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ORIGINAL RESEARCH

Tru9I Variant as a Novel Genetic Marker for Vitamin D Deficiency in Alopecia Areata

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Introduction: Alopecia areata (AA), is a common autoimmune nonscarring alopecia. Vitamin D is involved in various biological processes such as immune regulation, cellular growth, and specialization, as well as the maintenance of the hair cycle. We aimed to explore the impact of different Tru9I variant genotypes on serum vitamin D levels and vitamin D receptor (VDR) gene expression.

Methods: Case–control study that included 72 individuals diagnosed with AA, along with age and sex matched healthy controls of 72 individuals. Blood samples were obtained to measure Vitamin D level and VDR gene expression focusing on Tru9I variant genotypes.

Results: Our findings indicate, for the first time, a possible association between the "U" allele and low vitamin D levels, along with altered activity of the VDR gene as observed in patients with AA.

Conclusion: This suggests a complex causal relationship between genetic factors and vitamin D in AA. Interestingly, "u" allele was found to be significantly more prevalent in the healthy control group than in the patients group, raising the possibility of its protective mechanism against the development of this disease in healthy individuals.

Keywords: Tru9I variant, vitamin D, VDR gene expression, alopecia areata

Introduction

Alopecia areata (AA) is a common autoimmune non-scarring hair loss disease affecting around 2% of the general population at some point during their lifetime. This condition not only causes distress and anxiety for patients but also elevates the risks of developing psychological and psychiatric complications.[1] The underlying mechanism of hair loss in AA is thought to be linked to the destruction of hair follicles as a consequence of the loss of immune privilege within the hair follicles. Numerous factors have been implicated in the pathogenesis of AA, including autoreactive effector T cells and mast cells, CD8+ nature killer group 2 member D (NKG2D)-positive cytotoxic T cells (CD8+NKG2D+cytotoxic T cells), Janus kinase/signal transducers and activators of transcriptional signaling (JAK/STAT) pathways, regulatory T cells (Tregs), immune checkpoints, and oxidative stress.[1,2] However, despite extensive research, a complete understanding of the pathogenesis of AA remains elusive, and unfortunately, AA remains a curable disease but may be difficulty treated and may be recurrent.[3]

Vitamin D has been demonstrated to possess a crucial function in numerous intricate mechanisms pertaining to antioxidant, anti-fibrotic, anti-inflammatory, and immunomodulatory processes.[4,5] Consequently, the deficiency of this essential vitamin contributes significantly to the emergence and progression of diverse pathological conditions, such as respiratory infections, cardiovascular disorders, cancer, autoimmune disorders, and diabetes. The intricate interplay between vitamin D and these ailments underscores the pivotal role played by this vitamin in maintaining the overall health and well-being.[6] In recent studies, vitamin D deficiency has been consistently observed in individuals affected by

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AA.[7] Furthermore, the successful utilization of topical calcipotriol, a derivative of vitamin D, in the treatment of AA has garnered substantial attention.[8,9]

Vitamin D receptor (VDR) "a member of the nuclear hormone receptor super-family" after engaging with its ligand, undergoes translocation to the nucleus, thereby establishing a vital connection that orchestrates the control and regulation of numerous genes.[10] It is well known that the expression of VDR on keratinocytes is essential for establishing a normal hair cycle.[11] Positioned on chromosome 12q13, the VDR gene has been identified to harbor a substantial number exceeding 470 single nucleotide polymorphisms (SNPs). Among these, four SNPs in the VDR gene, namely rs1544410, rs731236, rs7975232, and rs2228570, have been shown to exert influence over the activity, stability, and expression levels of VDR gene products. Consequently, these specific SNPs have the potential to induce alterations in the vitamin-D-VDR signaling axis, ultimately culminating in the manifestation of vitamin-D dysfunction.[12,13] The potential of VDR gene polymorphisms to enhance the susceptibility of autoimmune diseases like Graves' disease and psoriasis has been reported.[14] Only two studies had investigated the possible association of VDR gene polymorphisms and AA and both failed to find any association.[11,15] Of note, both studies did not involve VDR gene variant Tru9I.

A recent discovery involved the identification of a novel variant within the VDR gene, which has been given the name VDR Tru9I.[16] It is believed that the presence of polymorphisms in this specific region of the VDR gene may have an impact on the stability of its mRNA, potentially due to its association with other genetic variations.[17] Several researchers in our population have tried to study the role of VDR gene and different genetic factors in the alternation of serum vitamin D levels in different skin diseases[18–27] but Up until now, no previous research has been conducted to examine the role and significance of this variant in individuals with AA.

So, our primary objective of this case–control study was to conduct an in-depth examination of the Tru9I variant within the VDR gene, focusing specifically on its potential association with the risk for AA. Thereby, allowing for a comprehensive evaluation of the genetic variant and its potential implications on disease susceptibility. Furthermore, we aimed to explore the impact of different Tru9I variant genotypes on both the VDR gene expression and serum vitamin D levels, thereby providing a more comprehensive understanding of the potential mechanisms underlying the observed associations. By thoroughly investigating these factors, this research endeavor aimed to contribute valuable insights into the genetic and molecular underpinnings of AA, ultimately paving the way for the development of innovative therapeutic approaches and personalized treatment strategies for individuals affected by this disease.

Subjects and Methods

Subjects

We conducted a case–control study that included 72 individuals diagnosed with AA, along with age and sex matched healthy controls of 72 individuals. The sample size chosen for this study is robust and representative, ensuring that the findings can be applied to a larger population. To maintain the ethical integrity of the study, the researchers obtained the necessary approval from the esteemed Ethics Committee of Shaqra University with approval reference number: ERC_SU_202300025 and the research was carried out in accordance with the Declaration of Helsinki's rules. All participants signed written informed consent after receiving full explanation about study aim and process. All participants underwent a thorough medical history and physical examination.

Inclusion Criteria

Regardless of gender and age, patients with a clinically proven diagnosis of AA (localized, patchy or ophiasis types). Subjects who were off medications in the past three months were considered eligible for this study.

Exclusion Criteria

Alopecia totalis, alopecia universalis, and other causes of alopecia like telogen effluvium, tinea capitis, and trichotillomania. Patients taking vitamin D supplements or other vitamins for the past three months.

DNA Extraction and Genotype Analysis

DNA Extraction and Purification

Blood specimens were obtained using Na2EDTA as a coagulation inhibitor. Pursuant to the manufacturer's guidelines for blood methodology, hereditary deoxyribonucleic acid was isolated from 200 µL of entire blood utilizing the QIAamp® DNA BloodMini Kit (Qiagen, Hilden, Germany).

Genotypes Analysis of Tru9I U > u rs757343

The primer sequences utilized for the amplification of Tru9I rs757343 consisted of a forward primer, 5′-GCA GGG TAC AAA ACT TTG GAG-3′, and a reverse primer, 5′-CCT CAT CAC CGA CAT CAT GTC-3′. A DNA template ranging from 80 to 120 ng and 2.5 units of Taq polymerase from Gibco-Invitrogen were also included in the reaction mixture. The procedure involved initially heating the solution to 94°C for 2 minutes, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 69°C, and 30 seconds at 72°C. The final step entailed extending the reaction for 7 minutes at 72°C. Subsequently, the amplified PCR products were subjected to MseI restriction enzyme treatment at 37°C overnight. Upon analysis, it was observed that the wild-type ("UU") allele remained uncut, resulting in a band of 177 base pairs. ("uu") genotype, on the other hand, exhibited 91 and 86 base pair bands. Lastly, the heterozygous ("Uu") allele was cleaved into fragments of 177, 91, and 86 base pairs.[28]

RNA Extraction Gene Expression

RNA Extraction and Purification

Total RNA was obtained from blood samples by means of the Qiagen QIAamp RNA Blood Mini Kit (Catalog number: 52304), and the quality of the RNA was assessed via the employment of the Nanodropper 2000 device (ThermoScientific).

cDNA Synthesis

The accomplishment of transforming RNA into its complementary DNA (cDNA) is carried out by employing the cDNA Reverse Transcription Kit, which is produced by Invitrogen, a well-known enterprise in the realm of Life Sciences.

Quantitative Real-Time PCR

Expression analysis of the VDR gene (Human ensemble gene ID: ENSG00000111424) was conducted via quantitative real-time PCR utilizing a Bio-Rad Cycler, Maxima SYBER Green Q PCR Master Mix, cDNA, and specific primers. The forward primer sequence was 5′-CATGCATT TGTCTTTGTAATGTCAC-3′ and the reverse primer sequence was 5′-AGGAGTTC CCCGAAGAAGG-3′. To ensure accuracy, normalization was performed based on the expression level of the internal control gene beta-actin, using the forward primer sequence 5′-TGTATGAAGGCTTTTGGTCTCC-3′ and the reverse primer sequence 5′-CTGGTCTCAAGTCAGTGTACAGGT-3′.[29] The relative mRNA expression of the target gene was determined by calculating the average threshold cycle (Ct) numbers obtained from triplicate amplification reactions, and the magnitude of change in mRNA expression was assessed employing the standard $2^{-(\Delta\Delta ct)}$ method.[30]

Biochemical Analysis

Serum concentrations of 25-hydroxyvitamin D were assessed utilizing the Roche Cobas e411 system, a diagnostic tool developed by Roche Diagnostics in Switzerland. These measurements were then categorized as adequate (>20 ng/mL), inadequate (12–20 ng/mL), or deficient (<12 ng/mL). In accordance with the guidelines set forth by the Institute of Medicine's Food and Nutrition Board.[31]

Statistical Analysis

Arlequin software (version 3.1) and SNPstats were employed to compute allele frequency and genotypes http://bioinfo.iconcologia.net/SNPstats. The data were analyzed using SPSS software version 17. Employing a significance level of 0.05, two-sided tests were conducted. Various statistical tests, including independent *t*-tests, chi-square tests, and One-way ANOVA, were utilized to compare groups and explore relationships among the data.

Table 1 Demographic and Clinical Characteristics of Both Patients and Controls

Variables	AA Patients N=72	Healthy Controls N=72	p-value
Age (mean ± SD)	24.6 ± 3.6	26.5 ± 2.6	0.897
Male (N/%)	46 (64%)	42 (58.4%)	0.831
Female (N/%)	26 (36%)	30 (41.6%)	0.551
Alopecia areata type - Localized patchy - Multiple patchy - Ophiasis	 33 (44.7%) 26 (37.3%) 13 (18%)	 — — —	 NA NA NA
Serum vitamin D level (ng/mL) (mean ± SD)	11.6 ± 3.4	22.8 ± 6.5	0.001
VDR gene folding change ($2^{-\Delta\Delta ct}$) (mean ± SD)	2.6 ± 0.66	6.5 ± 1.2	0.001
VDR gene folding change ($2^{-\Delta\Delta ct}$) (mean ± SD)	2.6 ± 0.66	6.5 ± 1.2	0.001

Note: Highly significant p≤0.001.
Abbreviations: N, number; %, percentage; SD, standard deviation; AA, alopecia areata; VDR, vitamin D receptor; NA, not applied.

Results

Demographic and Clinical Data for Patients and Controls

The characteristics of demographic and clinical data are presented on Table 1. Of particular significance, we observed that the group of healthy controls displayed significantly elevated concentrations of serum vitamin D as well as increased expression of the VDR gene in comparison to the patients group (p = 0.001).

Genotypic and Allelic Frequencies for Genetic Tru9I U > u (rs757343) Variant in VDR Gene

The co-dominant model, which was expertly applied to the Tru9I U > u variant, astutely revealed noteworthy variations between the two groups, thereby substantiating the presence of a statistically significant relationship with a p-value as minuscule as 0.001. Remarkably, this identical outcome was observed in the recessive models, further corroborating the notion that there exists a significant variance between the control and AA patient groups. However, it is important to note that no such significant variation was observed in the Dominant model, p = 0.31 (Table 2A).

Upon undertaking a meticulous examination of the observed allele frequencies for the identification of the Tru9I U > u variant led to the remarkable observation that the U allele exhibited a higher frequency among AA patients in comparison to individuals without the disease. This significant finding was supported by p-value of 0.001. Conversely, the u allele, which stands in stark contrast to its U counterpart, was found to be more common in the healthy controls p = 0.001 (Table 2B).

Correlate and the Alternation in Serum Vitamin D Levels and VDR Gene Expression with Tru9I U > u (rs757343) Genotype Variants in Patients with AA

For the genotype variant U/U of the Tru9I U > u, there exists a correlation with a reduction in the levels of vitamin D as well as the expression of the VDR gene, whereas the u/u variant is linked to elevated levels of vitamin D and increased expression of the VDR gene, as indicated by a P-value of 0.001 for both associations in (Table 3).

Table 2 Association of Tru9I Variant (rs757343) in VDR Gene in AA and Healthy Controls

A) SNP	Model	AA patients N (%)	Healthy controls N (%)	OR (95% CI)	p-value
Tru9I U > u (rs757343)					
u/u	Co-dominant	27 (37.5%)	33 (45.8%)	1	0.001*
U/u		14 (19.4%)	32 (44.4%)	0.53 (0.24–1.20) 5.41	
U/U		31 (43.1%)	7 (9.7%)	(2.06–14.21)	
u/u	Dominant	27 (37.5%)	33 (45.8%)	1	0.31
U/u- U/U		45 (62.5%)	39 (54.2%)	1.41 (0.73–2.74)	
U/u - U/u	Recessive	41 (56.9%)	65 (90.3%)	1	0.001*
U/U		31 (43.1%)	7 (9.7%)	7.02 (2.83–17.42)	
B) Allele frequency	**AA patients N (%)**		**Healthy controls N (%)**		**p-value**
Tru9I U > u (rs757343)					
u	68 (47%)		98 (68%)		0.001*
U	76 (53%)		46 (32%)		0.001*

Note: *highly significant p≤0.001.
Abbreviations: SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval, %, percentage.

Table 3 Comparison of Serum Vitamin D Levels and VDR Gene Expression with Tru9I U > U (rs757343) Variant Genotypes in AA

Tru9I U > u (rs757343) Genotype Variant	U/U (N=31)	U/u (N=14)	u/u (N =27)	p-value
Serum vitamin D level (ng/mL) (mean ± SD)	8.8 ± 2.6	12.4 ± 3.9	17 ± 3.8	0.001*
VDR folding change ($2^{-\Delta\Delta ct}$) (mean ± SD)	1.2 ± 0.63	2.2 ± 0.5	4.4 ± 0.8	0.001*

Note: *Highly significant. P≤0.001.
Abbreviations: N, number; SD, standard deviation.

Compare the Alternation in Vitamin D Levels and VDR Gene Expression in Different Clinical Variants

The assessment of the levels of vitamin D and the expression of the VDR gene in various clinical types of AA has revealed a mild significant discrepancy between the levels of vitamin D and the folding change of the VDR gene among patients with Localized patchy AA, multiple patchy AA, and ophiasis AA the p-values of 0.005 and 0.002 (Table 4). It is worth noting that ophiasis AA patients display lower levels of vitamin D and VDR gene folding change when compared to those affected by Localized patchy AA and multiple patchy AA.

Table 4 Comparison of Vitamin D Levels and VDR Gene Expression and Variable Clinical Types of AA Patients

Parameters	Ophiasis (N =13)	Multiple Patchy (N =26)	Localized Patchy (N =33)	p-value
Serum vitamin D level (ng/mL) (mean ± SD)	9.6 ± 3.2	12.3 ± 2.5	13.1 ± 4.7	0.005**
VDR folding change ($2^{-\Delta\Delta ct}$) (mean ± SD)	1.8 ± 0.6	2.2 ± 0.6	3.1 ± 0.7	0.002**

Note: **Mild significant differences p≤0.005.
Abbreviations: N, number; SD, standard deviation.

Table 5 Comparison of Vitamin D Levels and VDR Gene Expression with Different Genotypes of Tru9I U > U Genotype Variant According to Clinical Types of AA

A. Ophiasis				
Tru9I U > u (rs757343) variant	**U/U (N=10)**	**U/u (N=3)**	**u/u (N= 0)**	**p-value**
Serum vitamin D level (ng/mL) (mean ± SD)	7.5 ± 2.7	11.8 ± 3.7	–	0.005**
VDR folding change ($2^{-\Delta\Delta ct}$) (mean ± SD)	1.1 ± 0.5	2.5 ± 0.6	–	0.001*
B. Multiple patchy				
Tru9I U > u (rs757343) variant	**U/U (N=6)**	**U/u (N=5)**	**u/u (N=15)**	**p-value**
Serum vitamin D level (ng/mL) (mean ± SD)	9 ± 2.5	13.1 ± 3.6	15 ± 3.9	0.001*
VDR folding change ($2^{-\Delta\Delta ct}$) (mean ± SD)	1.2 ± 0.6	2 ± 0.4	3.4 ± 0.9	0.001*
C. Localized patchy				
Tru9I U > u (rs757343) variant	**U/U (N=15)**	**U/u (N=6)**	**u/u (N=12)**	**p-value**
Serum vitamin D level (ng/mL) (mean ± SD)	10 ± 2.6	12.2 ± 3.8	19 ± 3.7	0.001*
VDR folding change ($2^{-\Delta\Delta ct}$) (mean ± SD)	1.4 ± 0.7	2.2 ± 0.5	5.4 ± 0.7	0.001*

Note: **Mild significant differences p≤0.005. *Highly significant p≤0.001.
Abbreviations: N, number; SD, standard deviation.

In the case of ophiasis AA, there are only two genotypes of Tru9I U > u U/U and U/u. On the other hand, in the case of multiple patchy and localized AA, three genotypes are observed, namely U/U, U/u, and u/u. Our research has shown that the U/U genotype is associated with lower levels of serum vitamin D and VDR gene folding change in all clinical types. Conversely, the u/u genotype is associated with an increase in serum vitamin D and VDR gene folding change, with significant variation (Table 5).

Discussion

Gene polymorphisms continue to be the focus of researchers trying to interpret the variations in risk factors for developing important diseases. VDR gene Tru9I polymorphism has never been investigated before in AA to assess their potential association with the disease.

In the present study, we investigated for the first time the association between VDR gene Tru9I polymorphism and AA. Our findings indicated that VDR gene expression was significantly higher in healthy controls than in the patients group. Moreover, our data revealed that True9I co-dominant and recessive genotypic models were significantly higher in healthy controls than in the patients group.

Importantly, our data indicated that the "U" allele was significantly more prevalent in the patients group than in the control group, suggesting its potential role in the development of AA. On the other hand, the "u" allele was found to be significantly more prevalent in the healthy control group than in the patients group, raising the possibility of its protective mechanism against the development of this disease in healthy individuals. Such protective association was reported before by Hassab et al, who found that VDR gene variants "f", "A", and "t" of FokI, as well as VDR gene, haplotypes "BAt" and "fAt" may have a protective effect against the development of multiple sclerosis in the Egyptian population.[32]

It is worth noting that True9I variant u/u genotype was not detected in ophiasis type AA, while it was present consistently in patchy and localized types of AA. This is furtherly supported by our findings that in AA patients, u/u genotype was significantly associated with an increase in both levels of serum vitamin D and VDR gene expression in

comparison to U/U genotype, which was significantly associated with decreased levels of serum vitamin D and VDR gene expression. This is opposite to the previous two studies conducted by Akar et al, who found no association between VDR gene polymorphism and AA.[11,15] However, a linkage between VDR gene polymorphism and other diseases like sporadic colorectal adenomas and Omicron variant of COVID-19 has been reported.[13,33]

Joko et al propose that VDR may play an unidentified role in hair follicle homeostasis, which is independent of active vitamin D.[34] Furthermore, alopecia has not been observed in patients with point mutations within the VDR ligand-binding pocket, indicating that VDR's regulation of hair follicle homeostasis may function independently of its ligand.[35]

Conclusion

Up to our knowledge, our study is the first study in which the results clearly suggest the possible role of VDR gene polymorphism Tru9I in AA. We were able to find that "U" allele was significantly more prevalent in patients with AA. Also, our results detected that U/U genotype was significantly associated with decreased both levels of serum vitamin D and VDR gene expression in patients with AA (localized, patchy or ophiasis type alopecia areata). Keeping in mind that this could be an era of innovative therapeutic approaches and personalized treatment, further studies with larger groups and different populations should be conducted.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Disclosure

The authors report no conflicts of interest in this work.

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