

Original Article

ASSOCIATION OF *FOKI* AND *BSMI* RFLP GENOTYPES IN VDR GENE WITH HASHIMOTO'S THYROIDITIS IN ERBIL PROVINCE

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ABSTRACT

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Genetic variations in the Vitamin-D-receptor (VDR) gene have been associated with the pathogenesis of several immune-mediated conditions, notably Hashimoto's thyroiditis (HT). This work was undertaken to assess whether the widely studied *FokI* (rs2228570) and *BsmI* (rs1544410), two well-characterized VDR polymorphisms, are associated with susceptibility to HT among females in Erbil Province in the Iraqi Kurdish region. The design of this study was case-control, with 50 female patients diagnosed with HT and 50 healthy female controls, both age-matched (24–59 years). With the guidance of a qualified endocrinologist, various biochemical indicators have been methodically evaluated such as TSH, FT4 and anti-thyroid peroxidase (A-TPO) and Vitamin D3. Genotyping of both polymorphic loci was accomplished via the PCR-restriction fragment length polymorphism (PCR-RFLP) approach. Neither genotype nor allele distributions were differed significantly between groups for either polymorphism ($P > 0.05$). This result was also confirmed by Fisher's exact test, which revealed no significant relationship in genotypic and allelic distribution ($P > 0.05$). Hardy-Weinberg equilibrium was maintained for the *FokI* locus in both groups ($P > 0.05$). For the *BsmI* polymorphism, however, no similar difference was found, as HWE was only seen in patients, but not in controls ($P > 0.05$). Taken together, the data provide no evidence linking *FokI* or *BsmI* variants to Hashimoto's thyroiditis susceptibility in females at Erbil Province, and VDR variant frequencies were not different meaningfully between cases and control.

Keywords: Hashimoto's thyroiditis, vitamin-D-receptor gene polymorphism, *FokI* (rs2228570), *BsmI* (rs1544410).

1. INTRODUCTION

Based on the definition, a genetic polymorphism is the simultaneous occurrence of two or more alleles with a frequency greater than 1% in the population studied, and these alleles are genetically different and occupy the same position on the genome (He *et al.*, 2017; Zhang *et al.*, 2021). Variation in this variety comprises single-nucleotide polymorphisms (SNPs), insertion/deletion variants (InDels), both minisatellite and microsatellite repeats, and copy number variants which can change the phenotypic traits and disease susceptibility of an individual. All of the above variant types can produce phenotypic trait variability with difference of susceptibility to pathologies (Hitomi & Tokunaga, 2017). These differences in genetics have been shown to have a vital role in how genes are expressed and how well they operate, for instance, in the activity of the protein, regulation of the gene and alternative splicing, epigenetic changes and the environmental effects (Jin *et al.*, 2018; Johnson *et al.*, 2008). Polymorphisms in regulatory genomic regions (gene promoters, enhancers and UTRs) might modify a gene transcription or post-transcriptional processing and may modify the amount of gene expression quantitatively (Yip *et al.*, 2024). Furthermore, polymorphisms have been implicated in the risk of developing a diversity of autoimmune diseases; for instance, rheumatoid arthritis, in addition, inflammatory bowel disease, systemic lupus erythematosus, hyperglycemia or diabetes mellitus and autoimmune thyroid disease (AITD), in particular Hashimoto's thy-

roiditis (HT) (Giovinazzo *et al.*, 2017).

Alternatively, HT is termed as "chronic autoimmune thyroiditis" or "lymphocytic thyroiditis" (Yilmaz *et al.*, 2025). Pathologically, the thyroid tissue is gradually attacked by lymphocytes and destroyed (Ihnatowicz *et al.*, 2020), making it the most frequent autoimmune disease worldwide (Cayres *et al.*, 2021). The disease was first defined in 1912 by Hakaru Hashimoto (Younger, 2017), and was officially categorized in 1957 in the autoimmune thyroid disorder (AITD) group (Connelly *et al.*, 2022). The prevalence of HT varies by region and socioeconomic level: 0.9-7.9% in men, and 4.8-25.8% in women (Mikulska *et al.*, 2022). Though environmental factors account for an estimated 20–30% of the probability of progression in Hashimoto's thyroiditis, genetic factors contribute much greater in the etiology of the disease (Ragusa *et al.*, 2019). Vitamin D is believed to have immunomodulating properties, and combined with genetic factors it is believed to be a multifaceted contributor to the pathogenesis of Hashimoto's syndrome. Polymorphisms in the VDR gene may have certain effects in relation to HT susceptibility, where some polymorphisms confer additional risk for HT and others confer some immune protection (Feng *et al.*, 2013; Yazici *et al.*, 2013).

The VDR is a nuclear receptor of the transcription factors that modulate the intracellular signaling molecules for 1,25-Dihydroxycholecalciferol (1 α ,25(OH)₂D) or Calcitriol. Expression of VDR has been observed in several peripheral blood mononuclear cells, encompassing T lymphocytes, B lymphocytes and antigen-

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presenting cells (Starchl *et al.*, 2021). Originally, the VDR protein encoded by the VDR gene is located on the long arm of 12th chromosome (12q13.11) and more than 900 allelic variants that have been mapped to the VDR locus (Hanna *et al.*, 2021; Ibrahim *et al.*, 2021). The most common and researched of these SNPs are called *FokI* and *BsmI*, and through extensive scientific investigations, hundreds of such SNPs were identified within the VDR gene (Al-Ghafari *et al.*, 2020; Vidigal *et al.*, 2017). The restriction endonucleases that sense the restriction sites in genomic DNA,

FokI (from *Flavobacterium okeanokoites*) and *BsmI* (from *Bacillus smithii*) are named for the SNPs (Messaritakis *et al.*, 2020).

FokI polymorphism sits at the start of the VDR gene and generates a shorter expression of VDR protein with a three-amino acid deletion that is associated with greater transcriptional activity. The *BsmI* polymorphism is a covert genetic variant in the 3' untranslated region of intron 8, which does not produce a subsequent amino acid modification in the protein, yet can exert some transcriptional regulation on the stability of VDR mRNA and transcription thereof (Chauhan & Medithi, 2021). Genetic polymorphisms (variations within the regulatory domain of the VDR gene) have previously attracted considerable attention and have been extensively studied systematically for a wide variety of pathologic conditions, such as autoimmune thyroid disease (AITD). A total of sixty VDR SNPs have been found, two being of unique scientific interest regarding AITD: *FokI* rs2228570 and *BsmI* rs1544410 (Shaikh *et al.*, 2023). The primary purpose of this research was to determine whether there is a meaningful connection between the most clinically relevant polymorphisms, *FokI* (rs2228570) and *BsmI* (rs1544410) VDR gene variants, and the susceptibility to Hashimoto's thyroiditis (HT) from Erbil Province females.

2. MATERIAL AND METHOD

Study design

For this study, a case-control design was used. The samples were taken from Galiawa Diabetes and Endocrinology Teaching Center, Erbil province, Kurdistan Region of Iraq, from 1st October 2024 to 1st May 2025 and from a private laboratory, Erbil province. The subjects of the study were 50 women aged 24-59 years with Hashimoto's thyroiditis (HT) and 50 age matched women control subjects. In case of high anti-TPO antibody levels, low levels of radioactive iodine uptake, low levels of FT4 and high levels of TSH, the HT diagnosis was confirmed by an experienced endocrinologist, together with clinical signs and symptoms. If any of the individuals had a personal history of autoimmune disease or clinical and biochemical features of HT or other autoimmune diseases, they were excluded from the control group.

Sample Collection

All subjects and patients who had completed structured questionnaires, which included personal demographic data (gender, age, marital status) and relevant clinical parameters (BMI, family history, other autoimmune diseases), were included in the present analysis. 4 mL of venous blood samples were aseptically collected from each enrolled participant, placed in EDTA coated blood collection tubes to prevent blood from coagulating, and frozen at a point of -20°C . The other 2 mL aliquot was placed in the gel tubes that allowed to coagulate at room temperature for 1/2 hour. The clotted blood samples were then centrifuged at 3,500 revolutions per minute (rpm) for 10 minutes to ensure good separation of serum from the blood cells. The serum fractions were then stored in a series of Eppendorf tubes at -70°C until further hormonal and biochemical analyses were performed at Zheer Private Laboratory.

Genomic DNA for whole blood samples were taken according to the manufacturer's instructions applied to the Add Prep Genomic DNA Extraction Kit (Add Bio, Korea), prepared for the use with patients and healthy controls. The DNA concentration and purity were assessed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA). Specific primers were selected for genotyping the *FokI* and *BsmI* loci: Forward primer: 5'-AGCTGGCCCTGGCACTGACTCTGCTCT-3'; Reverse primer:

5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3'; Forward primer: 5'-CAACCAAGACTACAAGTACCGCGTCAGTGA-3'; Reverse primer: 5'-AACCAGCGGGAAGAGGTCAAGGG-3'. These sequences were taken from earlier studies (El Gendy *et al.*, 2019; Majid *et al.*, 2024; Youssef *et al.*, 2019). Each polymerase chain reaction (PCR) mixture was prepared in total volume of 20 μL (each forward and reverse primer at 1 μL concentration) together with 10 μL of a 2x PCR master mix provided to researchers (Add Start Taq Master, Add Bio, Korea). Moreover, we used 2 μL of template DNA and 6 μL nuclease-free water to get the mixture.

For thermal cycling involving the *FokI* locus, optimal conditions involved an initial denaturation step at 95°C for five minutes and then, 35 cycles of denaturation at 95°C for twenty seconds, annealing at 62°C for forty seconds, extension at 72°C for twenty seconds. Last extension of 7 minutes was made at 72°C . In like manner, the amplification process for *BsmI* was repeated 5 minutes at 95°C , circularly passing through denaturing events of 25 seconds at 95°C , anneal time of 40 seconds at 60°C followed by 30 seconds extension sessions at the final temperature of 72°C , with another extension period of seven minutes set to 72°C . To obtain all resulting PCR products, and separated for lives with 1.5% agarose gels with ethidium bromide through Biobase electrophoresis (Biobase China) under 4 V/cm for 55 min conditions. Band visualization was facilitated with UV light on a Biobase transilluminator. Tubes included 265 bp *FokI* and 825 bp *BsmI* products were subjected to restriction enzyme digestion, using the endonucleases *FokI* and *BsmI* (Thermo Scientific, Lithuania), strictly in accordance with manufacturer protocols. For digestion related to *FokI*, a volume of tenths samples of the amplicon was combined in 2 μL of Fast Digest buffer, 1 μL of Fast Digest *FokI* enzyme, 17 μL nuclease free water (totaling 30 μL), and then incubated at 37 degrees Celsius for the five minutes. Digestions in *BsmI* witnessed the addition of a ten-microliter amplicon to an assemblage comprising 2 μL 10x Buffer R; 1.3 μL fMva1269I (*BsmI*) enzyme alongside 18 μL nuclease removed water resulting in a volume 31.3 sample induction over two hours. Also, a terminating incubation temperature set at 37 degrees Celsius. Finally, digested sample products of 10 microliter for each individual were transferred into agarose gels that contained four microliters ethidium bromide enabling visualization of fragmentation.

Data analysis

GraphPad Prism 9 was utilized for analyzing data that were presented in mean $\pm$ standard error of mean (SEM). Fisher's exact test was applied to compare frequencies and genotype distributions of SNP alleles of HT patients and control group. In addition, an independent samples student t-test for continuous variables was conducted, with HT used to compare with normal controls, and one-way ANOVA among the different subgroups. Shapiro-Wilk test used for the normality results. Hardy-Weinberg equilibrium (HWE) tests were subsequently used to assess if VDR genotype distributions at the investigated different loci were in HWE. Relative risk (RR) and 95% confidence intervals (CIs) measures were calculated for association between genotypic profile and the occurrence of disease. Results with the corresponding P value <0.05 were considered statistically significant.

3. RESULTS

Study Group Characteristics

A detailed comparative synopsis of the demographic characteristics of the HT patient group (n=50) and healthy control group (n=50) is given in Table 1. There was no significant difference found between the mean age of HT patients (40.26 ± 1.22 years) and controls (40.22 ± 1.21 years) ($P = 0.981$), indicating age matched distribution between both groups and thus reducing the likelihood of age dependent confounding effects. BMI was significantly higher in the HT group ($31.06 \pm 0.57 \text{ kg/m}^2$) than in the control group ($28.87 \pm 0.68 \text{ kg/m}^2$) ($P = 0.016$), indicating that overweight and obesity were significantly more prevalent in the HT group. As far as familial predisposition is concerned, a significantly higher percentage of patients

with HT (72%) had a positive family history of Hashimoto's thyroiditis compared with control subjects (36%) ($P = 0.0003$), strongly suggesting involvement of hereditary and familial determinants in the susceptibility to HT.

The concentrations of serum TSH were significantly higher ($P = 0.0001$) in the HT group ($9.43 \pm 0.15 \mu\text{IU/mL}$) than control ($2.45 \pm 0.11 \mu\text{IU/mL}$), indicating poor thyroid function in the HT patients. However, there was no significant difference between the patient and control groups for free T4 levels (10.73 ± 0.45 and 11.00

$\pm 0.47 \text{ pmol/L}$, respectively; $P = 0.828$). Anti-thyroid peroxidase (anti-TPO) antibodies were significantly higher in HT patients ($230.2 \pm 10.68 \text{ IU/mL}$) than in healthy subjects ($8.66 \pm 0.36 \text{ IU/mL}$) and the difference was highly statistically significant ($P = 0.0001$). Moreover, the level of serum 25(OH)D was relatively higher in the HT group ($19.23 \pm 0.51 \text{ ng/mL}$) than in the control group ($15.22 \pm 0.49 \text{ ng/mL}$), but the difference was not statistically significant ($P = 0.062$).

Table 1: Demographic, laboratory and clinical traits of HT patients, and controls presented as Mean $\pm$ SEM.

Variables	patients(n=50)	Controls(n=50)	P-value
Age	40.26 $\pm$ 1.22	40.22 $\pm$ 1.21	0.981
BMI (kg/m ²)	31.06 $\pm$ 0.57	28.87 $\pm$ 0.68	0.016
History of HT (%)	36(72%)	18(36%)	0.0003
TSH ($\mu\text{IU/mL}$)	9.43 $\pm$ 0.15	2.45 $\pm$ 0.11	0.0001
Free T4 (pmol/L)	10.73 $\pm$ 0.45	11.00 $\pm$ 0.47	0.828
Anti-TPO (IU/mL)	230.2 $\pm$ 10.68	8.658 $\pm$ 0.36	0.0001
25(OH)D (ng/mL)	19.23 $\pm$ 0.51	15.22 $\pm$ 0.489	0.062

P-values < 0.05 are indicated in bold. HT is Hashimoto's Thyroiditis, BMI is Body Mass Index, TSH is Thyroid Stimulating Hormone, Anti-TPO is Antithyroid Peroxidase and 25-hydroxyvitamin D3 is 25-hydroxyvitamin D3.

Polymerase Chain Reaction (PCR) and Genotyping: Gel Electrophoresis

The result of PCR-RFLP analysis for *FokI* genotypes FF, Ff and ff are shown in figure 1-A and 1-B. Similarly, the result of PCR-RFLP analysis for *BsmI* genotypes BB, Bb and bb are shown in figure 2-A and 2-B.

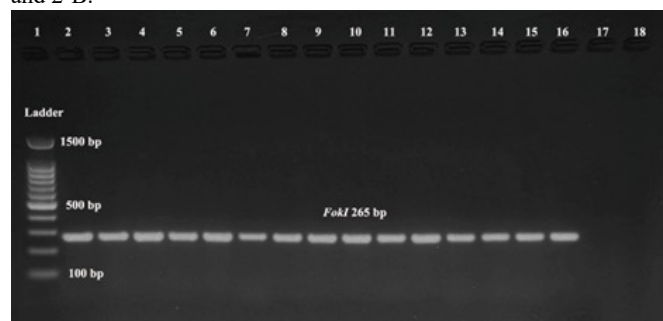


Figure 1-A: Agarose gel electrophoresis of PCR product for VDR gene. Lane1: DNA Ladder, Lanes 2-16: *FokI* (rs2228570) with expected size 265 bp against 100bp DNA Ladder, Lane18: negative control.

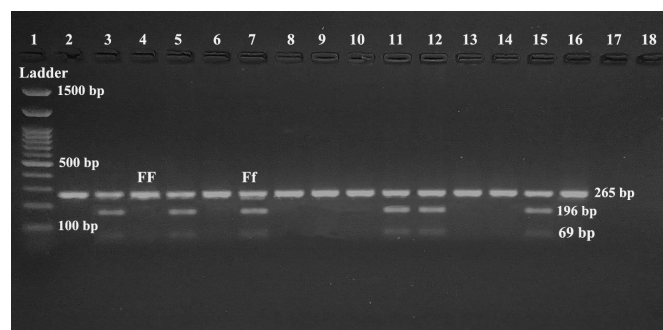


Figure 1-B: Genotyping outcomes for the *FokI* (rs2228570) polymorphism of the VDR gene Lane 1: DNA Ladder 100 bp; Lanes 2, 4, 6, 8, 9, 10, 13, 14, and 16: FF homozygous genotype; Lanes 3, 5, 7, 11, 12, and 15: Ff heterozygous genotype.

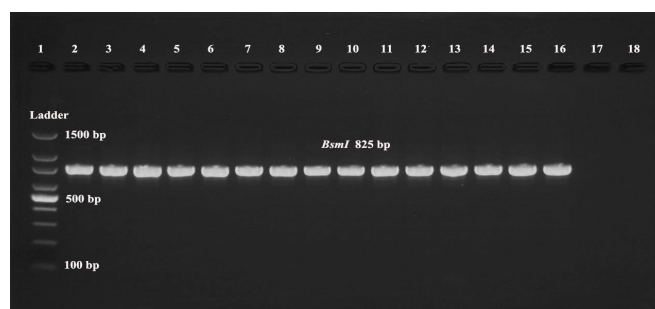


Figure 2-A: Agarose gel electrophoresis of PCR product for amplified VDR gene. Lane1: DNA Ladder, Lanes 2-16: *BsmI* (rs1544410) with expected size 825 bp against 100bp DNA Ladder, Lane18: negative control.

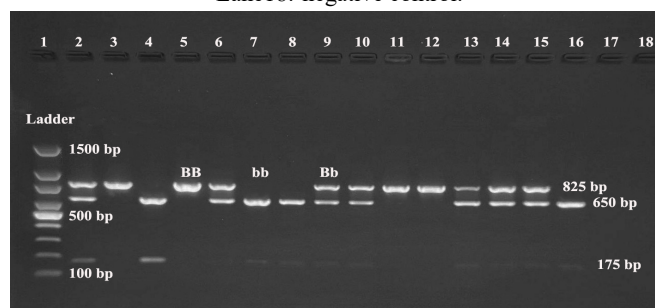


Figure 2-B: Genotyping outcomes for the *BsmI* (rs1544410) polymorphism of VDR gene. Lane 1: DNA Ladder 100 bp; Lanes 3, 5, 11, and 12: BB homozygous genotype; Lanes 2, 6, 9, 10, 13, 14, and 15: Bb heterozygous genotype; Lanes 4, 7, 8, and 16: bb homozygous recessive genotype.

Genotype and allele frequencies of *FokI* polymorphism

Table 2 displays the distribution of VDR-gene polymorphism genotype, and alleles frequencies as seen by the studied groups. As for *FokI* SNP (rs2228570), 32 of the 50 recruited patients had the homozygous dominant genotype (FF), 16 (32%) had the heterozygous genotype (Ff) and 2 (4%) patients had the homozygous recessive genotype (ff). The frequency of the 'F' allele was 0.80 and the frequency of the 'f' allele was 0.20. The FF genotype in the control (32 (64%) subjects), the Ff genotype was found in 18 (36%) subjects and 0 (0%) subjects had the (ff) genotype. The 'F' allele frequency was 82% and the 'f' allele was 18%. The genotype with the largest number of patients (64%) and controls (64%) (with both patient and

control groups) were FF genotypic, and no association was observed between this genotype and disease susceptibility ($RR = 1.00$). The frequency of heterozygous Ff genotypes was lower in patients (32%) compared to controls (36%) ($RR=0.84$). The results could potentially provide evidence for a protective role of the Ff genotype; it is not statistically confirmed. Importantly, the ff genotype was observed in a very small fraction of patients (4%) and was absent in the control group. Whilst RR was 5.21, was not significant ($P = 0.495$), and the extremely broad CI (0.25–107.93) cannot be used to draw any final conclusions. The differences in genotype distribution between

the two groups was checked by the Fisher exact test ($P > 0.05$). At allelic level, the majority of control (82%) individuals and 80% of patient adults had no significant variation in frequency of the F allele, even at a relative risk of 0.88 suggesting that there was the absence of a significant allele protection. Although it was found to have a higher incidence in the disease group (20%) compared to controls (18%) and an above marginal relative risk (RR) ratio of 1.14, the f allele and disease susceptibility were not associated significantly. Fisher's exact test showed that neither allele was statistically significant ($P = 0.857$).

Table 2: Genotypes and alleles frequencies of *FokI* VDR gene polymorphism are presented in Hashimoto's thyroiditis and control groups.

<i>FokI</i>	Gene frequency among patient group	Gene frequency among Healthy group	Relative Risk (RR)	Etiology or Preventive Fraction	Exact Fishers Probability (P)	95% Confidence Intervals (CI)
Genotype						
FF	32 (64%)	32 (64%)	1.000	0.000	1.000	0.45-2.24
Ff	16 (32%)	18 (36%)	0.84	0.059	0.833	0.37-1.90
ff	2 (4%)	0 (0%)	5.21	0.04	0.495	0.25-107.93
Allele						
F	80 (80%)	82 (82%)	0.88	0.010	0.857	0.43-1.77
f	20 (20%)	18 (18%)	1.14	0.02	0.857	0.56-2.30

Hardy-Weinberg Equilibrium Analysis of *FokI* Genotypes

VDR *FokI* genotype distribution was analyzed in HT patients and controls to determine if they were in HWE (Table 3). In the patient cohort, the observed genotype frequencies were as follows: FF: 32 (64%), Ff: 16 (32%), ff: 2 (4%). The observed frequencies were in agreement with the expected frequencies which indicated no deviation

from equilibrium. The value of the p-value for HWE was 1.000 which is not significant at 0.05. In control, the observed genotype frequencies were: FF: 32 (64%), Ff: 18 (36%), ff: 0 (0%). The anticipated genotype frequencies were: FF: 33.62 (67.24%), Ff: 14.76 (29.52%), ff: 1.62 (3.24%). The HWE p-value was 0.120, exceeding 0.05.

Table 3: HWE analysis of genotypic and allelic frequencies of VDR-gene (*FokI*) in healthy controls and Hashimoto's thyroiditis (HT) patients.

Study groups		VDR gene (<i>FokI</i>)					
		Genotypes			HWE p. value	Alleles	
		FF	Ff	ff		F	f
Hashimoto's thyroiditis patients	Observed	32 (64%)	16 (32%)	2 (4%)	1.000	80 (80%)	20 (20%)
	Expected	32 (64%)	16 (32%)	2 (4%)		NA	NA
Healthy Person	Observed	32 (64%)	18 (36%)	0 (0%)	0.120	82 (82%)	18 (18%)
	Expected	33.62 (67.24%)	14.76 (29.52%)	1.62 (3.24%)		NA	NA

NA: Not applicable

Genotype and allele frequencies of *BsmI* polymorphism

Genotype distribution and allele frequencies for gene polymorphism in VDR are presented in Table 4. Regarding *BsmI* SNP (rs1544410), the genotypic frequencies for the patient group were 17 (34%) BB, 19 (38%) Bb, and 14 (28%) bb. B and b alleles are 53% and 47%, respectively. However, the genotypic frequencies in the control group were 15 (30%) for BB, 15 (30%) for Bb and 20 (40%) for bb, the calculated allele frequencies were 45% (B) and 55% (b). BB genotypes were present in 34% of the patient cohort and 30% of the control group. Although the RR of 1.20 indicated a marginal trend towards increasing risk, this was not statistically significant ($P = 0.830$; CI: 0.52–2.77). Likewise, the Bb genotype was present in 38% of patients, versus 30% of controls; also, an elevated risk, however not statistically significant ($P = 0.527$; CI: 0.63–3.26). In contrast, the

bb genotype was detected in 28% of patients compared with 40% of controls. With an RR of 0.58, the distribution suggests a possible protective effect but was not significant ($P = 0.291$; CI: 0.25–1.34). Overall, a Fisher's exact test verified that wider genotype distribution had no significant relationship with disease susceptibility. Patients had a higher burden of B allele (53%) than controls (45%) in terms of allele distributions. This suggests a trend to increased risk but was not significantly different ($RR = 1.38$; $P = 0.322$; CI: 0.79–2.40). Conversely, the b allele, meanwhile, was more prevalent within the control group (55%) relative to the patient group (47%) and produced an RR of 0.73 that also did not reach statistical significance ($P = 0.322$; CI: 0.42–1.26). Results confirmed by Fisher's exact test indicated the differences in both allele frequencies were not significant ($P = 0.322$).

Table 4: Distribution of genotypes and allele frequencies of the *BsmI* VDR gene polymorphism in patients with Hashimoto's thyroiditis compared to control subjects.

<i>BsmI</i>	Gene frequency among patient group	Gene frequency among Healthy group	Relative Risk (RR)	Etiology or Preventive Fraction	Exact Fishers Probability (P)	95% Confidence Intervals (CI)
Genotype						
BB	17 (34%)	15 (30%)	1.20	0.057	0.830	0.52-2.77
Bb	19 (38%)	15 (30%)	1.43	0.114	0.527	0.63-3.26
bb	14 (28%)	20 (40%)	0.58	0.167	0.291	0.25-1.34
Allele						
B	53 (53%)	45 (45%)	1.38	0.145	0.322	0.79-2.40
b	47 (47%)	55 (55%)	0.73	0.151	0.322	0.42-1.26

Hardy-Weinberg Equilibrium Analysis of BsmI Genotypes

Genotype frequencies of the VDR *BsmI* polymorphism were evaluated in both HT patients and controls and the results are presented in Table 5. Within the patient group, the recorded genotype frequencies were as follows: BB: 17 (34%), Bb: 19 (38%), and bb: 14 (28%). The corresponding expected genotype frequencies were calculated as: BB: 14.05 (28.09%), Bb: 24.91 (49.82%), and bb: 11.05 (22.09%). The obtained p-value HWE was 0.093 which was greater than 0.05.

This result suggests that the distribution of genotypes among the patients is insignificantly different from the HWE.

Regarding the control group, the recorded genotype frequencies were: BB: 15 (30%), Bb: 15 (30%), and bb: 20 (40%). The expected genotype frequencies were calculated as: BB: 10.13 (20.25%), Bb: 24.75 (49.5%), and bb: 15.13 (30.25%). The p-value for the corresponding HWE was < 0.05. This result shows that the distribution of the genotype in the controls is far from Hardy-Weinberg equilibrium.

Table 5: HWE analysis for the genotypic and allelic distributions of the VDR gene (*BsmI*) in healthy individuals and patients with Hashimoto's thyroiditis.

Study groups		VDR gene (<i>BsmI</i>)					
		Genotypes			HWE p. value	Alleles	
		BB	Bb	bb		B	b
Hashimoto's thyroiditis patients	Observed	17 (34%)	19 (38%)	14 (28%)	0.093	53 (53%)	47 (47%)
	Expected	14.05 (28.09%)	24.91 (49.82%)	11.05 (22.09%)		NA	NA
Healthy Person	Observed	15 (30%)	15 (30%)	20 (40%)	0.005	45 (45%)	55 (55%)
	Expected	10.13 (20.25%)	24.75 (49.5%)	15.13 (30.25%)		NA	NA

NA: Not applicable

4. DISCUSSION

The objectives of this study were to elucidate whether there is genetic basis for the association of *FokI* (rs2228570) VDR and *BsmI* (rs1544410) polymorphisms and susceptibility to HT in people from Erbil Province. HWE, genotype frequencies and allele frequencies were studied simultaneously in all treated groups to establish the correlation between those markers and the studied conditions, HT for instance. There was no substantial difference in frequency of *FokI*, and *BsmI* in HT versus control group patients. This implies that, in this particular population, there was no meaningful contribution of either VDR polymorphism in generating traits related to hereditary susceptibility to Hashimoto's thyroiditis. The tests of Hardy-Weinberg Equilibrium (HWE) confirmed that HWE was not violated in the patients or controls. This confirms the genotyping approach thus provides evidence of free selection bias, population stratification and large genotyping errors. The *BsmI* polymorphism only showed a significant deviation on the HWE to the Control group only. Such discrepancy was not recognized by the scientists to have a biological significance, but was attributable to outside influences (population substructure, sample size or lack of sample size).

Moreover, genotypes and frequencies of the *FokI* (rs2228570) variant were not significantly different between HT patients and healthy control. The genotype and allele frequencies of *BsmI* (rs1544410) were also found not to be associated with HT.

Hanna *et al.*, (2021) reported no significant association between VDR *BsmI* polymorphism and a susceptibility to HT in an Egyptian population, similar to the findings of the present study. Similarly, Yazici *et al.*, (2013) reported no significant correlation between *BsmI* polymorphism and risk for HT in a Turkish population. Regarding the *BsmI* polymorphism, Stefanic *et al.*'s study Štefanić *et al.*, (2008) suggested in this area that between the B and b variants compared with the b allele the risk of HT might be reduced. Nevertheless, five further studies were unable to confirm this conclusion. Similarly, it was not found that the meta-analysis pooled results showed that the *BsmI* polymorphism was associated with HT, nor did it lead to differences between subgroups' respective results, or did the sensitivity analysis alter the trend of the aggregate (Wang *et al.*, 2017).

Majid *et al.*, (2024) reported significant differences between HT and healthy controls regarding the incidence of *FokI* and *BsmI* polymorphisms among Iraqi population. Wang *et al.*, (2017) conducted a comprehensive review of VDR SNP epidemiological research for Hashimoto's thyroiditis and determined that when stratifying by ethnic sub-populations, the SNP association with Hashimoto's was not significant; however, the SNP rs2228570 appeared to be a risk factor. In addition, Hanna *et al.*, (2021) also found significant association of *FokI* polymorphism with the predisposition to HT in Egyptian population followed by Yazici *et al.*, (2013) in Turkish population. Importantly, Majid *et al.* the results demonstrated that the "FF" genotype of

the VDR "*FokI*" was a significant association with the susceptibility in the Iraqi population to HT and this genotype was recognized as a risk factor Majid *et al.*, (2020)), and was the first investigation into VDR *FokI* polymorphism as a possible contributor to an increased susceptibility to Hashimoto's thyroiditis in the Iraqi population. However, the results from the current study corroborate the findings of (Yazici *et al.*, (2013); Majid *et al.*, (2020); & Hanna *et al.*, (2021)), where there is an association between *FokI* and HT.

Similarly, six out of eight previous studies on the distribution of *FokI* polymorphism in Hashimoto's patients and controls reported a positive association, while two studies (Guleryuz *et al.*, 2016; Meng *et al.*, 2015) did not. In a meta-analytical data, the prevalence of HT was higher in FF compared to Ff + ff subjects (OR = 1.72, $P = 0.019$), so that the presence of F allelic genotype could have a role in determining HT susceptibility in participants (OR = 1.44, $P = 0.010$). Ethnic section analysis also demonstrated an elevated risk of Hashimoto's in the Asian group (OR = 1.64, $P = 0.039$) but not in the Caucasian group and could potentially be attributed to variations in genetic background, lifestyle and environmental exposures among various ethnic groups (e.g. in diet and sun exposure). Overall, the results suggested a more effective measurement of HT was to compare the FF genotype and the Ff + ff genotype and to compare results. Furthermore, insufficient numbers of Caucasian subjects in the subgroup analyses may also have biased the results (Wang *et al.*, 2017).

The presence of polymorphic variants indicates that VDR gene expression is by nature normal Uitterlinden *et al.*, (2004). The association of the VDR-gene (rs2228570) with Hashimoto's disease was determined in Serbian (Djuovic *et al.*, 2015), Turkish (Yazici *et al.*, 2013), and Taiwanese groups (Lin *et al.*, 2006) respectively. Nonetheless, genetic studies and genome-wide association studies demonstrate an absence of significance for the individual's risk to Hashimoto's thyroiditis (ElRawi *et al.*, 2019). The differential inheritance of VDR gene polymorphisms found across study populations may be explained by the inter-ethnic variations and geographical variability (Semino *et al.*, 2000), and the differing results for rs2228570 on HT pathogenesis and progression should be treated with caution (Gao & Yu, 2018). In the current research, it was found that VDR *FokI* (Ff) genotype was statistically significant in patient plus control group ($P = 0.029$) which was similar to Yazici *et al.*, (2013) which found that the heterozygous, rs2228570 genotype had a statistically significant association with lower risk of Hashimoto's. On the contrary, Guleryuzi B. *et al.* (2016), on the contrary discovered no significant difference ($P = 0.28$) in the distribution of the Ff genotype in their study (24.2%) and control (32%) population.

Interpretation of these results is necessary. The relatively small sample size (50 patients and 50 healthy controls), may have limited the statistical power to detect potential associations between VDR gene polymorphisms and disease susceptibility. The studies also limit their generalizability to individuals living in other locations with varied environmental exposure and genetic make-up as they enrolled people

from Erbil Province only. The inclusion of females was also a constraint on the gender of the results although Hashimoto's thyroiditis is reported in men. In order to control for sampling bias, population stratification and/or genotyping errors, we consider the control group for the *BsmI* polymorphism, which had a non-obvious break from Hardy-Weinberg equilibrium. Finally, because the population of the patient cohort was of different demographics (newly diagnosed and previously treated patients) this can create the possibility of confounding the biochemical marker measurements with therapeutic intervention (treatment with thyroid hormone replacement or vitamin D supplementation). Vitamin D supplementation could represent a potential limitation, since supplementation status varied between the case group and the control group and detailed information regarding the dose, duration, and timing of supplementation was not available. Therefore, its potential influence on serum vitamin D levels could not be fully assessed.

CONCLUSION

To conclude, no significant variances were found in the polymorphism frequencies (*FokI* and *BsmI*) between HT patients and control group. These results showed that these individual genetic polymorphisms were not vital in developing HT in this population. Thus, further approaches with considerably larger sample sizes must be performed to sort out whether or not the VDR gene eventually plays a role in the onset of the pathogenesis of HT.

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Ethical Statement:

This research has been formally approved by the Human Research Ethics Committee (HREC) of the Department of Biology in the College of Science at Salahaddin University - Erbil (Approval No: 4S/349, Date:29/09/2024) before collecting any data from the participants. All participants gave verbal consent before taking blood sample from them.

Author Contributions:

Each author has agreed to be held accountable for every part of the article after reviewing the completed version that will be published.

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Authors confirm that they have no conflict of interest to declare.

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