

Is there any association between a vitamin D receptor gene polymorphism (*FokI*) and pemphigus vulgaris?

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Received: 10 October 2019
 Accepted: 14 February 2020

Background: Pemphigus vulgaris (PV) is an autoimmune bullous disease of the skin and mucous membranes caused by activation and proliferation of T cells, production of Th2 cytokine profile and pathogenic antibodies. Vitamin D is a probable immunodeviator to Th2, which its actions are mediated through the vitamin D receptor (VDR). *FokI* is the only single nucleotide polymorphism (SNP) leading to VDR protein with a different structure and function. For the first time, we focused on *FokI* VDR SNP to evaluate its potential role in the genetic susceptibility to PV, particularly in the Iranian population that has a high prevalence of pemphigus.

Methods: In this case-control study, DNA samples of 122 PV patients and 233 healthy controls were extracted, and *FokI* genotyping was performed using the PCR-RFLP method.

Results: The mean allele frequencies of F and f alleles in the PV and control groups were 75% and 25%, and 78% and 22%, respectively, showing no significant difference. The genotype frequencies for FF, Ff, and ff genotypes in the case group were 57.4%, 35.2%, and 7.4%, respectively. In the control group, the frequencies were 60%, 36%, and 4%, respectively. Statistical analysis showed no significant difference between the two groups.

Conclusion: The present study concluded the frequencies of F and f alleles as approximately 77% and 23% in the gene pool of the Iranian population. Additionally, it showed no association between the *FokI* alleles and PV in this population.

Keywords: *FokI*, pemphigus, polymorphism, vitamin D receptor

Iran J Dermatol 2020; 23: 49-55

INTRODUCTION

Pemphigus is an autoimmune blistering disease affecting the skin and mucous membranes.

Pemphigus vulgaris (PV) is the most common form of pemphigus. It is caused by autoantibodies directed against desmosomes, the principal adhesion structures of keratinocytes that are

essential to the stability of stratified epithelia. Binding of autoantibodies leads to destruction of desmosomes, loss of adhesion between epidermal cells (acantholysis), and formation of epidermal blisters¹.

Numerous studies have investigated the immunologic background of these antibody-mediated bullous diseases, with various methods and controversial results, but most of them emphasize on T-helper 2 (Th2) cytokines predominance or at least a mixed Th1/Th2 cytokine balance. It has been proposed that Th2-derived cytokines can modulate the acantholytic process, and this Th2-like response contributes to pemphigus lesions^{2,3}.

Many studies have shown a lower level of vitamin D among PV patients compared to healthy controls⁴⁻⁸, and the relationship between vitamin D level and disease severity^{4,8,9}. It has been suggested as an etiological factor that may trigger autoimmunity¹⁰. In addition, it has been revealed that vitamin D metabolites may play a protective role in keratinocytes from detachment and apoptosis¹¹.

Vitamin D is a potential immunomodulator with various effects on the immune system. It is a probable immunodeviator to Th2, and its actions are mediated through the vitamin D receptor (VDR). The VDR gene is localized on 12q12-14, consists of 11 exons, and spans 75 kb. The active form of vitamin D, 1, 25-dihydroxyvitamin D3 (1,25(OH)2D), binds with high affinity to specific VDRs located in the nucleus of target cells acting as a transcription factor. They regulate the transcription and expression of genes in different vitamin D-responsive tissues, including the epidermis and various immune cells expressing VDR¹². It has been demonstrated that VDR/1,25(OH)2D interferes with the signaling of transcription factors such as NFAT, NF- κ B and AP-1 in a dose-dependent way¹³. These transcription factors act in the regulation of immunomodulatory genes, such as for numerous cytokines, effector enzymes, adhesion molecules and growth factors¹⁴. The diversity in 1,25(OH)2D-regulated genes and the presence of VDR in multiple cell types reflect the pleiotropic actions of the molecule.

For example, 1,25(OH)2D suppresses cell cycle progression and activation of T lymphocytes and reduces their secretion of cytokines such as IL-2, IL-12, tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) (Th1 cytokines), which are

the hallmark of vitamin D effect on the immune system. These cytokine changes are important in the pathogenesis of several autoimmune diseases like PV¹⁵. In Th2 cells, 1,25(OH)2D also increases the production of IL-4¹⁶. Moreover, 1,25(OH)2D potentially influences maturation, differentiation, migration, and activation of antigen-presenting cells such as dendritic cells and shaping their cytokine secretion patterns¹⁷. Therefore, a possible immune deviation from Th1 to Th2 responses can be postulated. Many researchers have documented that vitamin D can regulate the ongoing immune reaction away from a Th1 toward a Th2 response^{16,18}.

In humans, several polymorphic variants of the VDR gene have been shown, which are scattered throughout the complete VDR gene region. Among them, the *FokI* single nucleotide polymorphism (SNPs) as a T/C transition polymorphism (ATG to ACG) at the first of two potential translation initiation codons in exon 2 is the only one resulting in a VDR protein with a different structure and function¹⁹. Moreover, *FokI* is the only polymorphism not linked to any of other VDR polymorphisms²⁰. In the presence of the C allele (designated F), translation initiates at the second ATG site and lacks the three NH₂-terminal amino acids of the full-length VDR protein. On the contrary, in the presence of the T allele (designated f), translation initiates at the first ATG site and synthesizes the full-length (427 amino acids) VDR protein¹⁹.

This polymorphism leads to formation of long f-VDR protein (presence of either two ATG start codons) or short F-VDR (only one start codon owing to a T-to-C substitution) that have different immunological consequences. The short F-VDR results in a higher NFAT- and NF- κ B-driven transcription capacity and a more active immune system than the long f-VDR. Therefore, it can be hypothesized that the VDR *FokI* polymorphism can play a role in the pathogenesis of immune-mediated diseases²¹. Numerous studies have evaluated the association between inheritance of *FokI* polymorphism and genetic susceptibility to various illnesses. They include different autoimmune diseases such as type 1 diabetes mellitus, rheumatoid arthritis, multiple sclerosis, autoimmune thyroid diseases, autoimmune hepatitis, psoriasis, vitiligo, alopecia areata, recurrent aphthous stomatitis, and skin cancer in the field of dermatology²¹⁻²³. Nevertheless, an obvious and conclusive association has not yet been identified.

The role of vitamin D in immunomodulation and pathogenesis of autoimmune skin diseases is an open new topic, which is highly discussed.

Thus, considering the autoimmune basis of PV and its specific immunologic pattern, and immunomodulatory effects of the vitamin D-VDR complex, along with lack of any association study of vitamin D receptor polymorphisms and this autoimmune skin disease, as well as regarding the fact that Iran is a region with a higher PV prevalence¹, we aimed to evaluate the role of one of VDR polymorphisms, which is the *FokI* polymorphism in the translation initiation codon of the VDR gene in Iranian patients with PV compared to healthy controls. Determination of the frequency of this polymorphism and its possible relation to PV can improve the knowledge about the genetic background of the diseases.

PARTICIPANTS AND METHODS

Participants and study design

The study was conducted within a year on PV patients referred to Razi Hospital, which is a tertiary referral dermatology clinic at Autoimmune Bullous Diseases Research Center, Tehran University of Medical Sciences, Tehran, Iran. All patients with PV were included until sample size was reached. Diagnosis of PV was made based on the clinical, histological and direct immunofluorescence study of cutaneous biopsies. Demographic and clinical data of the patients were recorded. Unrelated healthy individuals were also randomly selected as the control group. Patients with any systemic autoimmune diseases such as diabetes mellitus and thyroid disorders, and multiple sclerosis were excluded from this study. In controls, people, who had a history of autoimmune blistering diseases or a family history of such disorders in first-degree relatives or being affected by any other systemic disease, were excluded. This study was approved by the Ethical Committee of Tehran University of Medical Sciences. Written informed consent was obtained from the patients and controls before sampling.

DNA extraction and genotyping of the *FokI* polymorphism

Genomic DNA was extracted from 5 ml peripheral

blood collected in EDTA vacutainers by the modified salting out method. DNA was stored at -20°C until use. Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) were performed for genotyping of single nucleotide substitution at the *FokI* (rs2228570) position of the VDR gene. VDR gene polymorphic *FokI* site (exon 2) was amplified by PCR. The primers 5'-AGCCAGCTATGTAGGGCGAATCAT-3' (VDR-F) and 5'-TCTCTTGGGTGAGGAAGCTGTGAT-3' (VDR-R) were used (Pishgam Biotech Co., Tehran, Iran) to amplify exon 2. The PCR conditions were 30 cycles at 95°C for 1 min for denaturation, 1 min at 64°C for annealing and 1 min at 72°C for the extension. The PCR products were analyzed on 2% agarose gels containing ethidium bromide (0.5mg/ml) and visualized under a UV transilluminator. A 100 bp ladder (Fermentase, Germany) was used as a marker. Exon 2 PCR products were then digested with the *FokI* restriction endonuclease enzyme at 37°C for 1 h. Digested products were run on 2% agarose gels (voltage 120v) and then stained with silver nitrate. The genotypes were classified as *FF* homozygotes (absence of the *FokI* site resulted in one fragment of 557 bp); *Ff* heterozygotes, fragments of 557 bp, 494 bp, and 63 bp; and *ff* homozygotes (presence of the *FokI* sites resulted in two fragments of 494 bp and 63 bp). Two additional cutting sites existed for the *FokI* enzyme other than the *FokI* site resulting in two 128 and 63 bp bands in all digested samples with any genotypes (Figure 1).

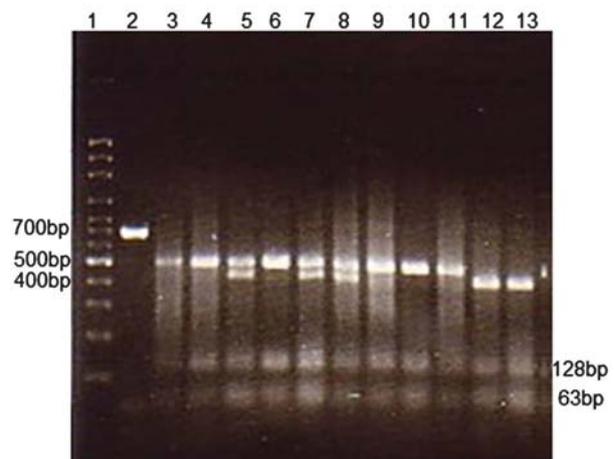


Figure 1. Restriction fragment length polymorphism (RFLP) results of *FokI* polymorphism on agarose gel electrophoresis. Lane 1, 100 bp DNA ladder. Lane 2, undigested PCR product. Lanes 3,4,6,9,10,11 *FF* homozygote genotypes. Lanes 5,7,8 *Ff* heterozygote genotypes, and lanes 12,13 *ff* homozygote genotypes

Statistical methods

Statistical analysis was performed using the SPSS software Version 17.0 (SPSS Inc). Chi-Square test and Yates' correction were used to compare the frequencies of genotypes from patients and controls. If five or fewer persons were present per group, Fisher's exact two-tailed test was used. P-value less than 0.05 was considered statistically significant.

Ethical considerations

The written informed consent was obtained from all participants.

RESULTS

In this study, a total of 122 Iranian patients with PV were recruited. They were 74 females and 48 males, age range 16–73 years, mean age 44.5 years, and mean age of disease onset 40.8 years. The patients were from different geographical regions of Iran and were referred to Razi Hospital. The distribution of three clinical phenotypes of the disease was as follows: mucous membrane form without skin involvement, 32 patients; both skin and mucous membrane involvement, 76 patients; and skin involvement without mucosal involvement, 11 patients.

FokI genotyping was carried out in the group of patients with PV and 233 healthy control subjects. Homozygous cleavage by *FokI* produced two fragments, 63 and 494 bp, whereas the heterozygotes displayed all three 557, 63 and 494 bp bands; therefore, three genotypes *FF*, *Ff* and *ff* were specified. Table 1 shows the frequencies of *FF*, *Ff* and *ff* genotypes in PV patients and control healthy subjects. The distribution of *FokI* genotypes in the PV group demonstrated no statistically significant differences compared to the healthy control group ($P = 0.35$). Allele frequencies of F and f were 75%

Table 1. Allele frequencies of *FokI* polymorphisms according to the age of disease onset in patients with PV.

Genotype	Age of onset			P-value
	≤40 years	>40 years	Total	
FF	41 (63.1%)	28 (50%)	69 (57%)	0.13
Ff	18 (27.7%)	25 (44.6%)	43 (35.5%)	
ff	6 (9.2%)	3 (5.4%)	9 (7.4%)	
Total	65 (100%)	11 (100%)	76 (100%)	

and 25% in the patient group, and 78% and 22% in the control group, respectively. The comparison of patients and controls revealed no significant differences in F and f alleles frequencies ($P = 0.31$). In Tables 1 and 2, you can see the allele frequencies based on the patients' age of disease onset and site of involvement, respectively. We concluded that the frequencies of F and f alleles were approximately 77% and 23% in the gene pool of the Iranian population (Table 3). The comparison of *FF*, *Ff* and *ff* genotypes among three phenotypic groups of the patients and between the two age groups (≤ 40 and > 40 years) at the disease presentation demonstrated no significant difference ($P = 0.49$ and 0.13 respectively).

DISCUSSION

According to epidemiological studies conducted in different ethnic populations and family studies, the genetic background can influence the occurrence of pemphigus²⁴. A relatively strong

Table 3. Genotype and allele frequencies of *FokI* polymorphisms in patients with PV and controls.

<i>FokI</i> genotype	Patients	Controls	P-value
FF	70 (57.4%)	141 (60%)	0.35
Ff	43 (35.2%)	83 (36%)	
ff	9 (7.4%)	9 (4%)	
Total	122 (100%)	233 (100%)	
Allelic frequency of <i>FokI</i>			
F	183 (75%)	365 (78%)	0.35
f	61 (25%)	101 (22%)	
Total	244 (100%)	466 (100%)	

Table 2. Allele frequencies of *FokI* polymorphisms according to the site of involvement in patients with PV.

Genotype	Pemphigus type			P-value
	Mucosal	Cutaneous	Mucocutaneous	
FF	21 (65.6%)	5 (45.4%)	42 (55.3%)	0.49
Ff	10 (31.3%)	4 (36.4%)	28 (36.8%)	
ff	1 (3.1%)	2 (18.2%)	6 (7.9%)	
Total	32 (100%)	11 (100%)	76 (100%)	

genetic predisposition to PV exists in certain ethnic groups such as Mediterranean, Persian and Ashkenazi Jewish descents¹. PV is an antibody-mediated autoimmune blistering disease that is probably caused by Th2 cells, and the vitamin D-VDR complex is identified as a strong immune-regulator, which can deviate immune response to the Th2 cytokine profile. In recent decades, cascade of studies has been launched to assess the role of VDR polymorphisms in the genetic background of different diseases.

Although there were numerous association studies evaluating the VDR gene polymorphism and various diseases, including various autoimmune diseases of different body systems, to the best of our knowledge, this is the first study investigating the association between the VDR gene polymorphism and the genetic susceptibility to PV. A number of polymorphisms have been identified in the VDR locus: an exon 2 initiation codon polymorphism detected with *FokI* restriction enzyme, *BsmI*, *Tru9I*, and *ApaI* restriction fragment length polymorphisms located between exons 8 and 9, the *TaqI* located in exon 9, and a *PolyA* polymorphism downstream of the 3'-untranslated region. Nevertheless, as previously mentioned, *FokI* is the only one leading to the VDR protein with a different structure and function as well as the only VDR polymorphism not linked to other VDR polymorphisms^{20,25}.

Overall, it has been documented that the VDR *FokI* polymorphism can affect immune system behavior, with more active immune cells in the presence of the short F-VDR protein, thereby playing a possible role in immune-mediated diseases²¹. Therefore, we focused only on the *FokI* site. We found no relationships between PV and the *FokI* RFLP VDR genotype in Iranian population. Our results suggest that this SNP, despite its associations with many other autoimmune disorders²⁶⁻³⁰ and dermatological conditions^{22,31-33} is not involved in the pathogenesis of pemphigus vulgaris and even the frequencies of the genotypes are similar in different phenotypes of the disease. It also does not affect the disease age of onset.

A number of previous reports are available on the VDR *FokI* polymorphism in Iranian population, and two studies investigated autoimmune diseases. Naderi *et al.*³⁴ indicated that the frequencies of *FF*, *Ff* and *ff* genotypes were 54%, 38.7%, and 7.3%, respectively, in Iranian control subjects, and the

frequencies of F and f alleles were 73% and 27% in their study. They suggest a probable association of the *FokI* polymorphism in the VDR gene, and the susceptibility to Crohn's disease in the Iranian population. Mohammadnejad *et al.*³⁵ found the frequency of 55%, 40% and 5% for *FF*, *Ff* and *ff* genotypes, respectively in the control group, and the frequencies of F and f alleles were 75% and 25% in their study. No significant association was observed between *FokI* VDR SNP and the susceptibility to type 1 diabetes mellitus in their survey in the Iranian population. The genotypes and allele frequencies found in our control group for *FokI* were similar to those seen in the control group of both above-mentioned studies, supporting validation of our results. Since there are many articles regarding association of dermatologic disorders with VDR gene polymorphism, which could be related to the disease susceptibility or clinical courses³⁶.

CONCLUSION

Our findings didn't identify the role of the VDR gene in the pathogenesis of PV, but we investigated only one site among various sites of the VDR gene and only the vulgaris variant of the pemphigus group of diseases. More extensive surveys on other polymorphisms of the VDR gene, such as *BsmI*, *Tru9I*, *ApaI*, *TaqI* and *PolyA*, and on other pemphigus variants such as pemphigus foliaceus are necessary. Furthermore, to conclude that there is no relationship between VDR gene polymorphism and PV, the VDR *FokI* should be further studied in other populations and larger groups, since a marked ethnic difference has been revealed in the VDR genotype. However, this preliminary study may provide insights into the VDR gene and pathogenesis of PV.

Acknowledgements

This study was supported by a research grant from Tehran University of Medical Sciences, Tehran, Iran (code 8811166001). We would like to thank Ms. Roya Moosavi for helping in patient recruitment in the study and Rasoul Akram Hospital Clinical Research Development Center (RCRDC), Iran University of Medical sciences for editorial assists.

Conflict of Interest: None declared.

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