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Vitamin D binding protein genotype frequency in familial Mediterranean fever patients

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Objective: Familial Mediterranean fever (FMF) is an autosomal recessive disorder characterized by recurrent short episodes (1–3 days) of inflammation and fever. FMF is associated with *MEFV* gene mutations but some patients with FMF symptoms do not have a mutation in the coding region of the *MEFV* gene. Vitamin D binding protein (VDBP) has important functions, including transporting vitamin D and its metabolites to target cells. Circulating levels of vitamin D are decreased in several inflammatory conditions, including FMF. Thus, we hypothesize that VDBP may play a crucial role in FMF pathogenesis, in addition to the *MEFV* gene.
Method: *VDBP* genotyping was performed by polymerase chain reaction (PCR)–restriction fragment length polymorphism in 107 FMF patients and 25 healthy individuals without FMF or family history. For this, after amplification of genomic DNA, PCR products were digested with restriction enzymes *HaeIII* and *StyI* and evaluated electrophoretically.
Results: We observed a statistically significant difference in the frequency of the 1F–2 genotype. The frequency of allele 2 was significantly higher and allele 1S was significantly lower compared to the [*MEFV*(−)] group and healthy controls ($p = 0.034$, 0.001, and 0.012, respectively). We observed a significant association between the presence of allele 2 and amyloidosis ($p = 0.026$) and arthritis ($p = 0.044$) in the [*MEFV*(−)] group.
Conclusion: Our results suggest that FMF symptoms in the absence of *MEFV* gene mutations may be due to the presence of *VDBP* allele 2. Therefore, *VDBP* genotype may explain the symptoms in FMF [*MEFV*(−)] patients.

Familial Mediterranean fever (FMF) is an autosomal recessive disorder characterized by recurrent short episodes of inflammation and fever. The *MEFV* gene was identified through positional cloning as the causative gene of FMF in 1997 by two independent groups. The *MEFV* gene is composed of 10 exons and encodes a 781 amino acid protein known as pyrin (1, 2). Four functional domains have been identified in the pyrin protein as the pyrin domain (PYD), zinc finger domain (bBox), coiled coil (CC) domain, and a B30.2/SPRY domain (3). The main function of pyrin is to control innate immune responses by regulating inflammasome formation (4). To date, more than 60 activating mutations have been identified in the pyrin gene, most of which are located in the B30.2 coding region of the gene (5). Although FMF has been associated with *MEFV* gene mutations and is considered as autosomal recessive, there are also patients displaying FMF symptoms who do not have any mutation in the coding region of the *MEFV* gene and no clear genotype–phenotype association can be made (6). This suggests that additional genes or factors play a role in developing the FMF phenotype.

The plasma protein, vitamin D binding protein (VDBP), also known as Gc-globulin, has been shown to exert several physiologically important functions, such as modulating the inflammatory response, macrophage activation, differentiation, osteoclast stimulation, and interaction with the T and B cells, in addition to the transport of vitamin D and its metabolites to target cells (7, 8). VDBP binds both active and inactive forms of vitamin D and transports 85–90% of circulating vitamin D metabolites (7). The gene that codes for VDBP is located on the chromosome 4q11–13 region and spans 35 kb. The gene is composed of 13 exons and 12 introns and is one of the most polymorphic genes in humans. The product of the *VDBP* gene is a 458 amino acid protein with vitamin D binding (residues 35–49) and actin binding (residues 373–403) domains, and a 16 amino acid leader sequence. Two common substitutions in exon 11 result in three possible isoforms designated as 1F (rs7041-T/rs4588-C), 1S (rs7041-G/rs4588-C), and 2 (rs7041-T/rs4588-A) (9). Owing to amino acid substitutions, the 1S and 2 isoforms are distinguished from the 1F isoform by significant post-translational glycosylation

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differences (10, 11). It is also well known that the deglycosylated form of VDBP is able to promote activation of macrophages and osteoclasts (12).

Growing evidence indicates that vitamin D may have immunoregulatory properties. Circulating levels of vitamin D have been shown to decrease in several inflammatory conditions, including FMF (13–15). Therefore, in the current study, our aim was to assess the *VDBP* gene isoforms in FMF patients in association with *MEFV* gene mutations and FMF symptoms.

Method

In this cross-sectional study, patients were recruited from Istanbul University–Cerrahpasa, Cerrahpasa Medical Faculty, Rheumatology Clinics. After physical and clinical examination by the rheumatologist, peripheral blood of 107 patients [68 (63.6%) females, 39 (36.4%) males] who applied for MEFV gene analysis to the Molecular Genetics Laboratory, Cerrahpasa Medical Faculty between April 2015 and June 2016 was used. The diagnosis of FMF was made according to the clinical findings in light of previously published and highly accepted diagnostic criteria (16, 17). All of the patients met the Tel-Hashomer diagnostic criteria. Inflammation was evaluated according to the manifestation of peritonitis, arthritis, and/or pleurisy. Patients with signs suggestive of autoinflammatory diseases other than FMF and patients who did not meet the criteria were excluded from the study. Blood samples of 25 healthy volunteers [(12 females (48%), 13 males (52%)] who were also examined by the rheumatologist were used as controls. None of the control subjects had FMF and/or a family history. To make sure of this, the FMF gene analysis was also performed for the control subjects.

Genomic DNA was isolated using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. To investigate *MEFV* mutations, the coding exons and flanking intronic sequences were analysed by direct sequencing. *VDBP* genotypes were determined by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP).

VDBP genotyping

The extracted DNA was amplified using 10 pmol primers (forward 5′-TAA TGA GCA AAT GAA AGA AG-3′ and reverse 5′-AAT CAC AGT AAA GAG GAG GT-3′) in a mixture (25 μL) containing 0.2 mM dNTP, 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 0.08% Nonidet P-40, 2.5 mM $MgCl_2$, 1.25 U Taq polymerase (MBI, Fermentas, Lithuania), and 300 ng genomic DNA. The size of the amplification product was 388 bp and genotyping was performed by digesting with either *Hae III* or *Sty I* restriction enzymes. The digestion products were subjected to 3% agarose gel electrophoresis and the genotypes were determined under ultraviolet light. The 1S allele has a *Hae III* restriction site but is not digested by *Sty I*. Allele 2 has only a *Sty I* restriction site, whereas 1F has neither the *Hae III* nor the *Sty I* site. Therefore, following digestion with *Hae III*, fragments of 295 and 93 bp are obtained for the 1S allele, and 304 and 84 bp fragments are observed for allele 2 after digestion with *Sty I*. When the amplicon is not digested by any enzyme then the allele type is determined as 1F.

Ethics

The study was approved by the Medical Faculty Ethics Committee (approval number 83045809/604.01/02-173216) and was performed in accordance with the ethical standards laid down in the 2013 Declaration of Helsinki. Signed informed consent was obtained from all patients.

Statistics

Statistical analyses were performed using the R Studio software. The associations between all data were analysed using the chi-squared test and Fisher's exact test. All tests were two sided and $p < 0.05$ was considered as statistically significant. 'Pwr' and 'ggplot2' packages were used for power analysis of the chi-squared test. We performed the Shapiro–Wilk normality test to check the data distribution.

Results

In total, 107 FMF patients and 25 healthy individuals without a history of FMF were enrolled in the study.

Table 1. Clinical parameters of the familial Mediterranean fever patients.

Clinical parameters		No. of patients (%)
Gender	Female	33 (63.5)
	Male	19 (36.5)
Peritonitis	Yes	35 (67.3)
	No	14 (26.9)
	Unknown	3 (5.8)
Fever	Yes	29 (55.8)
	No	22 (42.3)
	Unknown	1 (1.9)
Arthritis	Yes	39 (75)
	No	12 (23.1)
	Unknown	1 (1.9)
Amyloidosis	Yes	14 (26.9)
	No	37 (71.2)
	Unknown	1 (1.9)
Family history	Yes	9 (17.3)
	No	42 (80.8)
	Unknown	1 (1.9)
Inflammation	Yes	20 (38.5)
	No	31 (59.6)
	Unknown	1 (1.9)
Age (years), mean ± sd	21.73 ± 16.56353	

Table 2. *MEFV* gene mutation distribution in *MEFV*(+) patients.

MEFV mutations		No. of patients (%)
Homozygote mutations	V726A/V726A	1 (1.8)
	M680I/M680I	1 (1.8)
	M694V/M694V	6 (10.9)
	E148Q/E148Q	1 (1.8)
Heterozygote mutations	M680I/N	5 (9.1)
	E148Q/N	14 (25.5)
	V726A/N	4 (7.3)
	M694V/N	8 (14.5)
Compound heterozygote mutations	M680I/V726A	5 (9.1)
	M694V/M680I	4 (7.3)
	M694V/E148Q	2 (3.6)
	M694V/V726A	3 (5.5)
	M694V/R408Q	1 (1.8)

The demographic and clinical characteristics of the patients are presented in Table 1.

In 24 FMF patients both alleles of the *MEFV* gene were mutated and in 31 patients only one allele was mutated. Compound heterozygote mutations were present in 15 out of 24 patients (Table 2). The remaining 52 patients did not have any *MEFV* gene mutation. When we analysed the *VDBP* rs4588 and rs7041 polymorphisms in these patients and healthy controls, six genotypes were identified. As shown in Table 2, 1S–1S was the most frequent genotype in the *MEFV* mutation-carrying [*MEFV*(+)] group, whereas the most frequent genotypes were 1S–2 and 1S–1S in the *MEFV* mutation-negative [*MEFV*(−)] and healthy control groups, respectively. However, differences in the genotype distributions were not statistically significant between the [*MEFV*(+)] and [*MEFV*(−)] groups. When we compared the [*MEFV*(−)] group with healthy controls, we observed a statistically significant difference in the frequency of the 1F–2 genotype (p = 0.034).

Table 3. Vitamin D binding protein (*VDBP*) genotype and allele frequencies in the study population.

VDBP genotype	*MEFV*(+) (n = 55)	*MEFV*(−) (n = 52)		Control (n = 25)	
VDBP allele	n (%)	n (%)	p†‡	n (%)	p†§
1F–1F	3 (5.5)	1 (1.9)	0.317	3 (12)	0.317
1F–1S	15 (27.3)	13 (25)	0.705	5 (20)	0.059
1S–2	15 (27.3)	16 (30.8)	0.857	7 (28)	0.061
1F–2	3 (5.5)	7 (13.5)	0.206	1 (4)	0.034*
2–2	3 (5.5)	3 (5.8)	> 0.999	0 (0)	0.317
1S–1S	16 (29.1)	12 (23.1)	0.450	9 (36)	0.513
1S	62 (56.36)	53 (51)	0.401	30 (60)	0.012*
1F	24 (21.8)	22 (21.1)	0.768	12 (24)	0.086
2	24 (21.8)	29 (27.9)	0.492	8 (16)	0.001*

Data are shown as n (%).
†Statistical analysis performed using the chi-squared test.
‡*MEFV*(+) vs *MEFV*(–); §*MEFV*(–) vs Control.
*Statistically significant difference (p < 0.05).

Although we did not observe the 2–2 genotype in the healthy control group, the frequency of the 2–2 genotype was 5.6% among the FMF patients.

We also evaluated the allele frequencies in our study group. Among the three *VDBP* alleles, the most frequent allele was 1S in all groups (Table 3). The frequency of allele 2 was significantly higher and allele 1S was significantly lower in the [*MEFV*(−)] group compared to healthy controls (p = 0.001 and p = 0.012, respectively).

We also compared the clinical parameters by dividing each FMF group into two subgroups according to the presence or absence of allele 2. As a result of this comparison, we observed a statistically significant association between the presence of allele 2 and amyloidosis or arthritis in the [*MEFV*(−)] group (p = 0.026) (Table 4). However, no such association was present in the [*MEFV*(+)] group (p = 0.825).

Discussion

Following identification of the *MEFV* gene in 1997, FMF has been accepted as an autosomal recessive disease (1, 2). Since then, *MEFV* gene mutation analyses have been routinely performed for patients who are admitted to the hospital displaying FMF symptoms. However, most of the cases either are heterozygous or do not harbour any mutation in the coding region of the *MEFV* gene. Moreover, the disease is very heterogenous and the symptoms are different between patients who have the same *MEFV* genotype and as well as between members of the same family. This suggests that additional genes or mechanisms may function in FMF (18–20).

The main clinical manifestations of FMF are inflammation and fever. Inflammation is the body's response, by activating the immune system, to enable it to repair tissue damage or to defend itself against foreign organisms. The pyrin protein which is the product of the *MEFV* gene is mostly found in immune cells and functions as an inflammasome-forming receptor. However, the immune system is very complex and multiple molecules function in the formation of the inflammasome complex to induce the innate immune response. VDBP is an important component of the immune system (7, 8). There are four distinct physiological functions of VDBP. Its main function is the binding and transport of vitamin D and its metabolites to target cells. It also blocks the formation of the F-actin network by binding monomeric G-actin, which is released from dead cells (21). According to a report by Trujillo et al (22), when VDBP forms a complex with G-actin it functions as an active chemotactic cofactor. For a long time, depending on in vitro evidence, several reports indicated that VDBP functions as C5a chemotactic cofactor (23–25). Animal experiments have shown that VDBP recruits neutrophils to the inflammation site and, in addition to C5a, it functions as a cofactor for multiple

Table 4. Evaluation of vitamin D binding protein genotype distribution in familial Mediterranean fever mutation (–) and (+) patients with clinical characteristics.

Clinical characteristics		*MEFV*(–)			*MEFV*(+)		
		2 (–)	2 (+)	p	2 (–)	2 (+)	p
Gender	Female	17 (32.7)	16 (30.8)	0.773‡	22 (40)	13 (23.6)	0.833†
	Male	9 (17.3)	10 (19.2)		12 (21.8)	8 (14.5)	
Peritonitis	Yes	17 (32.7)	18 (34.6)	> 0.999‡	26 (47.3)	16 (29.1)	0.276‡
	No	7 (13.5)	7 (13.5)		8 (14.5)	5 (9.1)	
	Unknown	2 (3.8)	1 (1.9)		0 (0)	0 (0)	
Fever	Yes	14 (26.9)	15 (28.8)	> 0.999‡	19 (34.5)	14 (25.5)	0.427†
	No	11 (21.2)	11 (21.2)		15 (27.3)	7 (12.7)	
	Unknown	1 (1.9)	0 (0)		0 (0)	0 (0)	
Arthritis	Yes	19 (36,5)	12 (23.1)	0.044‡*	7 (12.7)	4 (7.3)	0.290‡
	No	6 (11.5)	14 (26.9)		27 (49.1)	17 (30.9)	
	Unknown	1 (1.9)	0 (0)		0 (0)	0 (0)	
Amyloidosis	Yes	3 (5.8)	11 (21.2)	0.026‡*	9 (16.4)	5 (9.1)	0.825†
	No	22 (42.3)	15 (28.8)		25 (45.5)	16 (29.1)	
	Unknown	1 (1.9)	0 (0)		0 (0)	0 (0)	
Family history	Yes	5 (9.6)	4 (7.7)	0.726‡	17 (30.9)	11 (20)	0.863†
	No	20 (38.5)	22 (42.3)		17 (30.9)	10 (18.2)	
	Unknown	1 (1.9)	0 (0)		0 (0)	0 (0)	
Inflammation	Yes	10 (19.2)	10 (19.2)	> 0.999‡	15 (27.3)	7 (12.7)	0.635‡
	No	15 (28.8)	16 (30.8)		18 (32.7)	14 (25.5)	
	Unknown	1 (1.9)	0 (0)		1 (1.8)	0 (0)	

Statistical analysis performed using
†chi-squared test; ‡Fisher's exact test.
*Statistically significant difference ($p < 0.05$).

chemoattractants (22). Depending on this important function of the VDBP in the immune system, we hypothesized that it may also function in the pathogenesis of FMF. The high frequency of allele 2 in patients who do not carry any *MEFV* mutations supports our hypothesis.

On the other hand, the *VDBP* gene encodes three different isoforms due to two polymorphisms at positions rs4588 and rs7041. These polymorphisms result in amino acid changes at codons 416 and 420. While the 1F and 1S alleles differ only in codon 416, allele 2 differs in both codons (9). Disaccharides or trisaccharides are linked to threonine residues in this region. Therefore, the absence of threonine in allele 2 at codon 420 prevents its glycosylation. In vitro studies have shown that VDBP is deglycosylated by T and B cells, and the deglycosylated form of VDBP acts as a macrophage activating factor, which functions in inflammation (10, 11). The high frequency of allele 2 in our [*MEFV*(–)] group indicates that this isoform may be responsible for symptoms such as amyloidosis and arthritis. The importance of allele 2 in FMF pathogenesis is also supported by the significantly lower frequency of allele 1S in *MEFV*(–) patients compared to controls.

Studies have shown that serum levels of 25-hydroxyvitamin D, one of the metabolites to which VDBP is bound, are reduced in adults and children with FMF, which has been associated with FMF attacks (13–16). Studies investigating associations between *VDBP* genotype and circulating 25-hydroxyvitamin D have revealed that higher circulating 25-hydroxyvitamin D concentrations are associated with the 1S allele (7, 26, 27). Thus, reports on low levels of 25-hydroxyvitamin D in FMF patients may be the result of high allele 2 frequency. Although vitamin D levels have been investigated in FMF patients, there is no study in the literature investigating the role of the *VDBP* gene in that context. However, a strong correlation has been shown between allele 2 and rheumatic fever in Arab children (28).

We hypothesize that in addition to the *MEFV* gene, VDBP may play a crucial role in the pathogenesis of FMF. Some patients are intolerant or resistant to colchicine treatment. However, a direct genotype–phenotype correlation between the *MEFV* gene and FMF has not been shown (6). Our results suggest that VDBP may be a strong candidate to explain the clinical symptoms in the [*MEFV*(–)] FMF patients. It is possible that vitamin D supplementation may be beneficial in [*MEFV*(–)] FMF patients harbouring the *VDBP* allele 2.

The present study has some limitations, such as the small sample size and lack of direct evidence. To prove the function and role of VDBP in FMF, there is a need for in vitro and in vivo functional studies.

Conclusion

In this preliminary study, the main purpose was to direct attention towards a possible role of VDBP in FMF. Our

data suggest that the role of the *VDBP* gene warrants detailed functional studies in FMF patients.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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