RESEARCH ARTICLE

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Apoptosis-associated Speck-like Protein Containing a CARD (ASC), TNF Like Factor 1a(TL-1a) and B Cell Chemoattractant Chemokine Ligand 13(CXCL-13) Expression Profiles in Familial Mediterranean Fever (FMF) Patients

ABSTRACT

Objective: This study was carried out to compare the expression levels of ASC(Apoptosis Associated Speck Like Protein Containing a CARD), TL-1a(TNF Like Factor 1a) and CXCL 13(B Cell Chemoattractant Chemokine Ligand 13) genes in FMF patients According to Tell-Hashomer Criteria and Genetic analysis result in Düzce University Research and Application Hospital with healthy controls and to determine their clinical significance in FMF.

Method: 36 patients (20 girls, 16 boys) and 12 healthy controls (7 girls, 5 boys) were included in the study. RNA was isolated from the peripheral blood of each individual and expression levels of ASC, TL-1a and CXCL 13 genes were determined. Routine biochemical parameters were also determined.

Results: CXCL 13 and TL-1a gene expression levels were significantly increased in patients with FMF, the expression level of the ASC gene was found to be increased in FMF patients, but not significantly.

Conclusion: The expression levels of these genes may be related to the pathogenesis of the disease and these genes could be used as a marker in the early diagnosis of the disease.

Keywords: FMF, ASC, CXCL13, TL-1a.

Ailesel Akdeniz Ateşi (AAA) Hastalarında Apoptosis-associated speck-like Protein Containing a CARD (ASC), TNF Like Factor 1a(TL-1a) ve B Cell Chemoattractant Chemokine Ligand 13(CXCL 13) Genlerinin Ekpresyon Düzeylerinin İncelenmesi

ÖZET

Amaç: Bu çalışma genetik analizi Düzce Üniversitesi Araştırma ve Uygulama Hastanesi'nde yapılan ve Tell-Hashomer Kriterleri'ne göre Ailesel Akdeniz Ateşi tanısı konan hastalarda ASC (Apoptosis Associated Speck Like Protein Containing a CARD), TL-1a(TNF Like Factor 1a) ve CXCL 13(B Cell Chemoatractant Chemokine Ligand 13) genlerinin ekspresyon düzeylerini sağlıklı kontroller ile karşılaştırmak ve AAA'daki klinik önemini belirlemek amacıyla yapılmıştır.

Gereç ve Yöntem: Çalışmaya 36 hasta (20 kız, 16 erkek) ve 12 sağlıklı kontrol (7 kız, 5 erkek) dahil edildi. Her bireyin periferik kanından RNA izole edildi ve ASC, TL-1a ve CXCL 13 genlerinin ekspresyon seviyeleri belirlendi. Rutin biyokimyasal parametreler de belirlendi.

Bulgular: AAA hastalarında CXCL 13 ve TL-1a gen ekspresyon seviyeleri anlamlı olarak artarken, ASC geninin ekspresyon seviyesinin arttığı ancak anlamlı olmadığı bulundu.

Sonuç: Bu genlerin ekspresyon düzeyleri hastalığın patogenezi ile ilişkili olabilir ve bu genler hastalığın erken tanısında bir belirteç olarak kullanılabilir.

Anahtar Kelimeler: AAA, ASC, CXCL 13, TL-1a.

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INTRODUCTION

FMF is a monogenic autoinfammatory disorder that is commonly observed in the Sephardic Jews (non-Ashkenaz Jews), Turks, Arabs and Armenians, and is primarily affects people of Mediterranean descent with a higher incidence. The disease is characterized by recurrent fever and recurrent peritonitis, pleuritis, arthritis or erysipelas-like rash (1).

In 1997, the International FMF Consortium and the French FMF Consortium identified the FMF gene using the positional cloning method simultaneously and independently of each other. This gene was called the MEFV gene, which consists of the initials of the Mediterranean FeVer, which means “Mediterranean fever, inspired by the region and symptoms of the disease. The MEFV gene is localized at 16p13.3 in the short arm of chromosome 16 and it has been reported that the 3.7 kilobase length gene consisting of 10 exons is encoding a transcription “marenostrine / pyrin which performs a protein synthesis of 781 amino acids (2).

The ASC(Apoptosis Associated Speck Like Protein Containing a CARD) gene is a cytosolic protein that encodes the binding protein consisting of two protein-protein interaction domains, the C-terminal caspase region (CARD) and the N-terminal pyrin region (PyD). It plays a key role in apoptosis and inflammation. In cell type-specific apoptosis, it supports caspase-mediated apoptosis including caspase-8 and caspase-9 (3).

The protein encoded by TL-1a is a cytokine of the tumor necrosis factor (TNF) ligand family. TNFRSF25 and TNFRSF6B are receptors for TL-1a. It acts as an autocrine factor to induce apoptosis in endothelial cells. It has been found that this cytokine also inhibits endothelial cell proliferation and can therefore function as an angiogenesis inhibitor. This gene supports Caspase activation (4).

CXCL-13(B Cell Chemoattractant Chemokine Ligand 13) is an antimicrobial peptide and chemokine that are strongly present in the follicles of the spleen, lymph nodes and Peyer's plaques. When B lymphocytes are confronted with T cells and macrophages, they provide migration by increasing calcium intake. It also allows B lymphocytes to return to follicles. CXCL-13 is chemotactic for B lenphocytes (5).

In addition to whole exome sequencing to reveal the roles of genes associated with different diseases in the etiopathogenesis (6-24), the detection of the expression levels of the genes also important for exactly understanding of their function and role to maintaining cellular homeostasis and viability (25,26). This study was conducted to determine the expression levels of ASC, TL-1a and CXCL-13 genes, demographic characteristics and to determine whether there is any relationship between expression levels of those genes and all of hemogram, sedimentation rate, CRP (C-reactive protein), Serum Amyloid A levels from patients and control group who applied to Duzce University Research and Application Hospital Pediatric Clinic with the diagnosis of FMF.

MATERIAL AND METHODS

The study included 36 patients with FMF and a control group of 12 subjects. The age, sex, weight, length, duration of the disease (years), kinship between the parents and family history of FMF disease were questioned. Patient duration, symptoms (abdominal pain, chest pain, joint pain, erythema, fever), diagnosis and colchicine use for many years, response to treatment, history of chronic kidney disease (history of amyloidosis), history of appendectomy, MEFV gene sequence analysis results were recorded. Routine tests such as leukocyte count, erythrocyte sedimentation rate (ESR), C reactive protein (CRP), Serum Amyloid A biochemical parameters were examined.

For the determination of ASC, TL-1a, CXCL-13 gene expression levels, 2 cc blood was taken from each individual into EDTA tube and RNA was isolated using PureLink® RNA Mini Kit. cDNA was synthesized from the isolated RNA using RT First Strand kit (Qiagen). The mRNA levels of the target genes (ASC, CXCL-13, TL-1a) of the patient group with FMF and the control group were determined by Real Time PCR. For the Real-Time PCR reaction, the TaqMan® Gene Expression Kit from Thermo Fisher Scientific Inc USA was used and the reactions were performed on the Applied Biosystems 7500 Fast Sequenced Detector (PE Applied Bio-systems, Foster City, CA) via Real-Time PCR. The GAPDH was used as the internal control. Expression levels of ASC, TL-1a, CXCL-13 genes were detected by quantitative real time polymerase chain reaction (qRT-PCR) method and standardized by expression of GAPDH gene.

Statistical analysis

SPSS 17.0 (SPSS, Inc., Chicago, Illinois, USA) statistical program was used to evaluate the data of the current study. Mann-Whitney U Test was used for paired comparisons. Additionally Kruskal-Wallis Test was used for more than two comparisons. Correlation test was used to investigate the relationship between two groups. The relationship between two categorical (noun or graded) variables were detected via the cross-tab analysis. Results are given as mean ± standard deviation. The statistical significance level (p value) was accepted as 0.05 in all tests.

RESULTS

The distributions of the MEFV Gene Variant in Patient with FMF were given in the table 1 (Table 1). There was no significant difference in age and sex between patients with FMF and healthy controls ($p = 0.13$ and $p > 0.05$, respectively) (Table

2). The mean disease duration was calculated as 4.9 years. Leukocyte count, Sedimentation rate, CRP and Serum Amyloid A levels were not significantly different between patients and healthy controls (p = 0.13, p = 0.14, p = 0.37 and p = 0.39, respectively) (Table 2).

Although there was no significant difference between patients with FMF and control groups in terms of ASC gene expression levels, this gene expression was higher in FMF patients than in the control group (p> 0.05) (Table 2) (Figure 1).

The expression levels of TL-1a and CXCL-13 were found to be significantly higher in the FMF patient group than in the control group (p = 0.02 and p = 0.03, respectively) (Table 3) (Figure 2,3). A significant correlation was found between ASC expression level and CXCL-13 expression level and between CXCL-13 and TL-1a expression level (p = 0.05 and p = 0.000). There was no significant relationship between ASC and TL-1a expression level (p = 0.48) (Table 4) (Figure 4,5,6)

When the relationship between the expression levels of genes and complaints of the cases to be taken into consideration; The relationship between CXCL-13 and fever (p = 0.00; r: 0.45), the relationship between CXCL-13 and abdominal pain (p = 0.01; r: 0.35), the relationship between CXCL-13 and mutation (p = 0.04; r: 0.29), the relationship between CXCL-13 and TL-1a (p = 0,000; r = 0.540), the relationship between TL-1a and fever (p = 0.01; r = 0 , 35), the relationship between TL-1a and abdominal pain (p = 0.01; r = 0.34), the relationship between TL-1a and symptom year (p = 0.01; r = 0.35) were statistically significant (Table 5).

Table 1. Distrubitions of *MEFV* Gene Variant in Patient with FMF

FMF Gene variation Tıpe	**Zygosity**	**Patient n (%)**
E148Q	Homozygous	6(16.6)
R202Q	Homozygous	9 (25)
M680I	Homozygous	3 (8.3)
M694V	Homozygous	9 (25)
V726A	Homozygous	1 (2.7)
M694V and R202Q	Homozygous / Homozygous	2 (5.6)
M694V and M680I	Homozygous / Homozygous	2 (5.6)
M694V and E148Q	Heterozygous / Heterozygous	2 (5.6)
E148Q and M680I	Heterozygous / Heterozygous	2 (5.6)

n_Number of patients
FMF: Familial Mediterranean Fever

Table 2. Demographic, clinical and laboratory characteristics of FMF patients and controls

Parameters	Control average±SD	Patient average±SD	P Value	Z Value
Age(M±SD)(min-max)	8.8±4(3-15)	10.8±4(3-18)	0.13	-1.50
Genders	7 (%58.4)5 (%41,6)	20 (%55.5)16 (%44.5)	0.74	-0.33
Height (cm)	132.4±21.9(109-165)	139.3±22.0(85.1-168.8)	0.3	-1.03
Weight (kg)	33.383±18.102(15-68.70)	36.894±15.282 (12,70-65,40)	0.43	-0.78
Fever (+)	1	18	**0.01**	-2.52
Abdominal pain (+)	2	28	**0.00**	-3.74
Joint pain (+)	1	24	**0.00**	-3.46
Erythema (+)	0	2	0.41	-0.82
Chest pain (+)	1	12	**0.09**	-1.67
Symptom year	-	4.9±4(0-15)	**0.00**	-4.30
Colchicine year	-	1.8±2(0-10)	**0.01**	-2.76
Colchicine response (+)	0	19	**0.01**	-2.67
Cr. Renal Failure (+)	0	2	0.41	-0.82
Appendectomy(+)	0	1	0.56	-0.57
Parent kinship(+)	2	2	0.42	-0.81
Family history(+)	2	23	**0.01**	-2.46
Mutation(+)	0	36	0.00	-5.24
CRP(0-0,5 mg/dl)	0.2±0.2 (0,01-0,93)	0.3±0.9 (0,01-3,83)	0.38	-0.88
Sedimentation(0-20) mm/h)	9.7±10.9 (2-40)	13.3±11.7 (1-45)	0.15	-1.44
SAA(1000-5000ng/ml)	3540.7±2425.7 (283-8314)	3302.9±3532.7 (208-10940)	0.39	-0.85
Leukocyte count	9.4±2.6 (5,40-13,80)	8.1±2.5 (5,20-17,10)	0.13	-1.51
ASC(FoldChange)($2^{-\Delta CT}$)	5.50±3.65 (1,74-13,17)	6.12±4.32 (1,03-15,45)	0.79	-0.26
CXCL13(FoldChange) ($2^{-\Delta CT}$)	0.67±0.67 (0,16-2,66)	2.37±2.50 (0,03-9,99)	**0.03**	-2.09
TL1a(FoldChange) ($2^{-\Delta CT}$)	0.41±0.23 (0,07-0,87)	1.28±1.09 (0,03-3,48)	**0.02**	-2.26

mean±standart deviation:(M±SD) minimummaximum: min-max

Table 3. Comparison of ASC, CXCL-13 and TL-1a Expression Levels in Control and FMF Patient Groups

Parameters	Control average±SD (min-max)	Patient average±SD (min-max)	P	Z
ASC(FoldChange)($2^{-\Delta\Delta C}{}_t$)	5.50±3.66 (1.74-13.17)	6.12±4.33(1.03-15.45)	0.79	-0.26
CXCL-13(FoldChange) ($2^{-\Delta\Delta C}{}_t$)	0.678±0.67 (0.16-2.66)	2.38±2.50 (0.03-9.99)	**0.03**	-2.09
TL1a(FoldChange) ($2^{-\Delta\Delta C}{}_t$)	0.41±0.23 (0.07-0.87)	1.28±1.09 (0.03-3.48)	**0.02**	2.26

mean±standart deviation:(M±SD) minimum-maximum: min-max

Table 4. Association of ASC, CXCL-13 and TL-1a Expression Levels with Other Parameters in FMF Patient Group

Parameters	ASC		CXCL-13		TL-1a	
	P	r	P	r	P	r
Age	0.86	0.30	0.86	0.30	0.53	0.11
Height	0.78	0.49	0.54	0.10	0.53	0.11
Weight	0.93	-0.01	0.83	0.04	0.85	0.03
Fever	0.52	0.11	**0.03**	0.36	0.11	0.27
Abdominal pain	0.94	0.01	0.28	0.19	0.12	0.26
Joint pain	0.50	-0.12	1.00	0.00	1.00	0.00
Erythema	0.34	-0.16	0.89	0.02	0.95	-0.01
Chest pain	0.87	-0.03	0.38	0.15	0.79	0.04
Symptom year	0.45	-0.13	0.88	-0.03	0.31	0.17
Colchicine year	0.48	-0.12	0.96	-0.01	0.87	-0.03
Colchicine response	0.60	-0.09	0.89	-0.02	0.94	0.01
Cr. Renal Disease	0.45	0.13	0.64	-0.08	0.95	-0.01
Appendectomy	0.17	-0.24	0.32	0.17	0.23	-0.20
Parent Kinship	0.45	0.13	0.27	-0.19	0.26	0.19
Family History	0.43	0.14	1.00	0.00	0.94	-0.01
Mutation	0.64	-0.08	0.57	0.10	0.46	-0.13
CRP	0.23	-0.20	0.99	-0.00	0.88	0.03
Sedimentation	0.12	-0.26	0.39	0.15	0.33	0.17
SAA	0.29	-0.18	0.66	-0.07	0.24	0.20
Leukocyte count	0.11	-0.27	0.95	0.01	0.48	0.12
ASC	-	1.00	**0.05**	0.32	0.48	0.12
CXCL-13	**0.05**	0.32	-	1.00	**0.00**	0.50
TL1-a	0.48	0.12	**0,00**	0,50	-	1.00

Table 5. The Relationship Between ASC. CXCL-13 and TL-1a Expression Levels and Other Parameters

Parameters	ASC		CXCL-13		TL-1a	
	P	r	P	r	P	r
Age	0.79	-0.04	0.56	0.09	0.15	0.21
Height	0.81	-0.04	0.37	0.13	0.17	0.20
Weight	0.54	-0.09	0.58	0.08	0.25	0.17
Fever	0.66	0.06	**0.00**	0.45	**0.01**	0.35
Abdominal pain	0.66	-0.06	**0.01**	0.35	**0.01**	0.34
Joint pain	0.54	-0.09	0.21	0.18	0.31	0.14
Erythema	0.36	-0.13	0.73	0.6	0.64	-0.012
Chest pain	0.71	-0.06	0.11	0.24	0.57	0.08
Symptom year	0.57	-0.08	0.11	0.23	**0.01**	0.35
Colchicine year	0.46	-0.10	0.38	0.12	0.60	0.07
Colchicine response	0.55	-0.09	0.39	0.12	0.53	0.09
Cr. Renal diseaese	0.41	0.12	0.76	-0.04	1.00	0.00
Appendectomy	0.14	-0.21	0.23	0.17	0.23	-0.17
Parent Kinship	0.80	0.03	0.26	-0.16	0.24	0.17
Family History	0.81	0.03	0.11	0.23	0.42	0.11
Mutation	0.91	-0.01	**0.04**	0.29	0.17	0.20
CRP	0.20	-0.18	0.53	-0.09	0.95	0.00
Sedimentation	0.17	-0.20	0.29	0.15	0.26	0.16
SAA	0.29	-0.15	0.49	-0.10	0.61	0.07
Leukocyte Count	0.37	-0.13	0.63	-0.07	0.88	-0.02
ASC	-	1.00	0.11	0.23	0.29	0.16
CXCL13	0.11	0.23	-	1.00	**0.00**	0.54
TL1a	0.29	0.16	**0.00**	0.54	-	1.00

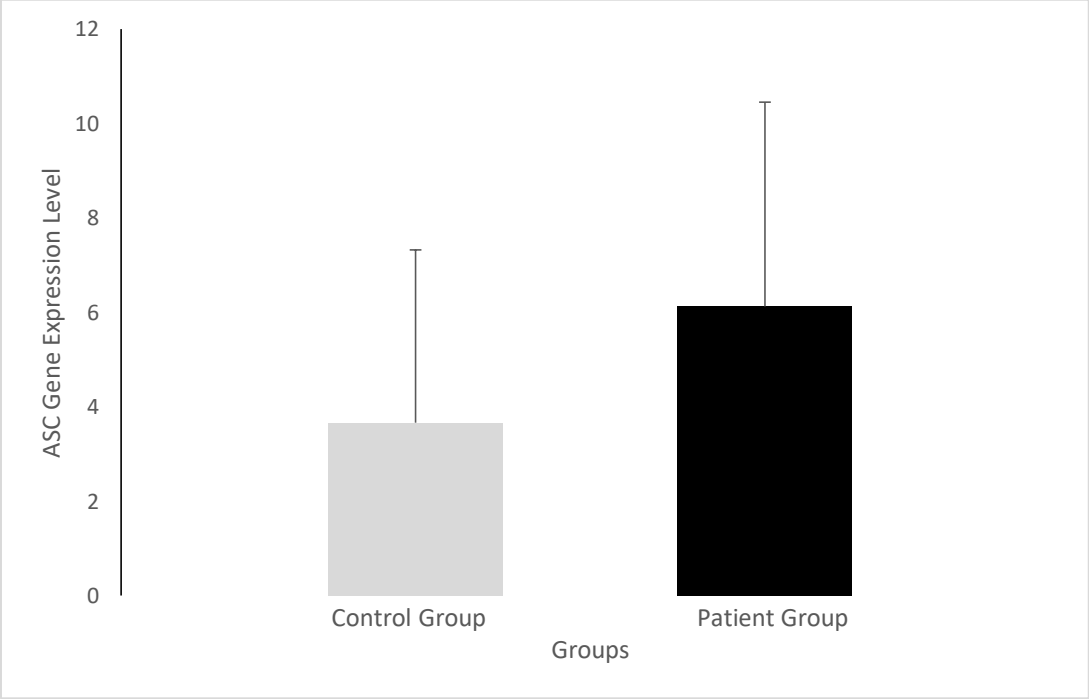

Figure 1. Comparison of ASC expression levels in patients and control groups

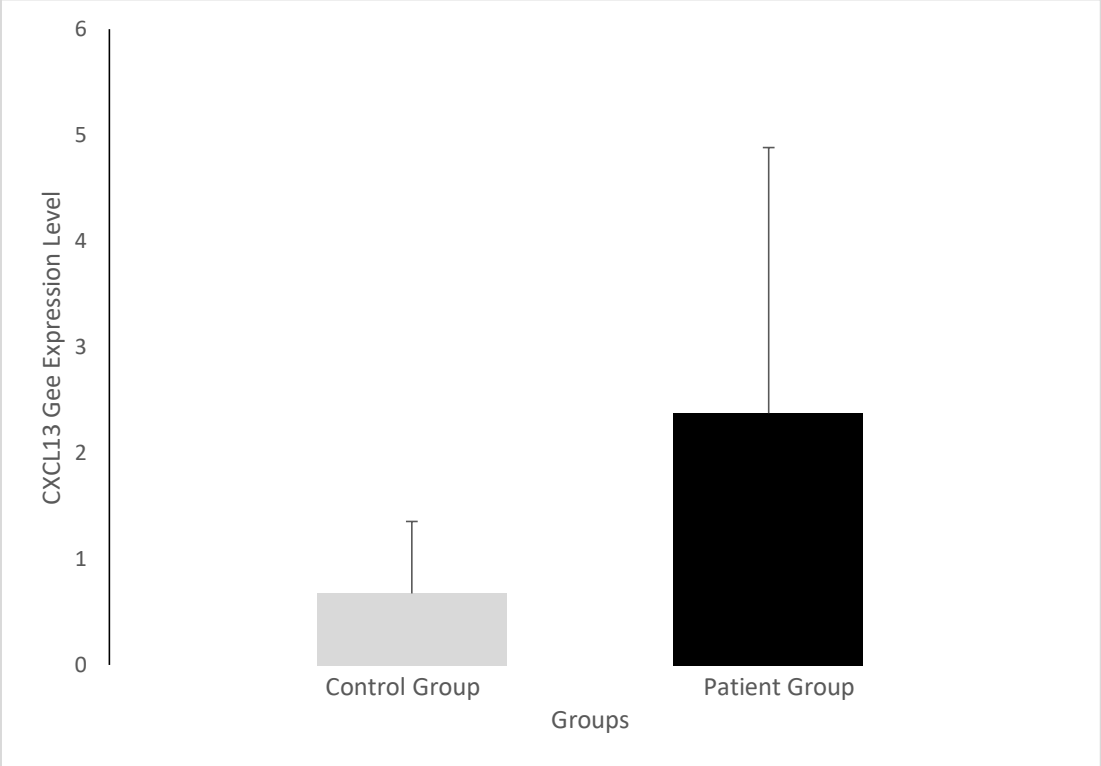

Figure 2. Comparison of CXCL-13 expression levels in patients and control groups

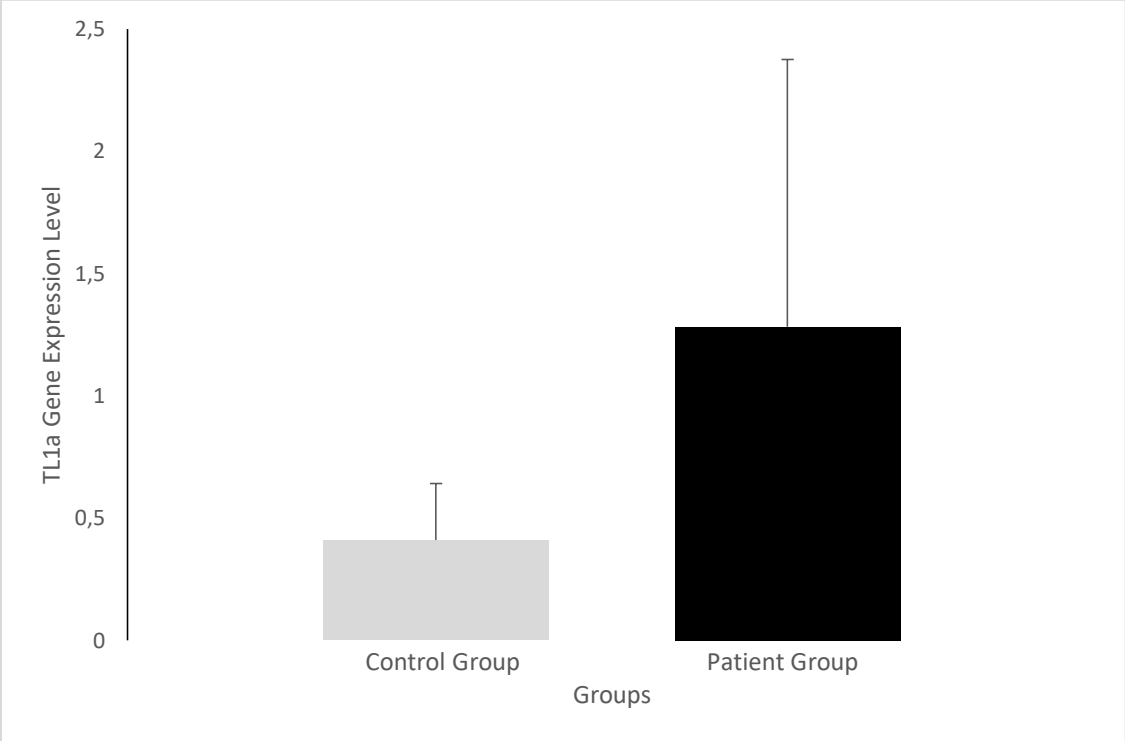

Figure 3. Comparison of TL-1a expression levels in patients and control groups

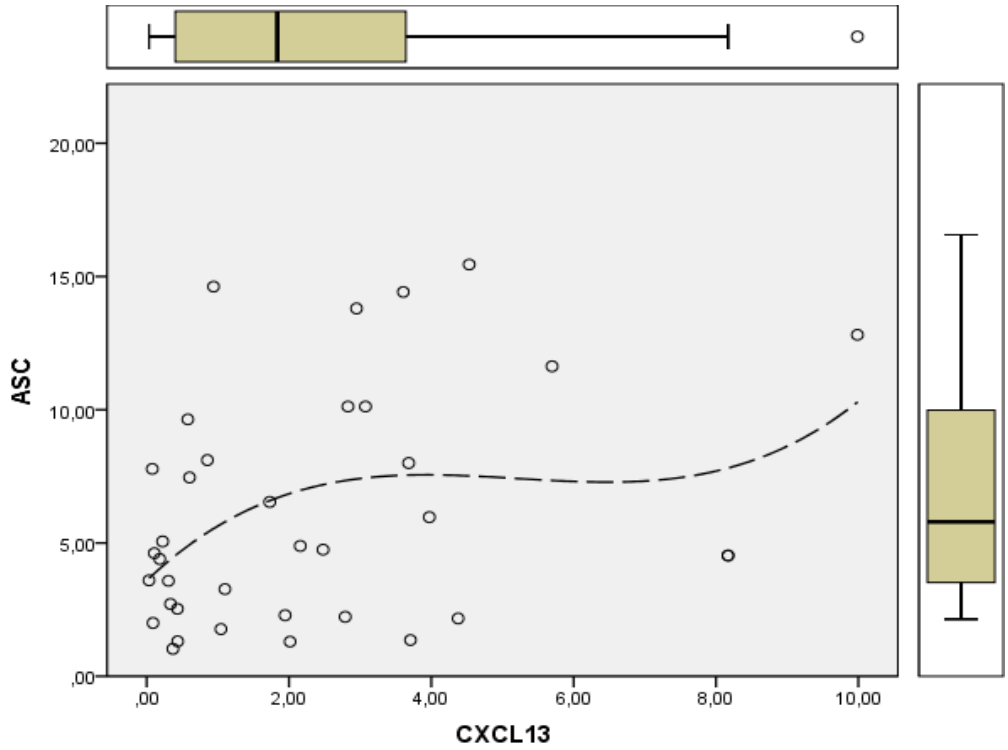

Figure 4. Cubic Table of the Relationship Between CXCL-13 and ASC

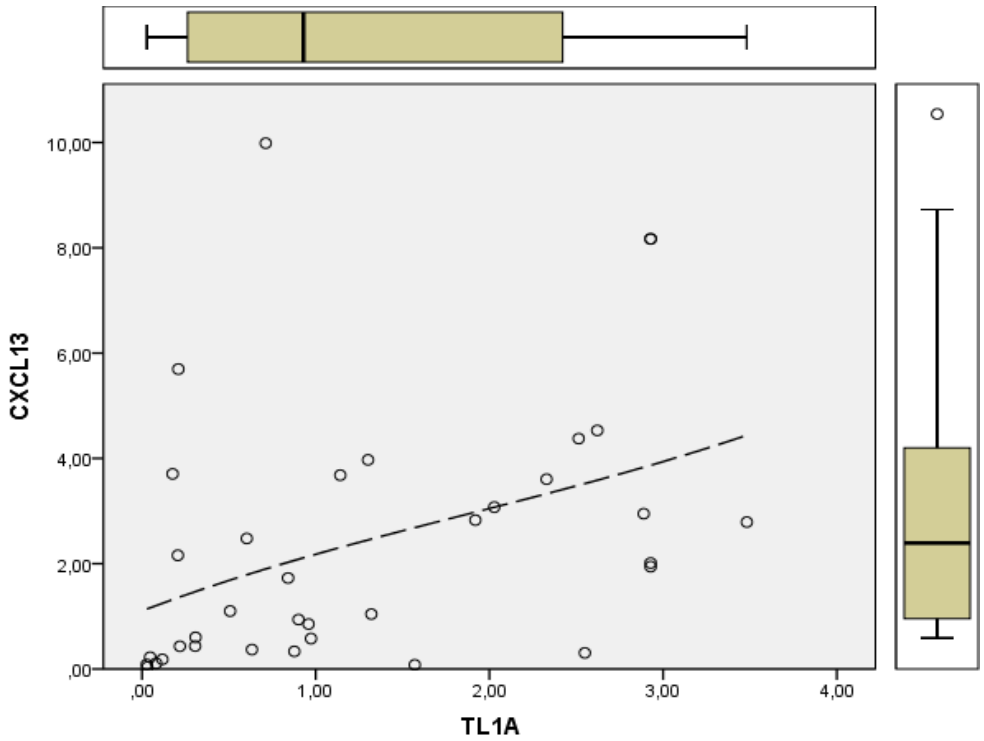

Figure 5. Cubic Table of the Relationship Between TL-1a and CXCL-13

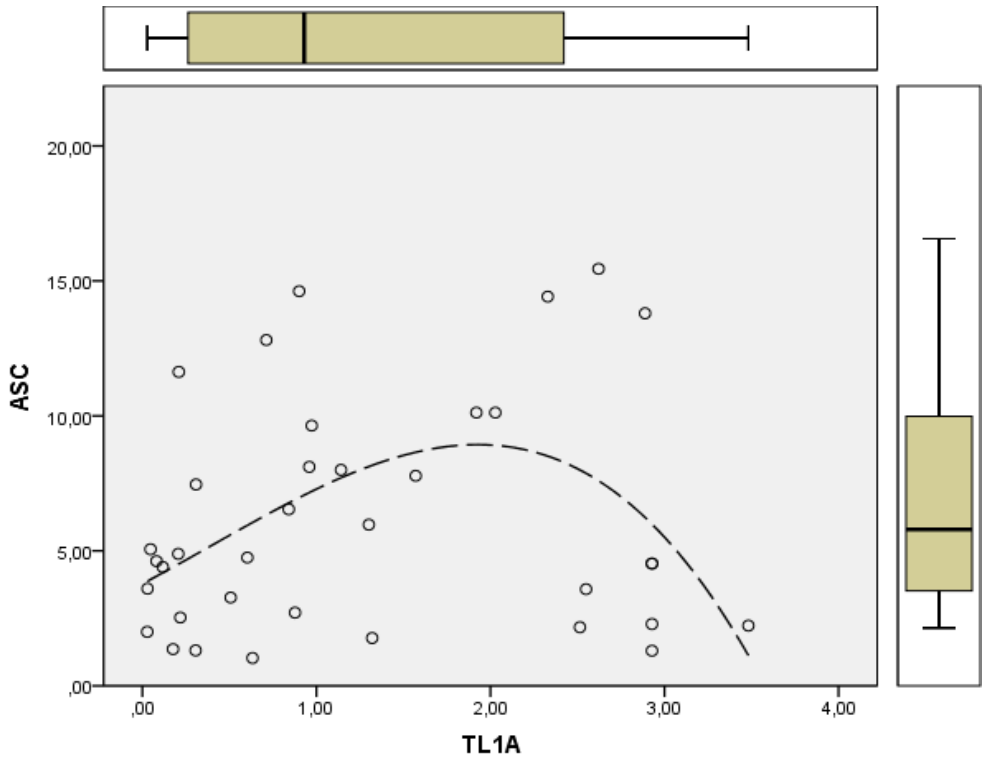

Figure 6. Cubic Table of the Relationship Between TL-1a and ASC

DISCUSSION

In addition to common variants, the novel variant such as 334-335 DelG (P. Glu112fs, C. 334-335delg) (27), K447M (P.LYS447MET, C.1340 A>T) (28), and rare variant such as A89T (p.Ala89Thr, c.265G>A) (29), E167D (30), 761_764dupCCGCp.Asn256Argfs70, c.761_ 764 dup CCGC (31), S288Y (P.SER863TYR, C.863 C>A) (32) were detected in patients with FMF.

It was reported that a case with Klippel-Feil Syndrome, bilateral Sprengel Deformity, congenital unilateral renal agenesis and a heterozygous mutation M680I(G>C) in the MEFV gene should be followed for kidney failure during her life for

amyloidosis risk (33). Patients with hereditary disease that can affect the kidney, if FMF is accompanied, closer monitoring is required for chronic kidney damage. Also it was reported that because the FMF patients with chest pain and at least one MEFV gene variant have increased risk for cardiac problems, these patients should be routinely followed up for cardiac problems (34). The understanding of both the contribution of the specific variant to clinical findings of FMF and genotype-phenotype correlation are important (1).

The symptoms of FMF can be confused with many diseases. Although clinical, laboratory and genetic assays help in making a diagnosis, there is no single diagnostic test that allows a definitive diagnosis. For this reason, it is very important to develop new markers and tests that will help to make an accurate diagnosis in the early period. That's why we planned to work now. There was a significant relationship between fever and CXCL-13 mRNA expression level ($p = 0.03$). However, there was no significant relationship between CXCL-13 mRNA expression level and both abdominal pain and joint pain ($p = 0.27$ and $p = 1.00$, respectively). According to this result, the increase in CXCL-13 mRNA expression level causes fever in patients and does not affect the occurrence of abdominal pain and joint pain symptoms.

Grossman et al. has shown that regular use of Colchicine for at least 12 months cause significant reduction in the symptoms of patients (35). In our study, a significant relationship was found with colchicine year and colchicine response in accordance with the literature. According to this result, colchicine treatment has been shown to be effective and colchicine is an effective drug in the treatment of FMF ($p = 0.00$).

Delibas et al. shown that the serum amyloid A(SAA) level was highest in the M694V gene mutation and erysipelas-like rash was more common than other mutations (36). In a study performed on gastric amyloidosis by Said et al., endoscopic evaluation revealed erythema of the gastric mucosa in some of the patients and amyloid deposition was the most common in the muscularis mucosa (37).

In our study, a significant relationship was found between erythema and Serum Amyloid A levels ($p = 0.05$). Although this suggests that erythema may be secondary to amyloid deposition in the skin, further studies are needed to fully explain this condition.

Significant relationship between symptom year and parental consanguinity was determined. According to this result, the symptoms of patients with familial mutation history were thought to start earlier than those with spontaneous mutations and the duration of symptoms was longer ($p = 0.01$).

Kasifoglu and colleagues in the study of FMF patients with end-stage renal failure in the family history of amyloidosis and end-stage renal failure revealed a significantly higher (38). In our current study, a significant relationship was found between family history and chronic kidney disease ($p = 0.04$). It is thought that the symptoms of familial mutation begin early and the risk of chronic kidney disease due to amyloid accumulation is increased in patients with a family history.

Yu et al. found that CRP and SAA levels of patients with sepsis were significantly higher than those without sepsis (39). In a study by Schellekens et al. found that CRP, SAA and leukocyte count were significantly increased in patients with acute appendicitis (40). In our study, there was a significant relationship between serum amyloid A level and CRP level and leukocyte count ($p = 0.00$). This shows us that SAA level is a good parameter for evaluating the systemic inflammatory response.

In the literature, to our knowledge only one study of ASC mRNA gene expression level in FMF patients has been found. In a study of 165 FMF patients in 2012, expression level of ASC mRNA was found to be significantly higher in MEFV gene mutation positive patients than negative, but there was no significant difference between the expression levels of ASC mRNA between MEFV mutation positive groups (41). Consistent with this study, ASC mRNA expression level was increased in the patient group compared to the control group, but this increase was not statistically significant ($p = 0.79$) (Table 3). This shows that ASC may play a role in the pathogenesis of FMF.

The best of our knowledge, there has not been any study about the CXCL-13 and TL-1a mRNA expression levels in patients with FMF. In the current study, we found that both CXCL-13 and TL-1a expression levels increased significantly in the patient group compared to the control group ($p = 0.03$; $p = 0.02$) (Table 3). This shows that CXCL-13 and TL-1a gene may have an important function in the pathogenesis of FMF disease.

As a result of this study; It may be said that ASC, TL-1a and CXCL-13 gene expressions were related to each other, that one could be the precursor or the product of the next step in the biochemical pathway and that these genes, their precursors or products could be targeted in the treatment of the disease. However, further studies are needed to better clarify the subject and to better manage the treatment strategy of the disease.

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