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ORIGINAL ARTICLE PT&R

Are follistatin-like protein 1 and follistatin-like protein 3 associated with inflammatory processes in patients with familial Mediterranean fever?

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ABSTRACT

OBJECTIVE: Follistatin-like protein 1 (FSTL-1) and follistatin-like protein 3 (FSTL-3) are glycoproteins whose associations with inflammatory cytokines were reported in previous studies. However, it is not yet known whether they have an effect on the pathogenesis of familial Mediterranean fever (FMF). We aimed to detect the FSTL-1 and FSTL-3 levels and to determine their relationship to the attack status and mutation types in patients with FMF.

METHODS: Fifty-six FMF patients and 22 healthy controls (HCs) were included in the study. Serum FSTL-1 and FSTL-3 levels were measured with the enzyme-linked immunosorbent assay method from collected serum samples. In addition, the MEditerranean FeVer (MEFV) gene mutation types of the patients were noted.

RESULTS: Serum FSTL-1 levels were significantly higher in FMF patients than in HCs (p=0.005). However, there was no significant difference in FSTL-1 levels between patients in the attack period (n=26) and in the attack-free period (n=30). FSTL-3 levels were similar between FMF patients and HCs or patients in the attack period and in the attack-free period. Furthermore, the MEFV mutation type and attack status had no significant effect on FSTL-1 and FSTL-3 levels (p>0.05).

CONCLUSION: Our results suggest that FSTL-1 may be associated with the pathogenesis of FMF, rather than FSTL-3. However, neither serum FSTL-1 nor FSTL-3 seems to be good markers to reflect inflammatory activity.

Keywords: Familial Mediterranean fever; follistatin-like protein 1; follistatin-like protein 3; inflammation; interleukin-1 beta.

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Familial Mediterranean fever (FMF) is an autosomal recessive, inherited, inflammatory disease that presents with self-limiting attacks of fever, polyserositis, arthritis, and skin lesions [1, 2]. It is more prevalent in people of Middle Eastern and Mediterranean origin, such as Turks, Jews, Arabs, Greeks, and Armenians [2, 3]. FMF is caused by mutations in the MEditerranean FeVer (MEFV) gene which is located on chromosome

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16 and encodes a protein named pyrin or marenostrin [4]. In recent years, studies revealing the role of pyrin in inflammatory processes and demonstrating the use of interleukin-1 (IL-1) inhibitors as an effective treatment alternative in colchicine-resistant FMF patients have enabled us to better understand the pathogenesis of the disease [5]. Pyrin regulates IL-1β production by interacting with multi-protein complexes called inflammasomes, which play important roles in the innate and adaptive immune systems [6]. Previously, pyrin was known to prevent caspase-1 activation by binding to components of the inflammasome such as pro-caspase-1 and nucleotide-binding domain, leucine-rich repeat, pyrin domain-containing 3 (NLRP3) [7]. Accordingly, mutated pyrin was thought to have reduced inhibitory effects. Recent studies have shown that the under appropriate stimuli, pyrin oligomerizes with other cellular proteins to form a macromolecular complex called the pyrin inflammasome, which mediates the release of IL-1β by caspase-1 activation, independent of NLRP3. Due to MEFV gene mutations, pyrin inflammasome formation is facilitated as a result of the deterioration of the relationship between pyrin and other proteins or enzymes [8].

Follistatin-like protein 1 (FSTL-1) is a soluble glycoprotein commonly expressed by non-hematopoietic mesenchymal cells. It has been proven to be involved in multiple signaling pathways and biological processes. The expression levels or patterns of FSTL-1 change in various diseases, including cardiovascular disease, cancers, and systemic autoimmune diseases [9]. Increased serum FSTL-1 levels have been found to be correlated with disease activity in various autoimmune diseases, such as juvenile idiopathic arthritis, rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE); therefore, FSTL-1 has been considered to play a role in the inflammatory response. In a collagen-induced arthritis model, endogenous FSTL-1 expression has been reported to be induced by pro-inflammatory cytokines, particularly IL-1β, in addition to tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β), and IL-6 [10, 11]. On the other hand, an interesting feature of FSTL-1 is that it increases IL-1β secretion through the NLRP3 inflammasome, possibly due to a positive feedback effect during inflammation [12].

Follistatin-like protein 3 (FSTL-3) is a glycoprotein that belongs to a different family than FSTL-1 and is lesser known than FSTL-1. FSTL-3 fundamentally regulates the functions of TGF-β superfamily members, particularly activin A and myostatin. In addition, because the FSTL-3 promoter region contains a nuclear factor-kappa B binding site, it is connected with TNF-α-associated inflammation. Moreover, recent studies have shown that FSTL-3 is positively correlated with low-grade inflammatory conditions, such as obesity and osteoarthritis [13, 14].

Highlight key points

- Follistatin-like protein 1 (FSTL-1) and follistatin-like protein 3 (FSTL-3) are glycoproteins whose associations with inflammation and inflammatory diseases were reported in previous studies. However, its importance in inflammatory processes in patients with familial Mediterranean fever (FMF) has not been investigated before.
- FSTL-1 may be associated with the pathogenesis of FMF and FSTL-3 may not be an FMF-related protein.
- Neither serum FSTL-1 nor FSTL-3 seem to be good markers to reflect inflammatory activity.

To the best of our knowledge, the association between IL-1β-mediated inflammation in patients with FMF and FSTL-1/FSTL-3 has not been investigated so far. This study aimed to determine whether circulating FSTL-1 and FSTL-3 are associated with the different stages of inflammation and the inflammatory mediators in patients with FMF, in addition to the effect of the underlying genetic (MEFV gene) mutation. Therefore, we designed a human cross-sectional study involving healthy controls (HCs) and patients with FMF in the acute-attack and attack-free periods. We hypothesized that the FSTL-1 levels would increase in patients with FMF during the acute-attack period because of its close relationship with IL-1β in the inflammatory pathway and ongoing low-grade inflammation during the attack-free period would result in elevated FSTL-3 levels.

MATERIALS AND METHODS

The present study was conducted between November 2016 and March 2018 in the Erciyes University Faculty of Medicine, Department of Physical Medicine and Rehabilitation. Before the evaluation, the study details were clearly explained to the patients and controls, and informed consent forms were received. The Helsinki principles were followed, and the study was approved by a local ethics committee (approval no. 2016/578).

Patients aged between 18 and 55 years who met the Tel-Hashomer criteria [15] were included in the study. Patients who had inflammatory diseases other than FMF, chronic diseases (e.g., diabetes mellitus or hypertension), malignancy, acute/chronic infection, or obesity (body mass index [BMI] >30 kg/m^2); who were pregnant; who consumed alcohol; and who received treatments other than colchicine were excluded from the study. Hospital workers who did not have a history of inflammatory diseases or evidence of systemic disorders, obesity, or pregnancy were included in the HC group. In total, 56 patients with FMF (29 females and 27 males) and 22 HC subjects (12 females and 10 males) met the inclusion criteria.

A standard patient evaluation form was completed by each participant. The form included the demographic features of the patients and HCs and information about the disease courses, treatments, and family histories of the patients. The patients with FMF were divided into two groups according to the attack status on the basis of their complaints, clinical findings, and acute phase reactants: Acute-attack and attack-free groups. Active disease (acute attack) was defined as including all FMF symptoms: Fever, abdominal and chest pain, arthritis/arthralgia, skin lesions, and other symptoms. Blood samples of attack-free patients were collected by waiting at least 2 weeks after acute episodes. MEFV gene mutations in the patients with FMF were recorded. The mutations included the most common variants in exon 2 (E148Q, E167D, S179I, and R202Q), exon 3 (P369S and P350R), exon 5 (Y471X and F479L), and exon 10 (M694V, R761H, A744S, M680I, G632A, V726A, K695R, M694I, I692del, and K695N) previously evaluated in the Department of Genetics. The patients in the acute-attack and attack-free groups were further divided into two subgroups according to the presence of the M694V allele mutation (patients with the M694V mutation and patients without the M694V mutation). In addition, patients in the attack-free period were separated into subgroups of those with and without subclinical inflammation based on serum amyloid A (SAA) levels and >7 mg/L indicated the patients with subclinical inflammation.

After resting in a supine position for 15 min, 10 mL blood samples were collected from the antecubital vein of each patient (7 mL for routine laboratory tests and 3 mL for analyzing FSTL-1, FSTL-3, and SAA levels). Laboratory parameters of the patients were assessed (hemogram, erythrocyte sedimentation rate [ESR], biochemistry tests, C-reactive protein [CRP] and fibrinogen tests, and complete urinalysis) during routine outpatient clinic examinations, and the results were noted. Gel biochemistry tubes consisting of 3 mL blood samples were centrifuged at 3000 ×g at 4°C for 30 min. The serum samples were then placed in Eppendorf tubes and stored at -80°C. Serum FSTL-1 and FSTL-3 levels were measured using ELISA kits (Boster Biological Technology Co., LTD, USA), according to the manufacturer's protocols in the Erciyes University Faculty of Medicine, Department of Medical Biochemistry. SAA levels were measured by the nephelometric method with a normal reference range of 0–7 mg/L using the Siemens BN II Binding Site device (serial no. 291713).

Table 1. Demographic and laboratory examinations of patients with familial Mediterranean fever and healthy controls

Variables	FMF patients (n=56)	HCs (n=22)	p
Age, years	29.54±10.09	29.27±5.55	0.446
Female/male	29/27 (51.8/48.2)	12/10 (54.5/45.5)	0.826
BMI, kg/m^2	23.83±3.42	24.01±2.39	0.850
SAA, mg/L	237.38±330.01	3.96±1.05	0.001
FSTL-1, pg/mL	1178.68±1042.35	880.68±1299.05	0.005
FSTL-3, pg/mL	1646.58±677.96	1606.4±452.85	0.594

Data are expressed as n (%) if categorical, and mean±SD if numerical. FMF: Familial Mediterranean fever; HCs: Healthy controls; BMI: Body mass index; SAA: Serum amyloid A; FSTL-1: Follistatin-like protein 1; FSTL-3: Follistatin-like protein 3; SD: Standard deviation.

Statistical Analysis

The SPSS Windows Version 24.0 package (SPSS, Chicago, IL, USA) was used for statistical analysis. The normality of the distribution of data was tested using the Shapiro–Wilk test. The Mann–Whitney U-test was used to compare non-normally distributed data between the two independent groups. In addition, the Kruskal–Wallis test and the all-pairwise multiple comparison test were used to compare numerical data among more than two independent groups. Relationships between numerical variables were assessed using Spearman's correlation coefficient, while relationships between categorical variables were assessed using the Chi-square test. Descriptive statistics for numerical variables are expressed as means±standard deviations, while those for categorical variables are expressed as numbers and percentages $p<0.05$ was considered as statistically significant.

TABLE 2. Serum follistatin-like protein-1, follistatin-like protein-3, and serum amyloid a levels in patients with familial Mediterranean fever according to the attack status

Variables	Attack-free period (n=30)	Acute attack period (n=26)	HCs (n=22)	p
FSTL-1, pg/mL	1208.18±1013.72[a]	1169.46±1126.43[a]	880.68±1299.05[b]	**0.019**
FSTL-3, pg/mL	1436.94±416.7	1881.63±852.21	1606.4±452.85	0.121
SAA, mg/L	11.07±15.24[a]	499.9±326.43[b]	3.96±1.05[a]	**0.001**

Data are expressed as mean±SD. A statistically significant difference between groups is indicated by different lower-case letters in one row. SAA: Serum amyloid A; FSTL-1: Follistatin-like protein 1; FSTL-3: Follistatin-like protein 3; HCs: Healthy controls, SD: Standard deviation.

TABLE 3. Serum follistatin-like protein-1 and follistatin-like protein-3 levels in the attack-free period according to the serum amyloid A

Variables	Patients without subclinical inflammation (n=20)	Patients with subclinical inflammation (n=10)	p
FSTL-1, pg/mL	1398.43±1160.46	754.18±323.52	0.140
FSTL-3, pg/mL	1552.3±389.79	1197.93±356.6	**0.031**

Data are expressed as mean±SD. FSTL-1: Follistatin-like protein 1; FSTL-3: Follistatin-like protein 3; SD: Standard deviation.

RESULTS

We enrolled 56 FMF patients and 22 HCs into the study. The mean age of the patients and HCs was 29.54±10.09 years and 29.27±5.55 years, respectively. There was no significant difference between the two groups in terms of age, gender, and BMI (p>0.05). Serum FSTL-1 and SAA levels were significantly higher in FMF patients than in the control group, while serum FSTL-3 levels were similar to those of the control group (Table 1). The mean disease duration was 6.93±6.13 years and the percentage of colchicine use was 78.6% in the patient group. Nine of 12 FMF patients who did not use colchicine were newly diagnosed and started on colchicine treatment after blood samples were taken. Three patients were previously diagnosed with FMF but were not using colchicine during the evaluation. In addition, the use of colchicine, compliance with treatment, and the distribution of newly diagnosed patients did not differ significantly between the acute-attack and attack-free patient groups (p>0.05).

When we evaluated the patients with regard to the attack status, 30 patients (53.6%) were in attack-free period and 26 (46.4%) were in acute-attack period. Significantly higher levels of ESR, CRP, fibrinogen, white blood cell, neutrophil, and neutrophil/lymphocyte ratio were found in the acute-attack period, compared to the attack-free period (p<0.05 for all; data not shown). Serum FSTL-1 levels were statistically significantly higher in patients both with acute-attack and attack-free periods compared to HCs (p=0.019). However, in terms of FSTL-1, there was no statistically significant difference between patients with acute-attack and attack-free periods (p>0.05). In addition, patients with acute attack had significantly higher SAA levels than both patients with attack-free and HCs (p<0.05). In contrast, no significant difference was observed among the groups in terms of serum FSTL-3 levels (p>0.05) (Table 2).

To evaluate the effect of subclinical inflammation on serum FSTL-1 and serum FSTL-3 levels, patients in the attack-free period were divided into two subgroups. Although we did not detect any difference between the groups in terms of serum FSTL-1 levels, FSTL-3 levels were significantly higher in the patients without subclinical inflammation (Table 3).

In the patient group, the distribution of MEFV mutations was determined to be twelve (21.4%) who were M694V homozygous, seven (12.5%) who were M680I homozygous, seven (12.5%) who were M694V heterozygous, twenty one (37.5%) who were compound heterozygous, eight (14.3%) with other types of mutations, and one (1.8%) with no mutation. The two most common mutation types were compound heterozygous (37.5%)

Table 4. Serum follistatin-like protein-1, follistatin-like protein-3, and serum amyloid levels in patients with familial Mediterranean fever according to the attack status and mutation types

Variables	Attack-free period (n=30)		Acute-attack period (n=26)		p[1]	p[2]
	Patients with M694V mutation (n=18)	Patients without M694V mutation (n=12)	Patients with M694V mutation (n=17)	Patients without M694V mutation (n=9)		
FSTL-1, pg/mL	1163.15±1112.54	1214.47±865.25	1260.74±1297.73	1007.03±626.08	0.391	1.000
FSTL-3, pg/mL	1408.54±409.46	1472.62±425.19	2072.96±927.94	1549.22±512.87	0.723	0.287
SAA, mg/L	14.61±18.29	5.11±4.54	508.44±335.48	483.99±306.82	0.065	0.833

Data are expressed as mean±SD. P1 and P2 indicate intra-group comparisons in the attack-free period and the acute attack period, respectively. FSTL-1: Follistatin-like protein 1; FSTL-3: Follistatin-like protein 3; SAA: Serum amyloid A; SD: Standard deviation.

and M694V homozygous (21.4%). According to the mutation types, we did not detect a statistically significant difference in the serum FSTL-1, FSTL-3, and SAA levels during the attack-free period and the acute-attack period ($p>0.05$) (Table 4).

Various correlations were applied to evaluate FSTL-1 and FSTL-3. There was a moderate positive correlation between FSTL-1 and FSTL-3 levels in all groups (i.e., attack-free period, acute attack period, and HC) ($p<0.05$). In the attack-free period, FSTL-3 showed a weak negative correlation with SAA ($r=-0.375$, $p=0.041$) and ESR ($r=-0.396$, $p=0.033$). However, no correlation was found between FSTL-3 and BMI ($p>0.05$). In the acute-attack period, there was weak negative correlation between FSTL-1 and symptom onset age ($r=-0.424$, $p=0.035$), whereas ESR showed a weak positive correlation with FSTL-1 ($r=0.432$, $p=0.031$) and a moderate positive correlation with FSTL-3 ($r = 0.517$, $p=0.008$).

DISCUSSION

The present study is the first to reveal that FSTL-1 levels are elevated in serum samples from FMF patients relative to normal controls. Furthermore, we show that serum FSTL-1 levels in FMF patients are higher than those of the HCs in both the acute-attack period and the attack-free period. However, we could not detect any differences in the attack status and mutation types. In the acute-attack period, we could not determine a significant correlation between FSTL-1 and other acute phase proteins using the detection of acute attacks, except for ESR. We also failed to find a significant difference in the serum FSTL-3 levels when comparisons were made between patients and healthy individuals or according to attack status. FSTL-3 levels were significantly higher only in the FMF (attack-free) patients without subclinical inflammation.

Although FSTL-1 functions at the molecular level are still unclear, the possible role in the pathogenesis of arthritis has been reported in previous studies [16]. Endotheliocytes are one of the main sources of FSTL-1 in human [17]. Li et al. [18] observed elevated serum FSTL-1 levels in patients with RA, SLE, Sjögren's syndrome, systemic sclerosis, ulcerative colitis, and polymyositis/dermatomyositis (PM/DM). In their review, they concluded that various autoantibodies in autoimmune diseases cause damage to endothelial cells and that inflammatory endothelial cells secrete FSTL-1. Furthermore, inflammation and damage to skeletal muscles are both considered to be the result of high FSTL-1 in PM/DM. CD54, an intercellular adhesion molecule, is commonly produced by the vascular endothelium. IL-1β, TNF-α, and IL-18 can induce CD54 expression. In the serum samples of FMF patients during the acute attack, Koga et al. [19] reported a close relationship between IL-18 and soluble CD54 (sCD54). Although they found that sCD54 levels higher in the FMF attack patients than in the HCs, they did not detect a significant difference between patients in acute attack and remission. In our study, we determined that there were higher serum FSTL-1 levels in the patient group (in both the attack-free and acute-attack periods) compared with the control group. Serum FSTL-1 levels, which did not change according to disease activity but were found to be significantly higher than in the HCs, were similar to the sCD54 results of Koga et al [19]. Hence, the el-

evated serum FSTL-1 levels in our FMF patients could be sourced from the vascular endothelium. In light of the data obtained through our study, future studies evaluating FSTL-1 levels together with endothelial markers or sCD54 could be designed.

Mutations in the genes encoding pyrin and NLRP3 (or cryopyrin), identified as inflammasome sensor proteins, lead to the development of FMF and cryopyrin-associated periodic syndromes (CAPSs), two well-known members of auto-inflammatory syndromes [20, 21]. In CAPSs, which represent a group of autosomal dominant inherited disorders consisting of neonatal onset multisystem inflammatory disorder (NOMID), Muckle-Wells syndrome, and familial cold autoinflammatory syndrome, persistently elevated SAA and CRP levels, and mostly neutrophilia, are observed due to the abnormal cryopyrin and impaired regulation of IL-1β production [22, 23]. In FMF, the relevant markers commonly increase during the acute-attack period. Gorelik et al. [24] found higher FSTL-1 levels and demonstrated persistent FSTL-1 levels despite anakinra (anti IL-1) treatment in patients with NOMID, compared to HCs. Although the mutations and inheritance patterns in the pathogenesis of FMF and CAPSs are different from each other, the increase in caspase-1 activity and its associated cytokine, IL-1β, is a common result. In fact, IL-1-blocking therapies have been used successfully in both CAPSs and colchicine-resistant FMF patients [20]. In our study, as in NOMID, FSTL-1 levels were significantly higher in patients with FMF than in the control group. On the other hand, serum FSTL-1 levels did not differ according to attack status. Unlike NOMID, inflammatory markers typically only remain high in FMF patients for 48–72 h. Therefore, the short-term inflammation in FMF may not stimulate mesenchymal cells enough to cause prominent FSTL-1 secretion.

When FSTL-3 levels were evaluated, no significant difference was found between FMF patients and HCs. Recently, FSTL-3 has been confirmed to be associated with inflammation. Plasma FSTL-3 concentrations were shown to be increased in response to TNF-α infusion [13]. In a study, serum and synovial fluid FSTL-3 levels were found to be positively correlated with the severity of knee osteoarthritis [14]. Brandt et al. [13] investigated the effect of obesity and obesity-related low-grade inflammation on FSTL-3 levels, and they concluded that increased levels of IL-6 in response to chronic low-grade inflammation in these patients might be the source of increased FSTL-3 levels.

Similar to FSTL-3, synthesis of SAA is regulated by IL-6. Chronic inflammation, which is sometimes seen during attack-free periods in FMF patients, is associated with amyloid A (AA) amyloidosis [25, 26]. Approximately 30% of FMF patients experience subclinical inflammation between acute attacks. Elevation of SAA levels can be detected in the attack-free period, despite colchicine treatment [27]. Cakan et al. [28] reported that it might be appropriate to control SAA levels in the attack-free period to evaluate subclinical inflammation. In the present study, we detected significantly elevated serum SAA levels during the acute-attack period, compared to the attack-free period or the HCs, which is compatible with the literature. Although the FSTL-3 levels in FMF patients with acute attack were relatively higher than those in the attack-free period, there was no statistically significant difference between the groups. In the attack-free period, we hoped to obtain high FSTL-3 levels in patients with high SAA levels and a positive correlation between FSTL-3 and SAA. However, we surprisingly found a poor negative correlation between FSTL-3 and SAA. Similar to FSTL-1, FSTL-3 levels did not change according to attack status. This may originate from short-term inflammatory stimulus in FMF, as opposed to diseases which cause persistent low-grade inflammation, such as obesity and osteoarthritis. Moreover, FSTL-3 may not be an FMF-related protein. Further studies are needed to confirm whether FSTL-3 plays a role in the pathogenesis of FMF, particularly in subclinical inflammation.

In the present study, we could not determine any differences of FSTL-1, FSTL-3, and SAA levels in patients who have the M694V mutation in either the attack-free period or the acute-attack period. Several studies have reported that the M694V allele mutation causes severe inflammation and increases amyloidosis risk. On the other hand, some authors have suggested that the amyloidosis risk in FMF patients may originate from causes other than the MEFV gene, such as the SAA gene [29]. Homozygous M694V mutation has been imputed as a risk factor for AA amyloidosis, together with frequent FMF attacks, long disease duration, and familial origin [30]. It has been attributed to several possible mechanisms, such as lower production of pyrin and deterioration in pyrin function after structural changes [31]. Based on our results, we above suggest that the elevated acute phase proteins in the acute-attack period of FMF might not have any effect on FSTL-1 and FSTL-3 levels. Similarly, severe inflammation and frequent attacks caused by the M694V mutation may not be associated with serum FSTL-1 and FSTL-3 levels in FMF patients.

Our study had some limitations. There was a relatively small sample size. The majority of our FMF patients had mutations in exon 10 and we could not evaluate serum FSTL-1 and FSTL-3 by taking into account different exon types. We also did not reveal the effect of the colchicine treatment and colchicine doses on FSTL-1 and FSTL-3 levels. Finally, due to the fact that our study was performed in a single center, our results cannot be generalized to the entire FMF population.

Conclusion

This study showed that the serum FSTL-1 levels were higher in FMF patients than in HCs. However, there was not a similar relationship for FSTL-3. Hence, neither the serum FSTL-1 nor the serum FSTL-3 levels seems to be good markers that reflect inflammatory activity in FMF patients. Furthermore, the MEFV mutation-type may not be associated with FSTL-1 and FSTL-3. We believe that FSTL-1 might be associated with FMF pathogenesis due to having a close connection with IL-1β production.

Ethics Committee Approval: The Erciyes University Faculty of Medicine, Clinical Research Ethics Committee granted approval for this study (date: 08.11.2016, number: 2016/578).

Conflict of Interest: No conflict of interest was declared by the authors.

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