokIgenotypes. The above lanes 1, 2, 3, 4, 5, 6 represents F + control and lanes 7, 8, 9, 10, 11, 12 represents f+ control. Lane 1, 7; 2, 8; 3, 9; 4, 10 shows homozygous FF genotype. Lane 5; 11, 6; 12 shows Ff genotype.

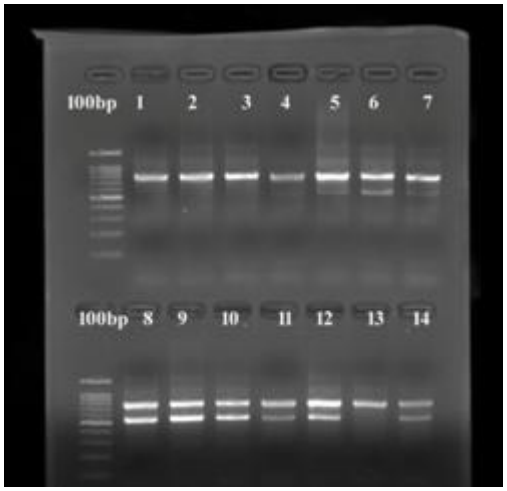

Figure 2: Agarose gel electrophoresis (2%) of ARMS - PCR technique of the amplified BsmIgenotypes. The above lanes 1, 2, 3, 4, 5, 6, 7 represents B+control and lanes 8, 9, 10, 11, 12, 13, 14 represents b+control. Lane 1, 8; 2, 9; 3, 10; 4, 11 shows homozygous bb genotype. Lane 5; 12, 7; 14 shows Bb genotype and lane 6, 13 shows BB homozygous.

3. Statistical Analysis

The data recorded on predesigned and pretested proforma was tabulated and the master chart was prepared. Microsoft Word and Excel were used to generate graphs and tables. All data were analyzed by specific statistical methods applicable to the various sets of data. The Hardy - weinberg equilibrium was performed using chi - square test by comparing the observed to the expected genotype frequencies. Other tests employed were Student t - test, ANOVA for quantitative data, and for qualitative data Fischer's exact test and Chi - square test was used. The association between the genotypes or variant alleles and their susceptibility to disease was assessed, and odds ratio (OR) with 95% confidence intervals and chi - square tests were calculated. The computer program, Statistical Package for Social Sciences (SPSS for Windows, version 21.0 Chicago, SPSS inc.) was used for analysis of data. P - value of <0.05 was considered statistically significant.

4. Results

Table 1: Age Distribution

Age of onset (in years)	Mean±SD	p - value
8 - 15	12.40±2.14	<0.0001
16 - 24	19.88±2.13	
25 - 32	26.25±1.50	

From Table1, it was seen that the majority of the subjects in both cases and controls (19 and 21) respectively belonged to 18 - 23 years of age group (47.5% and 52.5% respectively).25% of cases and 17.5% of control were in 12 - 17 years of age group.22.5% of both cases and controls were in the age group (24 - 29) years. The mean age for all cases was 20.4±5.12 years, whereas the mean age for all controls was 21.55±5.03 years. There was no significant difference in age distribution in both the groups.

Table 2: Age of onset of disease

Age	Case (N)	%	Control (N)	%	p - value
12 - 17	10	25	7	17.5	0.31383476
18 - 23	19	47.5	21	52.5	
24 - 29	9	22.5	9	22.5	
30 - 36	2	5	3	7.5	
Total	40	100	40	100	
Mean±SD	20.4±5.12		21.55±5.03		

Table 2 shows that the difference in mean age of onset of disease in different age group was significant. The mean age of onest in 8 - 15 years of age group was 12.40±2.14, in 16 - 24 years of age group it was 19.88±2.13 and in 25 - 32 years of age group it was 26.25±1.50. the overall mean age of onset was 16.77±5.20.

Table 3: Gender distribution

Sex	Case (N)	%	Control (N)	%
Male	20	50	21	52.5
Female	20	50	19	47.5
Total	40	100	40	100

From Table 3 it is observed that the percentage of the male and female in the cases group was 50% for both, whereas, in the controls group, 52.5% and 47.5% was the percentage of males and females respectively. The male: female ratio in the case and control groups was 1: 1 and 1.1: 1 respectively.

Table 4: Distribution of genotype of FokIpolymorphism of VDR in case and control

Genotype	Case		Healthy Control		p – value	Odds Ratio
	N	%	N	%		
FF	13	32.5	8	20	0.207	1.92
Ff	23	57.5	14	35	0.045	2.51
ff	4	10	18	45	0.001	0.135

The table 4 showed that out of 40 cases and 40 controls, 13 (32.5%) cases and 8 (20%) controls had homozygous FF genotype, 23 (57.5%) cases and 14 (35%) controls had heterozygous Ff genotype, whereas 4 (10%) cases and 18 (45%) controls had ff genotype. The difference in genotype distribution among cases and controls was significant (p value= 0.0145). The genotype distribution of FokI VDR gene was in Hardy Weinberg Equation (HWE) in both T1DM cases and the Healthy controls group.

Table 5: Distribution of genotype of BsmI polymorphism in case and control

Genotype	Case		Healthy Control		p – value	Odds Ratio
	N	*%*	*N*	*%*		
BB	7	17.5	13	32.5	0.126	0.44
Bb	11	27.5	18	45	0.007	0.25
Bb	22	55	9	22.5	0.003	4.2

The table 5 showed that out of 40 cases and 40 controls, 7 (17.5%) cases and 13 (32.5%) controls had homozygous BB genotype, 11 (27.5%) cases and 18 (45%) controls had heterozygous Bb genotype, whereas 22 (55%) cases and 9 (22.5%) controls had bb genotype. The difference in genotype distribution of Bb and bb among cases and controls was significant (p value= 0.007 and 0.003). The genotype distribution of BsmI gene was in Hardy Weinberg Equation (HWE) in both T1DM cases and the Healthy controls group.

5. Discussion

In this study we observed that out of 40 Type 1 DM cases, 13 subjects (32.5%) had FF homozygous genotype, and 27 subjects (67.5%) had Ff/ff genotype. Out of these 27 subjects, 23 (57.5%) had Ff genotype and 4 (10%) had ff genotype. Amongst the 40 healthy controls, 8 subjects (20%) had F homozygous FokI gene, and 32 subjects (80%) had Ff/ f homozygous. Out of these 32 subjects, 14 (35%) had Ff heterozygous and 18 (45%) had ff genotype. Regarding the BsmI, we found that 7 cases (17.5%) had BB homozygous genotype and 33 cases (82.5%) had Bb/bb genotype. Out of these 33 subjects the percentage of Bb and bb genotype was 27.5% and 55% respectively. Whereas 13 (32.5%) of controls had homozygous BB genotype, 18 (45%) had Bb and 9 (22.5%) had bb genotype. The genotype distribution of both FokI and BsmI gene polymorphism was in Hardy Weinberg Equation (HWE) in both T1DM cases and the Healthy controls group. On analysis of Genotypes (FF, Ff, ff) distributions, the distribution of heterozygous Ff and homozygous ff among cases and control was significant ($p<0.001$). It was found that the heterozygous Ff genotype doubles the risk of T1DM when compared to other genotypes ($p=0.045$; OR=2.51). Whereas the distribution of Bb and bb among cases and controls was significant ($p<0.05$). It was found that bb genotype increases the risk of T1DM compared to other genotypes ($p=0.003$; OR=4.20). The frequency of (F) allele was 61.25% and 37.5% among T1DM cases and controls respectively, whereas (f) allele frequency among cases and controls was found to be 38.75% and 62.5% respectively. Whereas the frequency of (B) allele was 31.25% and 55% in cases and control respectively and (b) allele frequency in cases and control was found to be 68.75% and 45% respectively. Our study shows that FokI (F) allele and BsmI (b) alleles increases the risk of T1DM. Previous studies had supported the role of FokI and BsmI gene polymorphism in development of T1DM, but the results were inconsistent. Positive association of FokI and BsmI polymorphism with risk of T1DM was reported by Rabab Ali *et al*[16] (2018) in Saudi population T1DM cases showed a significant increased frequency of the heterozygous genotype (Ff) than controls (33% vs 21%, OR = 1.9, 95% CI = 1.006 - 3.587, P =.04). On the other hand, cases showed significantly higher frequency of the BsmI homozygous (bb) and heterozygous (Bb) genotypes (25% vs 11.8%, P =.01, OR = 2.5, 95% CI = 1.18 - 5.31) & (45% vs 27.5%, P =.0, OR =2.1, 95 % CI = 1.20 - 3.89, respectively). The frequency of (b) allele was high in cases than controls. The findings in this study are in agreement with our study. S. H Abd - Allah*et al*[17] (2014), their study indicated that vitamin D deficiency and VDR BsmI and FokI polymorphisms were associated with T1DM in Egyptian children. They found that VDR BsmI Bb and bb genotypes and VDR FokI Ff and ff genotypes were associated with increased risk of T1DM (OR=2.3, 95% CI=1.3 - 4.2, P=0.005; OR=2.2, 95% CI=1.1 - 4.7, P=0.04; OR=1.8, 95% CI=1.03 - 3.04, P=0.04; OR=4.03, 95% CI=1.2 - 13.1, P=0.01 respectively). The findings are similar to our study. However the association of BsmI and T1DM was not reported in studies by M. A Rasoul*et al*[18] (2019). This finding is not in agreement with our study probably due to different Ethnicity and demographic data. In a meta analysis by N. Zhai *et a1*[19] (2020) the results of overall population rejected any significant association between VDR gene polymorphisms and T1DM risk. However, the pooled results of subgroup analysis revealed significant negative and positive associations between FokI and BsmI polymorphisms and T1DM in Africans and Americans, respectively. This is probably due to difference in demographic and ethnic variation.

6. Conclusion

In this case - control study done in North - East population of India, comprising 40 cases of T1DM and 40 Healthy controls, we found that vitamin D receptor gene (BsmI and FokI) polymorphism is associated with T1DM So we conclude that our study provides evidence regarding association between FokI and BsmI polymorphism of VDR gene and susceptibility to T1DM. While the underlying pathophysiologic mechanism explaining how genetic factors play a role in development of T1DM is not clearly understood, the involvement of VDR gene polymorphism may be a significant factor. So our findings reinforce the need for further association studies with larger subjects in order to replicate such findings. Furthermore, conflicting results from various studies on different populations make the association of VDR gene BsmI and FokI polymorphism

in T1DM still debatable. So a large case control study including T1DM and healthy individuals would provide a clearer picture on the association of VDR gene polymorphism and susceptibility to T1DM.

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