

# Vitamin D Receptor Gene polymorphisms and Genetic susceptibility to Hashimoto's Thyroiditis

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## Research Article

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# Abstract

## Background

The severity and complexity of autoimmune disorders is dependent on the genetic capability of individuals. Genetic studies have revealed association between polymorphisms of Vitamin D Receptor gene and individuals' predisposition to autoimmune diseases. Therefore, this study aimed to develop relationship between vitamin D receptor gene polymorphisms and hypothyroidism.

## Materials and Methods

A total of 144 individuals were studied, including 72 patients presenting with symptoms of Hashimoto's disease. The amplicon sequencing was performed on samples bearing M13 tail tags. Statistical analysis was carried out using SPSS software to establish correlations of genotypes and alleles among control and diseased individuals.

## Results

The mean concentrations of vitamin D were observed to be critically low in patients with Hashimoto's thyroiditis. Of the four SNPs studied, only rs7975232 was found to be significantly related with disease progression. Other three rs1544410, rs731236 and rs2228570 did not show significant correlation in the individuals studied.

## Conclusion

Altered VDR expressions because of various VDR polymorphisms have been shown to exhibit differently among various races and ethnic groups. Furthermore, these VDR polymorphisms also vary among populations in different environmental and genetic predispositions. This study suggests the homozygous CC genotype at rs7975232 to be more as a risk factor for development of Hashimoto's disease in the population studied.

## Introduction

Autoimmune thyroid diseases (AITD), including Graves' (GD) and Hashimoto's Disease (HD) are examples of organ specific auto-immune disease. Severity as well as the complexity of AITDs, is difficult to predict since they are dependent on the genetic capability of the patients to express, and produce immune regulatory factors (1).

In addition to genetic factors, the non-genetic factors such as dietary and environmental factors have also been shown to trigger the inflammatory pattern. Hashimoto's thyroiditis (HT) and Psoriasis, the two

of the common inflammatory diseases, affect the overall health as well as quality of life (2). HT is the most commonly occurring autoimmune disease with an annual incidence rate of 3.5 cases among 1000 individuals. Due to the thyroid gland damage, hypothyroidism is caused as a response, mediated by the auto antibodies. The disease progression and management mainly rely on the early diagnosis of HT (3).

Hashimoto's Thyroiditis, a T cell-mediated auto-immune disorder, is characterized by goiter, presence of circulating serum antithyroid peroxidase and/or antithyroglobulin antibodies, as well as intra-thyroidal infiltration of T and B cells with a predominance of CD4<sup>+</sup> Th1. This causes thyroid hypofunction to varying degrees. Various studies have shown that lower VitD levels appear to be closely associated with the risk of HT onset. Patients with HT are found to harbor a higher proportion of hypovitaminosis D, indicating that HT is more closely associated with vit-D deficiency (4).

Although the mechanism is not clear, vit-D has been shown to have protective effects on thyroid malignancies as well as auto-immune thyroid disease. The significant role of adequate vit-D levels has been supported by many researches. Vit-D supplementation is observed to be beneficial for reduction of inflammation (5).

Vit-D refers to a group of steroid compounds that include Vit-D2 (ergocalciferol) and Vit-D3 (cholecalciferol). Primarily it functions to regulate phosphor-calcium metabolism and also to promote bone homeostasis. Moreover, the discovery of the Vit-D-receptor (VDR) gene and the enzymes responsible for its metabolism show that this vitamin also influences several diseases (6). Approximately 200 genes in human body are functionally controlled by Vitamin D. Worldwide an ignored epidemic, the deficiencies of Vitamin D are associated with the risk of hypertension, infectious diseases, and autoimmune disease (7).

In recent past, the non-calcemic effects of vit-D has been studied, demonstrating an association of circulating vit-D levels with disease outcome. The cells of the immune system have shown the ability to synthesize the active metabolite of Vit-D which also exhibits immunomodulatory properties (8).

The main sources of Vitmain D in humans are skin production, dietary sources and supplementation. Both VitD2 and VitD3 are hydroxylated to calcidiol (25-hydroxyvitamin-D), the major circulating form of VitD, which is used as an indicator to measure VitD status in an organism. The half life of calcidiol is 2–3 weeks. The hydroxylation occurs in two steps: first being carried out in the liver whereas second hydroxylation is carried out in kidney. 1- $\alpha$ -hydroxylase, (CYP27B1) mediated the second hydroxylation where calcidiol is converted to calcitriol, which is the biologically active form of VitD. Calcitriol then binds with nuclear VitD receptor in the target cells, thereby regulating expression of more than 3–5% of human genome (200 genes). Both VDR and CYP27B1 are found in almost all body cells including immune cells as well as thyroid tissues The evolving association of lower levels of VitD with hypothyroidism may be attributed to the single primordial gene from which both the VitD 3 receptor as well as receptor for thyroid hormone are thought to evolve. This suggests strong homology between the two receptors (9).

Many of the genetic studies have shown the association between an individual's predisposition to autoimmune disorders and the polymorphisms of various enzymes and proteins. These enzymes and proteins including CYP27B1, CYP2R1, and CYP24A1, VDR, DBP are associated with VitD functions (10).

This study aims to establish the molecular relationship between vit-D levels and hypothyroidism in patients with Hashimoto's Disease as well as characterization of VDR gene polymorphisms in the local population.

## Materials And Methods

Sample collection:

A total of 144 samples were collected, including 72 healthy individuals and 72 patients presenting with Hashimoto's disease. Informed consents were also obtained from the individuals included in this study.

Inclusion Criteria:

Patients presenting with symptoms of HT were included in this study.

DNA Extraction:

DNA was isolated by using Phenol Chloroform (Organic) method in accordance with the guidelines given in Sambrook and Russell et al (11). Extracted DNA was run on 1% agarose gel at 500 mA of current with 70 volts for 60 minutes in gel electrophoresis and visualised under UV Trans-Illuminator bio Doc Analyzer. DNA quantity was measured using Multi Skan Go Instrument (Thermo Scientific) at 260/280nm.

PCR Amplification:

Extracted DNA was then amplified using Galaxy XP Thermal Cycler (BIOER, PRC). Primers were designed using Primer3 software (12) implemented in Geneious 8.1.9 (13). Polymerase chain reactions were run using three primers: a reverse primer, a forward primer containing M13 tail at its 5' end, and a third primer consisting of a barcode and M13 tail. A Total of 24 barcodes were used to tag / identify 24 individuals, while forward and reverse primers were used to attach to their respective loci. Table 1 shows the primer sequences.

The initial denaturation was carried out at 95°C for 10 min. Amplification was carried out at total of 40 cycles. Each cycle was carried out 95°C for 1 min, 54.8°C for 1 min and extension at 72°C for 1 min. A final extension was carried out at 72°C for 10 min.

Amplicon sequencing

For amplicon sequencing, a total of 144 individual PCR products (24 individuals x 6 loci) were pooled together in one tube. Six separate tubes (three tubes for the control group and three tubes for the diseases group) were sent to Azenta Inc. Hong Kong for the amplicon sequencing for 150 base pair paired end

sequencing run using Illumina HiSeq platform. Approx 1 GB data for each tube was requested. After the sequencing, the sequencing data in each tube (short reads) were binned / separated first on the basis of loci into six folders. Within each folder for individual locus, the data were further assorted on the basis of 24 barcodes for the individual persons.

Identification of SNPs and noting the homozygosity / heterozygosity status

Each individual locus for every individual person showed multiple coverage depth, hence the accuracy of the SNPs determined was high. For each individual person at each locus, the sequencing data in fasta format were copied in MS Excel. Around 20 bases adjacent to the SNP position were used to find exact match and count the number of alleles for every SNP. Depending upon the allele count, homozygosity and heterozygosity was determined and noted for all individual SNPs at all loci for all individuals.

Statistical analysis:

Chi square tests were applied to find out significance of correlations of genotypes and alleles for the individual loci using SPSS v28.0.1.

## Results

This study included a total of 144 individuals, including 72 healthy individuals and 72 patients presenting with Hashimoto's Thyroiditis. Table 2 shows the mean age of the diseased and control individuals along with their gender.

DNA Genotyping:

The extracted DNA samples were run on agarose gel with 1Kb ladder (Fig-1).

VDR SNP Polymorphisms:

VDR SNPs observed at four loci were rs1544410, rs731236, rs7975232 and rs2228570. Table 3 shows the genotypes obtained at each locus.

Both of the loci rs1544410 and rs731236 were observed to be heterozygous (CT and AT, respectively). There was however non-significant frequency distribution among diseased vs control individuals studied.

At rs7975232, diseased individuals exhibited heterozygous genotype (CA) at higher frequency, whereas the homozygous (CC) allele was observed at higher numbers in control individuals. The frequency distribution between diseased and control individuals was found to be significant at this locus.

At rs2228570, heterozygous allele was found to be most prevalent (GA). The frequency was however non-significant among control vs diseased individuals.

## Discussion

In autoimmune diseases, the body's immune system identifies host's own cells as foreign elements, thereby developing immune response against own cells. Both genetic predispositions as well as external triggers cause the disease to manifest the autoimmune diseases. Among external factors, low serum Vit-D levels have not only been shown to predict the course of autoimmune disease, but also the progression of existing autoimmune diseases (14). Vit-D supplementation seems beneficial in case of autoimmune thyroid disorders. More recently, the role of Vit-D has been shown to be far beyond perceptions, suggesting its role in regulation of immune system as well as in altering cell differentiation and proliferations. Thus, Vitamin D serves as one of the excellent contributors to various inflammatory and/or auto immune diseases (15).

The metabolization of vitamin D to its active form, and its interaction with a vitamin D receptor protein (VDR) determines the effectivity of the Vitamin D signalling. The processes of metabolization as well as enzymatic deactivation are mediated by different molecules. These are encoded by genes classified as vitamin-D related genes. Studies suggest the influence of SNPs within these genes on the serum levels of Vitamin D (16).

HD is observed to occur more in females than in males. A 10:1 female to male ratio is commonly observed in disease occurrence (17). In their study, Kamyshna et al have shown association between low vit-D status and HD, indicating a link between vitamin D deficiency and HD. The present study shows significantly decreased levels of Vitamin D in patients than in the control group studied. These results support the study conducted by Kamyshna et al suggesting the association between low serum vit D levels and hashimoto's disease (18).

Four polymorphisms of the VDR gene (rs7975232, rs2228570, rs731236, and rs1544410), have been linked to the risk of HD. However, different studies have produced inconsistent results. The present study aimed at the genotyping of functional VDR polymorphisms (were rs1544410, rs7975232, rs731236 and rs2228570) to elaborate their association with development of Hashimoto's disease in the local population.

In the present study, heterogenous allele frequency was prevalent in all the four loci studied. There was no significant frequency distribution among diseased versus control groups at rs1544410, rs731236 and rs2228570. However, the locus rs7975232 was observed to illustrate significance difference of allelic frequencies between the diseased vs control group. These results are also supported by the study of Maciejewski et al, who reported a weak association between rs7975232 and rs1544410 SNPs and volume of thyroid gland in autoimmune thyroiditis individuals (19).

Xia et al has reported altered VDR expressions because of VDR gene polymorphisms. Different studies conducted to investigate relationship of these polymorphisms with autoimmune disorders illustrate different results between different races and ethnic groups (20). VDR rs2228570 and rs731236 were significantly associated with risk of HD in a Turkish population whereas a significant association between rs2228570 and risk of HD risk has been reported in Serbian population (21, 22). Wang et al. has also suggested varied risk of prevalence of VDR polymorphisms among different ethnic groups; the risk

of HD in rs2228570 was shown to be higher in Asian Population, whereas Caucasians did not show the same (23). The inconsistency may be attributed to certain other factors, including different genetic backgrounds; different environmental factors as well as the difference in the lifestyles such as diet and exposure to sunlight.

Giovinazzo et al have reported no significant difference in either allelic or genotypic frequencies of the VDR SNPs studied in HD patients as compared to healthy controls (24). In the present study, the frequencies of the CC genotype at VDR locus rs7975232 were lower in the HD group than in control subjects. This data is not consistent with the previously published study by Inoue et al; 2014 who report a higher frequency of C allele in patients with autoimmune thyroid disease(1).

In our population, no previous studies have been published regarding the association of VDR polymorphisms in Hashimoto's patients. The current study therefore concludes the lower frequency of CC genotype at rs7975232 suggestive of risk of development of Hashimoto's Disease in local population.

## **Declarations**

### **Ethical approval:**

The study design and protocol were approved by Institutional Review Committee (IRC), Islamic International Medical College, Riphah International University, Islamabad. All the experimental work was carried out in accordance with relevant guidelines and regulations.

### **Availability of Data and materials:**

All sequencing data is available through the National Library of Medicine Sequencing Read Archive (SRA), accession number: Bio project PRJNA947482. Following are the details. Accession Title Links Status Release Date Created Updated Submission PRJNA947482 Vitamin D Receptor Gene and interleukin polymorphisms Bio Sample: 144, SRA: 144 Released 2023-03-30 2023-03-22T11:44:03.623Z 2023-03-30T08:32:49.353Z SUB12941017

### **Consent to publish:**

Not applicable.

### **Funding:**

No funding was received from any institute for the current study.

### **Conflict of Interest:**

All authors mentioned in this manuscript declare that they have no conflict of interest.

### **Author Contribution:**

As conceived and designed the study, sample selection & collected samples, performed analysis, collected the data, bioinformatics analysis, drafted the manuscript. AKN, Reviewed the manuscript, JAK, clinical diagnosis, sample selection. NF, drafted the manuscript, reviewed the manuscript, MM, Bioinformatics analysis. All authors read and approved the final manuscript.

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### **Author details:**

Not applicable.

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## tables

**Table 1. Primers designed for VDR SNP analysis:**

SNP	Forward primer	Reverse Primer
rs2228570	CACGACGTTGTAAAACGAGTTCCGGTCAAAGTCTCC	ATGTATGAGGGCTCCGAA
rs1544410	CACGACGTTGTAAAACGAAACAGGAATGTTGAGCCC	GAGACGTAGCAAAAGGAGAC
rs731236 and rs7975232	CACGACGTTGTAAAACGACGGATGTACGTCTGCAG	TCACTGGAGGGCTTTGG

\*This product size is based on the length of the nucleotides on human genome. Expected amplicon sizes for all loci were 24 bp (6 bp unique barcode plus 18 bp M13 sequence) longer than the length stated here.

**Table-2: mean age of control and diseased individuals:**

	Age	
Diseased (n=72)	m(n=16)	33±6
	f (n=56)	37±11
Controls (n=72)	m (n=10)	37±11
	f(n=62)	31±8

**Table-3: Genotype and allelic frequencies at the SNPs studied**

Allele	Genotype	Frequency in control	Frequency in HD	p value
rs1544410	CC	11(15.5)	9(12.5)	0.87
	CT	57(80.3)	60(83.3)	
	TT	3(4.2)	3(4.2)	
	C	79(55.6)	78(54.2)	0.80
	T	63(44.4)	66(45.8)	
rs731236	AA	27 (38.6)	26(37.3)	0.87
	AG	40 (57.1)	42(60)	
	GG	3 (4.3)	2(2.9)	
	A	94(67.1)	94(67.1)	1.0
	G	46(32.9)	46(32.9)	
rs7975232	CC	21(55.3)	8(22.2)	0.014*
	CA	9(23.7)	16(44.5)	
	AA	8(21)	12 (33.3)	
	C	51(67.1)	32(44.4)	0.005*
	A	25(32.9)	40(55.6)	
rs2228570	GG	23 (0.38)	25(0.38)	0.88
	GA	34(0.56)	38(0.58)	
	AA	4(0.07)	3 (0.04)	
	G	80(0.68)	88(0.67)	0.85
	A	38(0.32)	44(0.33)	

## Figures

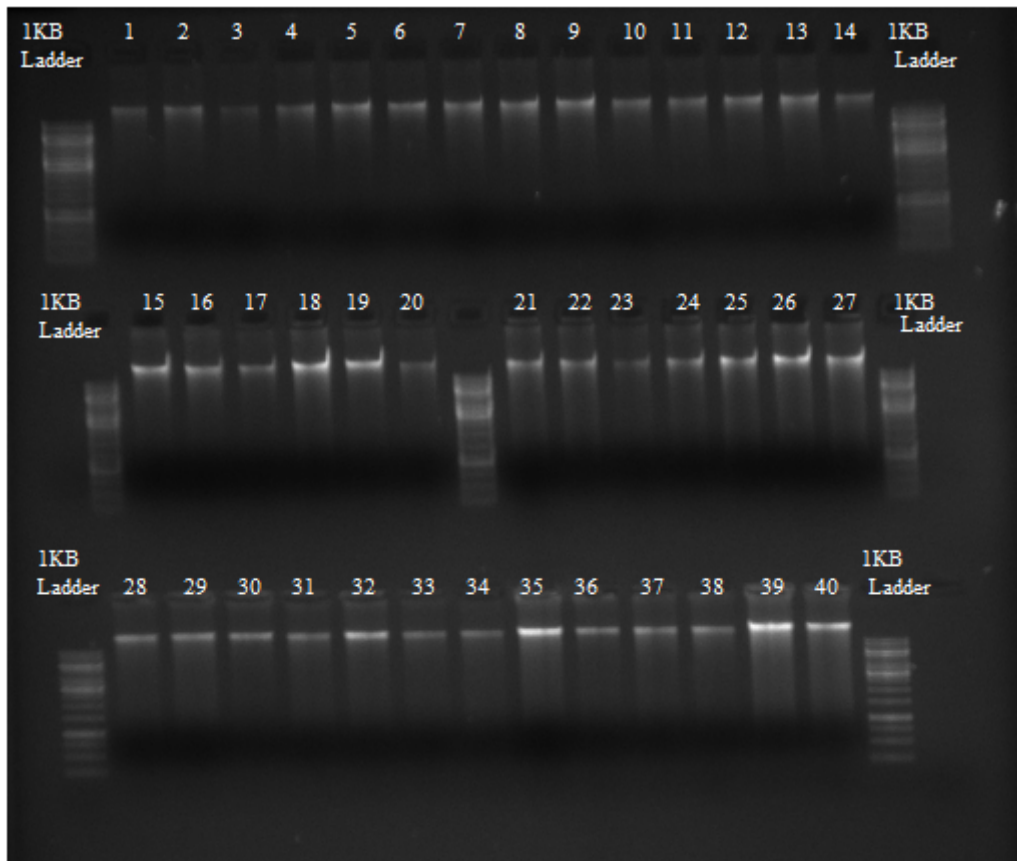


Figure 1

Genomic DNA with 1Kb Ladder

## Supplementary Files

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- [AITDSupplementarydataFile.xlsx](#)