RESEARCH ARTICLE

Evaluation of vitamin D receptor gene polymorphisms (*Fok-I* and *Bsm-I*) in T1DM Saudi children

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Background: Vitamin D deficiency conferred strongest susceptibility to pathogenesis of type 1 diabetes mellitus (T1DM). Altered gene expression and function have strong effect on VDR gene polymorphism.

Objectives: We aimed to check for the association of two single nucleotide polymorphisms (SNPs) in *VDR* gene (*Fok-I* and *Bsm-I*) with T1DM in Saudi children.

Subjects and Methods: Cross-sectional study included 100 T1DM Saudi children, plus 102 unrelated healthy subjects. PCR technique was used for detection of *Fok-I* and *Bsm-I* SNPs in *VDR* gene.

Results: Regarding the *Fok-I* polymorphisms, 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, $P = .04$). In the meantime, they showed significantly lower frequency of the homozygous (ff) genotype (64% vs 79%, OR = 0.51, 95% CI = 0.28-0.96, $P = .03$). Cases showed also a significantly lower frequency of the (f) allele than controls (80.5% vs 87.7%, OR = 0.57, 95% CI = 0.33-0.995, $P = .04$). On the other hand, cases showed significantly higher frequency of the *Bsm-I* 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). Cases showed also significantly higher frequency of (b) allele compared to control (47.5% vs 25.5%, $P = .0$, OR = 2.6, 95% CI = 1.74-4.02). Haplotype analysis showed an increased risk with the fB and fb haplotypes.

Conclusion: This study emphasizes a positive association between SNPs (*Fok-I* and *Bsm-I*) and T1DM among Saudi children with increased risk with the *Fok-I* F and *Bsm-I* b alleles.

KEYWORDS

Bsm-I, *Fok-I*, SNPs, type 1 diabetes mellitus, *VDR* gene

1 | INTRODUCTION

One of the major health problems in the kingdom Saudi Arabia is diabetes; it is ranked third worldwide.¹ The highest frequency of childhood diabetes is found in Al-Madinah city compared with other areas such as the Mediterranean Sea and the Middle East.²

One of the autoimmune diseases is T1DM that occurred in the pancreatic islets.¹ Pancreatic β cells express vitamin D receptor (VDR).³

The immune system is modulated by vitamin D, and its action is labor via the vitamin D receptor.⁴

Vitamin D contribute in cell regulation and differentiation.^{5,6} Hence, the immune-mediated destruction of β -cells insulin-producing influenced by deficiency of vitamin D. Moreover impairment of insulin secretion.⁷⁻⁹

Vitamin D deficiency is peculiar in Kingdom Saudi Arabia, especially children and adolescents.¹⁰ The high prevalence linked to low

exposure of skin to sunshine, black pigmentation, and lack supplementation of vitamin D,^{11,12} especially potential inherited factors like VDR polymorphism mutations.¹³

VDR gene included single nucleotide polymorphisms (SNP) which is located at chromosome 12q12-q14. One of them is Fok-I (rs10735810) in exon 2, and due to G/A change, the second one Bsm-I (rs1544410) in intron 8 results G/A change.¹⁴ Alteration in sequence of the initiation site leads to shorter protein in three amino acids, which believed to be more efficient than the wild type.¹⁵ Bsm-I (b) site is in introns, but change in intronic sequence may impact on protein expression. Despite that the functional effect of intronic Bsm-I SNPs still vague,¹⁵ the importance of these SNPs can be helpful as markers to other functional allele exist within this gene site.¹⁵

This study aimed to check for the association of two single nucleotide polymorphisms (SNPs) in VDR gene (Fok-I and Bsm-I) with T1DM in Saudi children.

2 | MATERIALS AND METHODS

2.1 | Subjects

This work is a case-control study that involved 100 Saudi children affected with T1DM (54 boys and 46 girls with mean age 10.33 ± 3.15 years with an age range of 2.3 and 17 years). Patients were selected according to the multinational project for childhood diabetes criteria for the diagnosis of diabetes World Health Organization (WHO).¹⁶ Cases were compared with 102 unrelated healthy adult volunteers (43 males and 59 females, ages above 35 years) as control group. We selected adult controls above 35 years with no type 1 diabetics. They were recruited from maternity and Children's Hospital, Al-Madinah, Saudi Arabia. The local ethics committee of hospital approved the project. This work has been performed in laboratories of Faculty of Applied Medical Science at Taibah University, Saudi Arabia. The age of onset of the disease and the first occurrence of symptoms were collected from the patient's medical records. All patients received a standardized well-accepted drug regimen. Exclusion criteria included cases with a clinically significant nephropathy or other complication.

2.2 | Detection of VDR gene polymorphisms (Fok-I and Bsm-I)¹⁷

EDTA tube was used for peripheral blood collection, and the generation DNA purification capture column kits (Gentra-system, USA) was used to extract DNA. Amplification of DNA by PCR technique

was carried out to The FOK-I and BSM-I polymorphism in exon 2 and intron 8, respectively. For Bsm-I (rs1544410) (A/G) polymorphism, each PCR was carried out in total volume of 25 μ L, including 10 μ L PCR Master Mix (2x) (Hotstart, ACE- BIOTECH, cat.#11M001-A, 11M001-B, 11M001-C), 8 μ L PCR distilled water, 2 μ L of each primer, and the senses primer in exon7 (5-CAACCAA GACTACAAGTACCGCGTCAGTGA-3), antisense primer in intron 8 (5-AACCAGCGGGAAGAGGTCAAGGG-3) (Bio-Basic Inc., Canada) and (3 μ L) extracted DNA. PCR program is shown in Table 1. After amplification, FLEP technique was carried out using restriction endonuclease BSM enzyme, digestion for PCR product of 850 bp was carried out through incubation at 37°C overnight and then electrophoresed in 1.5% agarose gel and UV trans-illuminator for visualization. Digestion products are of two fragments such as 650 and 200 bp for bb homozygous, homozygous BB for the absence of site give one band at 850 bp and heterozygous Bb at 850, 650, 200 bp (Figure 1).

The same steps were carried out for FOK-I (rs10735810) (C/T) polymorphism using the following primer: VDR-2a (5'-AGCTGGCCCT GGCCTGACTCTTGCTCT-3') & VDR-2b (5'-ATGGAAACACCTTGCTT CTTCTCCCTC-3') (Bio-Basic Inc., Canada). Amplification product was 265 bp, and PCR conditions are shown in Table 2. Digestion for PCR products was done using FOK-I endonuclease enzyme overnight at 37°C. Electrophoresis in 2% agarose gel. Homozygous FF genotype lacked a FOK-I restriction site and give 265 bp. Homozygous ff genotype gives 196 and 69 bp bands; while heterozygote Ff represents 265, 196, and 69 bp (Figure 2).

2.3 | Statistical analysis

Data were done by excel program (Microsoft Office; 2013), SPSS program version 20 (SPSS, Inc, Chicago, IL). Qualitative data were offered as frequency. Comparison between groups is performed using chi-square and Fisher's exact tests. While quantitative data were used to calculate mean and standard deviation, and Kolmogorov-Smirnov test used for normality of data. Student's *t* test and ANOVA tests were used to compare groups. Chi-squared test was used for Hardy-Weinberg equilibrium expectations. The Haplo-View program (version 4.2) used for estimation of haplotypes and linkage disequilibrium (LD),¹⁸ which uses the expectation maximization (EM) algorithm.

3 | RESULTS

This study represents 100 diabetic Saudi children (47 boys and 53 girls; means ages 10.33 ± 3.15 years), plus 102 healthy subjects unrelated

Cycle (s)	Denaturation		Annealing		Extension	
	Time	Temperature	Time	Temperature	Time	Temperature
1	5 min	94°C	0	25°C	0	25°C
2-37	1 min	94°C	1 min	64°C	90 s	72°C
38	-	-	-	-	10 min	72°C

TABLE 1 PCR program for BSM-I (rs1544410) (A/G) polymorphism

FIGURE 1 Agarose gel electrophoresis (2%) of PCR-RFLP technique of the amplified Bsm-I genotypes. Lane 1 shows homozygous (bb) genotype (mutation), where b allele at 650 and 200 bp. Lanes 2, 3, and 7 show heterozygous pattern (Bb) genotype at 850, 650, and 200 bp. Lanes 4, 5, and 6 show (BB) homozygous for the absence of mutation; where B allele at 850 bp

individuals of nearly matched age and sex as control group (29.4% males and 70.6% females); the main demographic and clinical features data are summarized in Table 3.

Regarding Fok-I polymorphisms, 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, $P = .04$). In the meantime, they showed significantly lower frequency of the homozygous (ff) genotype (64% vs 79%, OR = 0.51, 95% CI = 0.279-0.961, $P = .03$). Cases showed also a significantly lower frequency of the (f) allele than controls (80.5% vs 87.7%, OR = 0.57, 95% CI = 0.334-0.995, $P = .04$). On the other hand, cases showed significantly higher frequency of the Bsm-I homozygous (bb) and heterozygous (Bb) genotypes (25% vs 11.8%, $P = .01$, OR = 2.5, 95% CI = 1.177-5.311) and (45% vs 27.5%, $P = .0$, OR = 2.1, 95% CI = 1.203-3.888, respectively). Regarding allele frequency (b), allele showed higher frequency among cases compared to control (47.5% vs 25.5%, $P = .0$) with high-risk factor (OR = 2.6, 95% CI = 1.738-4.024) (Table 4).

Combined Bsm-I and Fok-I haplotypes (Fb and fb) significantly showed higher frequency, while fB showed significantly lower frequency among cases compared to control. After 10 000 permutations, only fB and fb haplotypes showed still significant levels (Table 5). As we observed that cases with DM type 1 had higher frequencies of the Fb and fb haplotypes than controls, we were interested to compare those who are carriers of these haplotypes vs others in relation to their clinical diameters including age, gender, BMI, and Hb A1C level. These data are represented in

TABLE 2 PCR program for FOK-I (rs10735810) (C/T) polymorphism

Cycle(s)	Denaturation		Annealing		Extension	
	Time	Temperature	Time	Temperature	Time	Temperature
1	5 min	94°C	0	25°C	0	25°C
2-37	30 s	94°C	30 s	61°C	1 min	72°C
38	-	-	-	-	10 min	72°C

FIGURE 2 Agarose gel electrophoresis (2%) of PCR-RFLP technique of the amplified Fok-I genotypes. Lanes 1, 5, 6, and 7 showed heterozygous pattern (FF) genotype, where F allele at 265 bp. Lane 2 showed (Ff) genotype at 265, 196, and 69 bp

TABLE 3 Clinical data (means $\pm$ SD) of studied cases and controls

	Cases N = 100	Controls N = 102
Age (y); mean $\pm$ SD	10.33 $\pm$ 3.15	35 $\pm$ 5.8
Age of onset (y); mean $\pm$ SD	7.32 $\pm$ 2.98	-
Gender; N(%)		
Males	47 (47)	43 (42.2)
Females	53 (53)	59 (57.8)
BMI; N (%)		
Underweight	12 (12)	
Normal	63 (63)	102 (100)
Overweight	12 (12)	
Obese	13 (13)	
HbA1c (%); mean $\pm$ SD	10.40 $\pm$ 1.97	5.2 $\pm$ 1.3

Table 6. It showed a non-significant difference between cases carrying the haplotypes Fb and fb vs others that signifies a non-association of this polymorphism with the clinical presentation of patients.

4 | DISCUSSION

The molecular pathogenesis of T1DM is still not fully understood although it was speculated to be a product of interactive immune, genetic, and environmental factors.^{19,20} Studies pertinent to vitamin

	Cases N (%)	Controls N (%)	P	OR, 95% CI
<i>Fok-I</i>				
FF	3 (3.0)	2 (2)	.68	1.54 (0.25-9.46)
Ff	33 (33.0)	21 (20.5)	.04*	1.90 (1.01-3.59)
ff	64 (64.0)	79 (77.5)	.03*	0.51 (0.28-0.96)
F	39 (19.5)	25 (12.3)	.04*	1.734 (1.01-2.99)
f	161 (80.5)	179 (87.7)		0.57 (0.33-0.995)
HWE	$\chi^2 = 0.26, P > .05$	$\chi^2 = 0.19, P > .05$		
<i>Bsm-I</i>				
BB	30 (30.0)	62 (60.7)	<.001**	0.27 (0.15-0.496)
Bb	45 (45.0)	28 (27.5)	.00**	2.16 (1.20-3.89)
bb	25 (25.0)	12 (11.8)	.01*	2.50 (1.18-5.31)
B	105 (52.5)	152 (74.5)	<.001**	0.378 (0.25-0.58)
b	95 (47.5)	52 (25.5)		2.64 (1.74-4.02)
HWE	$\chi^2 = 0.96, P > .05$	$\chi^2 = 7.84, P < .05^*$		

Fok-I gene polymorphism; Alleles F, f are expressed in percentage. *Bsm-I* gene polymorphism; alleles B, b. P, probability; OR, odds ratio; CI, confidence Intervals; χ^2 , chi-square. $P < .001$ extremely significant, $P < .05$ significant. * = significance; ** = highly significance.

TABLE 5 Distribution of *Fok-I* and *Bsm-I* combined haplotypes among studied controls and cases

<i>Fok-I</i> and <i>Bsm-I</i> Haplotypes	Cases N = 100 Frequency	Control N = 102 Frequency	P	pc
FB	0.118	0.095	.447	0.8295
Fb	0.398	0.227	.026	0.0798
fB	0.407	0.650	<.001	<0.001
Fb	0.398	0.227	<.001	0.0004

pc: corrected after 10 000 permutations.

D and VDR genotype association with T1DM showed different results. Environmental factors like sun exposure were also speculated to be a determinant factor particularly in Saudi Arabia where deficiency of vitamin D was reported to be a public condition among children.²¹

In this current study, we investigated the association of the *Fok-I* and *Bsm-I* polymorphism of the VDR gene with T1DM among Saudi children. The present study revealed that *Bsm-I* (bb, Bb) genotypes and (b) allele were risk biomarkers for T1DM in Saudi children. Similar observation in T1DM from Barcelona²² and families of Southern Indian manifested by the discriminatory transmission of the b allele is found.²³ On the other hand, B allele has been shown to be responsible for T1DM from Taiwan.²² Similar observations were also noticed among Japanese and Dalmatia population.^{24,25} Regarding the *Fok-I*, we found that the heterozygous (Ff) genotype was a risk biomarker for T1DM that doubles the risk when compared to other genotypes. Meanwhile the (ff) genotype and (f) allele seemed to be responsible for

TABLE 4 Genotype and alleles frequency of vitamin D receptor among Saudi diabetic children compared to controls

TABLE 6 Clinical parameters of cases of DM type 1 carrying the VDR *Fok-I* and *Bsm-I* Fb or fb haplotypes compared to other cases

Parameter	Fb, fb carriers N = 73	Others N = 27	P
Age (y); Mean $\pm$ SD	10.5 $\pm$ 3.1	9.8 $\pm$ 3.3	.374
Age of onset (y) Mean $\pm$ SD	7.5 $\pm$ 2.4	6.7 $\pm$ 2.1	.260
Gender N (%)			
Males	32 (43.8)	15 (55.6)	.369
Females	41 (56.2)	12 (44.4)	
Weight N (%)			
Under weight	10 (13.7)	2 (7.4)	.705
Normal	46 (63)	17 (63)	
Overweight	9 (12.3)	3 (11.1)	
Obese	8 (11)	5 (18.5)	
HBA1c(%); Mean $\pm$ SD	10.4 $\pm$ 2.1	10.4 $\pm$ 1.9	.980

the prevention from T1DM. Similar finding was also reported among Uruguayan and Japanese population.^{4,26,27}

In addition, a meta-analysis study has reported that increased risk of type 1 diabetes linked to F and B alleles especially in increased regional winter UVR; while high percent of controls carrying *Bsm-I* B allele appears at decreased winter regional UVR.²⁸ On the contrary of our finding, a study among Italian population reported that the ff genotype has been associated with T1DM.²⁹ Also, no associations were detected in the Portuguese, the Finnish population, and other population.³⁰⁻³²

Another study among Turkish, although it is a sunny country, researchers did not prove any difference in the F and B allele frequencies between T1DM cases and healthy ones.³²

These data might point to the potential impact of vitamin D deficiency associated with mutant VDR receptor gene on the development of T1DM. In fact, many observational studies have confirmed the possible association between vitamin D deficiency and T1DM.^{33,34} Other studies revealed that there is a link between serum 1, 25(OH) 2 D3 levels, and B/b alleles of VDR gene.^{34,35} In Bangladeshi subjects, reduced levels of vitamin D were noticed among the most vulnerable to T2DM.³⁶

Our study also showed that SNPs *Bsm-I*, *Fok-I*, haplotypes had moderate linkage disequilibrium, in both case and control subjects. Our study represented that the combined *Bsm-I* and *Fok-I* haplotypes (Fb and fb) significantly showed high frequency, but after 10 000 permutation, only (fB and fb) haplotypes preserved their significance.

The importance of VDR gene role in T1DM has also been stressed upon in some other previous research publications.^{17,37} Some studies proved that the affected Indian Asians offspring have discriminatory transmission of VDR b allele.¹⁷ The same was reported in a previous study among German population that revealed that SNPs *Bsm-I*, *Apa-I*, and *Taq-I* were in linkage disequilibrium.^{9,37} While, no association proved among broad familial studies in UK.³⁸

There was different estimation for VDR haplotypes combination.^{22,38,39} Genotype combinations BBAAtt have been proposed to be the strongest vulnerability to T1DM among Dalmatian population from South Croatia.²⁶ Interestingly, among German, BAT haplotype was detected to be a risk factor.⁴⁰ In Santiago, Chile population the haplotype combination BAT has higher frequency among cases while bAT was more prevalence among control; according to genotype combination association was found of AabbTT and aabbTT.²⁴

Although this study had some limitations particularly concerning the relatively small sample size together with a lack of an actual estimated levels of vitamin D metabolites and lack of genetic information concerning Saudi population, we could come to a conclusion of a definite association between the VDR gene included SNPs (*Bsm-I*) and T1DM especially (b) allele. SNPs *Bsm-I*, *Fok-I* haplotypes had moderate linkage disequilibrium, in both case and control subjects. Combination of genotypes haplotypes (fb) preserved their significance after 10 000 permutations. We recommend further wide scale studies involving multicenter interaction extending the work to all VDR polymorphisms and vitamin D estimation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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