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Vitamin D Receptor Gene Polymorphism FokI (rs2228570) among Egyptian Children with Type 1 Diabetes Mellitus

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ABSTRACT

Background: T1DM is an autoimmune disease marked by pancreatic beta-cell destruction, causing lifelong insulin dependence. While often diagnosed in childhood, 25% of cases occur in adults. Egypt ranks among top ten countries globally for diabetes prevalence (ages 20–79). Human VDR gene has over 14 polymorphisms; among them, FokI (rs2228570) has been studied for its potential role in T1DM, as vitamin D signaling influences insulin receptor gene expression through VDR interactions.

Objective: This study aims to investigate the association between the VDR gene FokI (rs2228570) polymorphism and susceptibility to T1DM among Egyptian children.

Subjects and methods: This case-control investigation assessed association between FokI (rs2228570) polymorphism and T1DM in 200 Egyptian children (100 with T1DM and 100 healthy controls) aged 1 month to 18 years. Participants were recruited from Mansoura University Children's Hospital between October 2023 and September 2024. Genotyping analysis was carried out utilizing real-time PCR technique.

Results: The mean ages of cases and controls were 9.48 ± 3.22 and 8.85 ± 3.71 years respectively ($p=0.216$) with comparable sex distributions ($p=0.322$). Genotype frequencies (GG/AG/AA) were 51%/45%/4% in cases and 59%/37%/4% in controls ($p>0.05$). Allele distributions showed no substantial variations (G: 73.5% vs. 77.5%; A: 26.5% vs. 22.5%; $p>0.05$). Both groups were in Hardy-Weinberg equilibrium.

Conclusion: FokI (rs2228570) polymorphism was not substantially correlated with T1DM in Egyptian children, indicating it may not play a major role in genetic susceptibility to T1DM in this population.

Keywords: Type 1 diabetes mellitus, Vitamin D receptor (VDR), FokI polymorphism, Genetic association.

INTRODUCTION

T1DM represents an autoimmune disorder distinguished by destruction of pancreatic beta cells, which are essential for insulin synthesis, thereby necessitating lifelong insulin therapy. Although T1DM typically presents during childhood, approximately 25% of individuals receive their diagnosis in adulthood. Despite rising prevalence of T2DM, T1DM remains more frequent form of diabetes in younger populations [1].

Egypt is encompassed within 21 countries and territories delineated by IDF Middle East and North Africa Region. It is distinguished as one of top ten nations globally with respect to prevalence of diabetes among adults aged 20 to 79 years, as well as absolute number of adults diagnosed with diabetes. In 2024, estimates indicated that approximately 69,281 individuals aged 0 to 19 years would be diagnosed with T1DM, within an age group population totaling 48,037,938, corresponding to a prevalence rate of about 0.14%. VDR, a component of steroid/thyroid hormone receptor superfamily, is encoded by VDR gene positioned at 12q13.11 on chromosome 12. This genomic region spans roughly 100,000 base pairs, with approximately 4,600 base pairs dedicated to coding VDR protein. Functioning as a ligand-regulated transcription factor, VDR activity may also be modulated by phosphorylation [2].

Human VDR gene harbors more than 14 recognized polymorphisms, each of which may modulate physiological response to vitamin D (calcitriol). Among these genetic variants, four SNPs have been focus of extensive research: ApaI (rs7975232), FokI (rs2228570), TaqI (rs731236), and BsmI (rs1544410) [3].

The mechanism through which vitamin D augments insulin sensitivity involves binding of $1,25(OH)_2D_3$ to VDR within insulin-responsive cells. The resulting $1,25(OH)_2D_3$-VDR complex subsequently associates with RXR. This combined complex binds to VDREs within promoter region of human insulin receptor gene, consequently facilitating augmented synthesis of IRs and upregulating transcription of insulin receptor genes [4]. Vitamin D exerts its effects through VDRs, which are expressed in numerous tissues, including pancreatic β cells. This distribution indicates that vitamin D may directly influence insulin response to hyperglycemia, as well as indirectly affect it by regulating calcium homeostasis. Four prevalent SNPs within VDR gene, located on chromosomes 12 and 14, have been identified: TaqI (rs731236), ApaI (rs7975232), BsmI (rs1544410) and FokI (rs2228570). Despite extensive investigations, specific relationship between T1DM and

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these VDR gene polymorphisms has yet to be conclusively determined [5].

This study aims to investigate the association between the VDR gene FokI (rs2228570) polymorphism and susceptibility to T1DM among Egyptian children.

PATIENTS AND METHODS

Study design: This case-control study investigated the association between FokI (rs2228570) polymorphism and T1DM in a cohort of 200 Egyptian children (100 cases with T1DM and 100 controls). Participants were recruited from endocrinology outpatient clinics over a one-year period from October 2023 to September 2024, while controls were enrolled from general outpatient clinics during visits for routine follow-up or minor infections. Both groups belonged to same ethnic background. Their ages ranged from 1 month to 18 years of both sexes. Diagnosis of T1DM was based on ISPAD criteria, and all controls shared same ethnicity [6].

Exposure data: FokI (rs2228570) polymorphism in VDR gene, also termed SCP, was first identified by RFLP analysis using FokI enzyme. FokI, positioned in exon 2, is a non-synonymous SNP that modifies amino acid sequence of VDR protein. C-to-T nucleotide substitution (ancestral T allele) results in a threonine-to-methionine change generating two alternative translation initiation sites and producing protein isoforms of differing lengths [7]. FokI restriction site (C allele) creates a novel start codon 9 bp downstream and produces a truncated 424-amino-acid VDR isoform with increased transactivation activity. In contrast, absence of FokI (T allele) leads to translation from canonical start site, yielding full-length 427-amino-acid VDR isoform with ~1.7-fold lower transactivation capacity and reduced stability [8].

Vitamin D may confer protection against T1DM by exerting immunomodulatory effects. It effectively inhibits activation of macrophages, restrains maturation of antigen-presenting cells, and suppresses differentiation of dendritic cells. These mechanisms regulate cytokine secretion and suppress expression of HLA class I molecules and Fas, thereby mitigating destruction of pancreatic β-cells [9]. While HLA class I and II loci are well established as major determinants in pathogenesis of T1DM, non-HLA genes also exert substantial influence. These include genes encoding proinflammatory cytokines (TNF-α, IL-1, interferon-γ, IL-6), transforming growth factor-β, anti-inflammatory cytokines (IL-13, IL-12, IL-10), as well as vitamin D-related factors (VDR, CYP27B1, CYP24A1, CYP2R1), all of which have been implicated as high-risk factors [10].

When vitamin D binds to its receptor, a member of nuclear hormone receptor superfamily, it forms a complex with RXR. This complex attaches to VDREs located within promoter regions of vitamin D-responsive genes and, through recruitment of co-activators or co-repressors to these VDREs, regulates transcriptional activity of corresponding target genes [11]. VDR gene is located on chromosome 12 at position 12q13.11 and spans approximately 75 kilobases of genomic DNA, comprising nine exons. The protein-coding region, primarily exons 2 through 9, encodes 427 amino acids that generate multiple VDR protein isoforms. These isoforms include distinct domains responsible for DNA binding (encoded by exons 2 and 3) and vitamin D binding (encoded by exons 5 through 9) [12]. The mechanism through which vitamin D enhances insulin sensitivity entails the binding of $1,25(OH)_2D_3$ to VDR within insulin-responsive cells. The resulting $1,25(OH)_2D_3$-VDR complex associates with RXR, and this combined complex attaches to VDREs in promoter region of human insulin receptor gene, thereby augmenting IR production and stimulating transcription of insulin receptor genes [4].

Outcome data: Genetic predisposition to T1DM was assessed through a case-control study conducted among Egyptian children, including 100 cases with T1DM and 100 controls. Genotyping for vitamin D receptor gene polymorphism FokI (rs2228570) was carried out using TaqMan SNP Genotyping Assay. The findings demonstrated no statistically significant link between FokI genotypes and T1DM susceptibility. Genotype distributions were 59% GG, 37% AG, and 4% AA in controls, compared to 51% GG, 45% AG, and 4% AA among T1DM cases, with no meaningful differences observed between the groups. Analysis of Hardy-Weinberg equilibrium indicated no significant deviations in either cohort ($p = 0.542$ for controls; $p = 0.121$ for cases). Allelic frequencies were similarly distributed: G allele appeared in 77.5% of controls and 73.5% of T1DM cases, while A allele was present in 22.5% and 26.5% respectively. Furthermore, no significant associations emerged between genotype distribution and age (controls: $p = 0.668$; cases: $p = 0.716$) or sex (controls: $p = 0.637$; cases: $p = 0.255$) within either group.

Investigations: Genotyping of vitamin D receptor gene polymorphism FokI (rs2228570) was performed using StepOne real-time PCR (Cat. No. 4371355) through a three-step process comprising DNA extraction, PCR amplification, and allele detection.

DNA extraction: For each participant, 2 ml of venous blood was collected into sterile EDTA anticoagulant tubes, and genomic DNA was subsequently isolated from whole blood using QIAamp DNA Blood Mini Kit (QIAGEN, Cat. No. 51104, USA). QIAamp extraction procedure involved four key steps: First, cell lysis using QIAGEN protease enzymes, second adsorption of DNA onto QIAamp silica-gel membrane through brief centrifugation and third washing of membrane with two sequential centrifugations. Finally, elution of purified

DNA from QIAamp spin column in a concentrated form using buffer AE [13].

Reagents preparation: QIAGEN protease stock solution (pK) was prepared by reconstituting lyophilized enzyme in specified solvent, with resulting solution stored at 2–8 °C or –20 °C to ensure stability. Buffer AL was maintained at 15–25 °C, thoroughly homogenized prior to use, and remained stable for up to one year. Buffers AW1 and AW2 were prepared by adding 25 ml and 13 ml of 96–100% ethanol respectively and stored at room temperature with a stability period of one year. Buffer AE was similarly stored at 15–25 °C retaining its stability for one year [14].

Procedure: To facilitate effective lysis, samples and buffers were first equilibrated to room temperature, and water bath was preheated to 56 °C. In a 1.5 ml microcentrifuge tube, 200 µl of blood was initially added, followed by 20 µl of QIAGEN protease (pK). Next, 200 µl of Buffer AL was incorporated, and mixture was homogenized by pulse vortexing for 15 seconds. The preparation underwent incubation at 56 °C for 10 minutes to maximize DNA yield, after which brief centrifugation was carried out to clear residual droplets from tube lid. Subsequently, 200 µl of 96–100% ethanol was added, mixed by pulse vortexing for 15 seconds, and centrifuged. The resulting solution was carefully loaded onto a QIAamp spin column, centrifuged at 8,000 rpm for 1 minute, and transferred to a sterile 2 ml collection tube, discarding flow-through. After incubation, spin column was placed into a clean 1.5 ml microcentrifuge tube, eluted with 200 µl of Buffer AE following a 1-minute incubation at room temperature, and centrifuged at 8,000 rpm for 1 minute. Earlier, 500 µl of Buffer AW1 had been applied and centrifuged at 8,000 rpm for 1 minute, with column then transferred to a fresh collection tube. This step was followed by addition of 500 µl of Buffer AW2 and centrifugation at 14,000 rpm for 3 minutes. The resulting purified DNA (4–12 µg) was collected and stored at –20 °C for subsequent analyses [14].

PCR amplification [15]: TaqMan Assay employed preoptimized PCR primer pairs alongside dual probes specifically designed for allelic discrimination or quantitative reverse transcription PCR (qPCR). Each assay comprises an unlabeled PCR primer pair and an Applied Biosystems™ TaqMan™ probe labeled at 5′ end with FAM™ and VIC™ dyes, and featuring an MGB and an NFQ at 3′ end. Reactions were assembled in 0.2 ml microcentrifuge tubes to a final volume of 20 µl, consisting of 10 µl TaqMan Genotyping Master Mix, 1.25 µl TaqMan Genotyping Assay Mix, 2 µl DNA template, and 6.75 µl DNase- and RNase-free water.

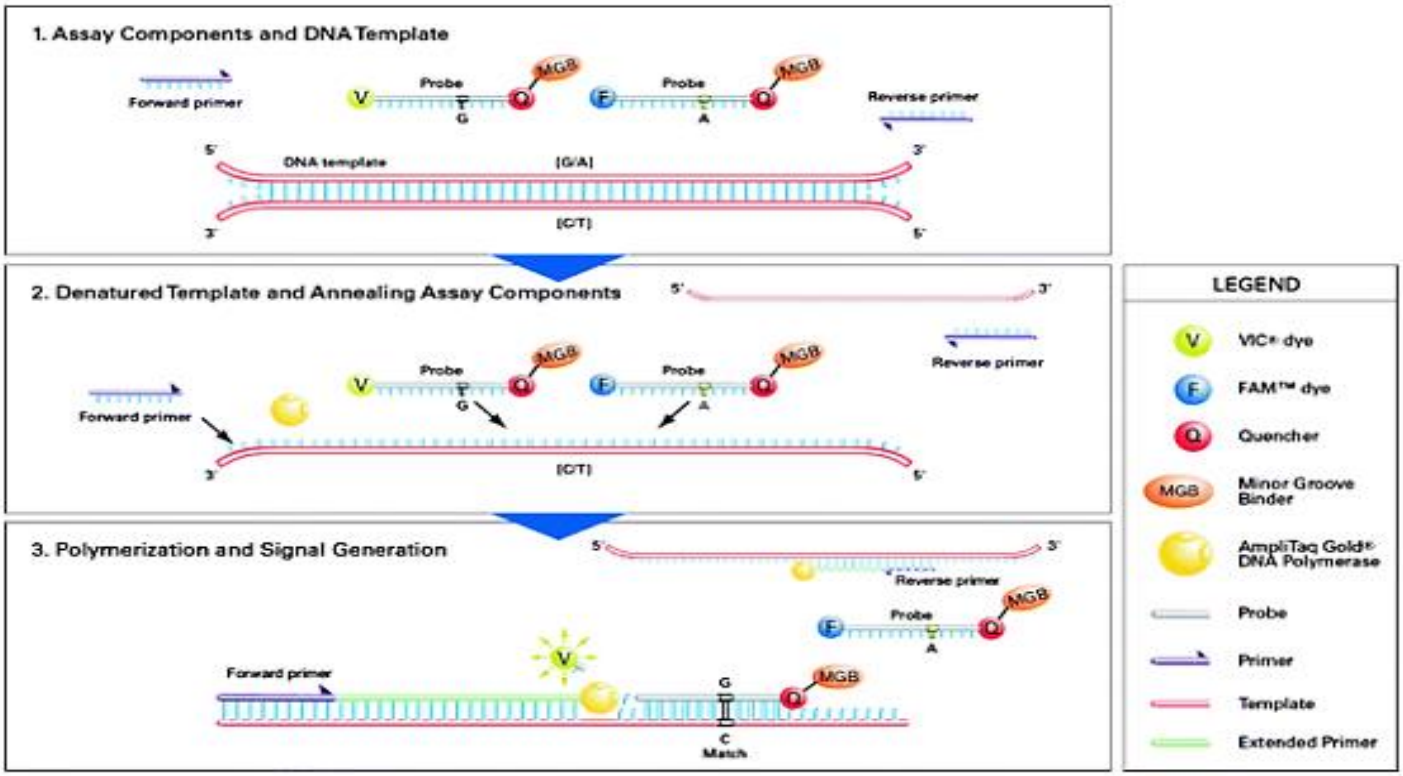

Figure (1): TaqMan SNP Genotyping Assay. (1) Components of the assay with target DNA template showing SNP alleles (in brackets). (2) Denaturation of DNA and annealing of assay components. (3) Signal generation enabling specific allele detection.

Detection of allele: By monitoring fluorescence changes from dye-labeled probes, genotyping assay determines presence or absence of a specific SNP. Genotype interpretation was as follows: Homozygosity for allele 1 was indicated by exclusive VIC dye fluorescence, homozygosity for allele 2 by exclusive 6FAM dye fluorescence and heterozygosity for alleles 1 and 2 by simultaneous fluorescence from both dyes [9] (Figure 1).

Ethical approval:

The study was approved by the Ethics Board of Mansoura University and an informed written consent was taken from each participant or their parents in the study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Statistical analysis

Statistical analyses were executed using SPSS version 26 (SPSS Inc., PASW Statistics for Windows version 26, Chicago, IL, USA). Qualitative variables were described by frequencies and percentages, while quantitative variables were reported as mean ± SD when normally distributed, or as median for non-normally distributed data. Assessment of data normality was performed using Kolmogorov-Smirnov and Shapiro-Wilk tests. A p-value ≤ 0.05 was considered indicative of statistical significance. For qualitative comparisons, Chi-square, Fisher's exact, and Monte Carlo tests were employed as appropriate. Mann-Whitney U test was utilized for comparing two groups with non-normally distributed data, while Kruskal-Wallis test was applied for comparisons involving more than two groups [16, 17].

RESULTS

No substantial variations were observed between groups in terms of age or sex (p = 0.216 and p = 0.322 respectively). Controls had a mean age of 8.85 ± 3.71 years, while case group's mean age was 9.48 ± 3.22 years. Regarding sex distribution, females constituted 53% of controls and 45% of case group (Table 1).

Table (1): Demographic characters of studied groups

	Controls (n=100)	Cases (n=100)	Test of significance
Age / years	8.85±3.71	9.48±3.22	Z=1.24 P=0.216
Sex			
Male	47(47)	55(55)	χ^2=1.28
Female	53(53)	45(45)	P=0.322

χ2=Chi-Square test, Z: Mann Whitney U test.

No substantial variations in genotype distribution were found between the studied groups. Within controls, 59% exhibited GG genotype, 37% AG, and 4% AA, compared to 51% GG, 45% AG, and 4% AA in case group. Hardy-Weinberg equilibrium analysis revealed no notable deviation from expected frequencies in either cohort (p = 0.542 for controls; p = 0.121 for cases). In terms of allele distribution, G allele was detected in 77.5% of controls and 73.5% of cases, while A allele was present in 22.5% and 26.5% of controls and cases respectively (Table 2).

Table (2): Comparison of genotypes between studied groups

Genotype	Controls (n=100)	Cases (n=100)	Test of significance	OR (95%CI)
GG	59(59)	51(51)	χ^2=1.29, P=0.256	0.723(0.414-1.26)
AG	37(37)	45(45)	χ^2=1.32, P=0.251	1.39 (0.791-2.45)
AA	4(4)	4(4)	FET=0.0, P=1.0	1.0 (0.243-4.11)
Hard Weinberg equilibrium	χ^2=0.371 P=0.542	χ^2=2.41 P=0.121		
G (R)	155(77.5)	147(73.5)	χ^2=0.865 P=0.352	1
A	45(22.5)	53(26.5)		1.24(0.786-1.96)

χ^2=Chi-Square test, FET: Fisher exact test, R: reference group.

No substantial association was observed between genotype distribution and age (p = 0.668) or between genotype and sex (p = 0.637) within controls (Table 3).

Table (3): Relation between genotype and demographic characteristics among controls

Among control group	Genotype			Test of significance
	GG N (%)	AG N (%)	AA N (%)	
Age / years Median	8(3-15)	10(3-16)	5.5(3-16)	KW= 0.807 P=0.668
Sex				
Male	29(49.2)	17(45.9)	1(25)	χ^2=0.903
Female	30(50.8)	20(54.1)	3(75)	P= 0.637

KW: Kruskal Wallis test, χ^2= Chi-Square test.

No substantial correlation was detected between genotype distribution and age (p = 0.716) or between genotype and sex (p = 0.255) within case group **(**Table 4).

Table (4): Relation between genotype and demographic characteristics among cases group

Among Cases group	Genotype GG N (%)	AG N (%)	AA N (%)	Test of significance
Age / years	10(3-15)	10(4-15)	9.5(3-12)	KW= 0.667 P=0.716
Sex				
Male	26(51)	28(62.2)	1(25)	χ^2= 2.74
Female	25(49)	17(37.8)	3(75)	P= 0.255

KW: Kruskal Wallis test, χ^2= Chi-Square test.

DISCUSSION

This study's outcomes indicated no notable variation in VDR gene FokI (rs2228570) polymorphisms among examined groups, indicating a lack of significant association with T1DM. This is consistent with previous studies, including an Egyptian study of 50 children with T1DM and 50 non-diabetic controls aged 6–12 years, which also reported no association using real-time PCR for genotyping [18], as well as similar findings observed in a Portuguese population [19]. A Brazilian study [20] involving 189 cases and 194 controls utilized PCR-RT for genotype analysis and found no correlation between FokI (rs2228570) polymorphism and T1DM. These findings align with multiple studies conducted in various populations, including Danish [21] and Pakistani [22] cohorts.

Conversely, an Egyptian study involving 120 children with diabetes and 120 participants aged 7 to 17 years identified substantial variations in VDR gene polymorphisms BsmI (rs1544410) and FokI (rs2228570) between groups, indicating a substantial relation with T1DM. Genotype analysis in this study was performed using PCR-RFLP methodology [23]. Similar findings were observed in Greek studies [24] and in subgroup analyses of American and African populations. The inconsistencies observed among similar studies conducted in Egypt could stem from factors such as limited and heterogeneous sample sizes, variations in genotyping methodologies, and environmental influences including dietary habits and ultraviolet exposure. In contrast to our findings, FokI (rs2228570) polymorphism demonstrated a significant distinction between diabetic and non-diabetic subjects and was markedly associated with T1DM onset in a Brazilian study. That study analyzed 180 diabetic children aged 9–16 years, classified according to presence or absence of thyroid autoantibodies, and employed PCR-RFLP for genotyping. A similar association was also reported in two Egyptian studies [25, 26].

It is essential to acknowledge that distribution of VDR polymorphisms and their association with T1DM development have been topics of significant controversy, both across diverse populations and within individual populations. For instance, in Egyptian population, findings related to FokI polymorphism have been inconsistent, with two studies reporting an association with increased susceptibility to T1DM [25, 26] , whereas a third study found no such correlation [27]. FokI polymorphism has been linked to an increased risk of T1DM among individuals of West Asian descent [28].

In two studies [29, 30], FokI was significantly associated with increased susceptibility to T1DM among Egyptian children, while another study from the same region [31] found no significant association, aligning with our findings. Furthermore, a large-scale meta-analysis conducted in Eastern Mediterranean region [31] reported no significant association between FokI and T1DM, suggesting possible regional or ethnic variability in genetic susceptibility. However, in contrast, a study conducted in an Indian population [32] demonstrated a strong association, with FokI genotype significantly more frequent among T1DM cases. The discrepancies observed among similar studies may be attributed to ethnic diversity, limited and heterogeneous sample sizes, differences in gene expression patterns, use of varying genotyping methodologies, and environmental factor variability.

CONCLUSION

In this study of Egyptian children, no significant association was identified between FokI (rs2228570) gene and risk of developing T1DM. These results indicated that this particular genetic variant may not play a substantial role in T1DM susceptibility within this population. Further research with larger cohorts and analysis of additional VDR polymorphisms is recommended to clarify potential genetic contributions to autoimmune diabetes in children.

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