High prolidase levels in patients with Familial Mediterranean Fever (FMF)

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Introduction. Familial Mediterranean Fever (FMF) is an autoinflammatory disease. Prolidase is a specific imidodipeptidase that plays a role in collagen degradation, and an important role in inflammation and wound healing. Hypoxia-inducible factor-1 α (HIF-1) is an important protein in the regulation of immunological response, hemostasis, vascularization. The aim of the study was to compare serum prolidase and HIF-1 α levels in patients with FMF in attack-free period and healthy control group.

Methods. Between August 2017 and December 2017, sixty patients diagnosed with FMF according to the criteria of the Tel-hashomer and admitted to Sivas Cumhuriyet University Medical Faculty, Internal Medicine Rheumatology Department and sixty healthy volunteers were enrolled in the study.

Results. Median serum prolidase levels were 72.1 (25.1–114.9) ng/ml in FMF group and 30.7 (21.3–86.2) ng/mL in healthy control (HC) group ($p = 0.018$). ROC analysis showed that the sensitivity was 65% and the specificity was 68.3% at serum prolidase levels 54.03 ng/mL ($p < 0.05$). The median serum levels of HIF-1 α in the FMF group was 482.0 (292.0–3967.0) pg/mL and 632.0 (362.0–927.0) pg/mL in the HC group ($p > 0.05$). There was no significant correlation between laboratory findings, sex, age, and prolidase ($p > 0.05$).

Conclusion. Serum prolidase enzyme levels in FMF patients with attack-free period were significantly higher than in the HC group. However, the role of prolidase and HIF1- α in the FMF disease needs to be clarified with more extensive and comprehensive studies.

Key words: Familial Mediterranean Fever (FMF), prolidase, HIF-1 α .

INTRODUCTION

Familial Mediterranean Fever (FMF) is an auto-inflammatory disease and it has been shown that uncontrolled interleukin (IL) 1 β secretion plays a role in its pathogenesis with the activation of caspase-1 from cells such as neutrophils, monocytes and macrophages [1, 2]. Prolidase is a cytosolic exopeptidase which cleaves the iminodipeptides containing proline or hydroxyproline at the C-terminal [3]. In a few studies in which the prolidase enzyme activity was assessed, it has been shown that enzyme activity increases due to collagen degradation in diseases with chronic inflammation [3]. Hypoxia-inducible factor-1 (HIF-1) α is a dimeric protein complex that plays a complementary role in the body's response to low oxygen concentration (response to hypoxia) [4]. HIF-1 is an important protein involved in the regulation of immunological response, hemostasis, vascularization and anaerobic metabolism [4]. While hypoxia-inducible factors (HIFs) are known to control T cells and macrophage activation, their function in B lymphocytes has not

yet been clearly identified. In macrophages, HIF-1 α enhances cell motility, and allows the release of pro-inflammatory cytokines [5, 6]. Recent studies have shown that HIF-1 α has an important role in the function of T helper cells (Th), regulatory T cells (T-reg), and Dendritic Cells (DCs) [7]. In our study, we aimed to compare Prolidase and HIF-1 α levels in patients with FMF and healthy individuals.

MATERIALS AND METHODS

PATIENTS

Patients who were admitted to Cumhuriyet University Faculty of Medicine (CUFM) Hospital Department of Internal Medicine-Rheumatology outpatient clinic between August 2017 and February 2018 and who were diagnosed with FMF according to Tel-Hashomer criteria but in attack-free period (at least 2 weeks after the last attack) were included in our study. All of FMF patients were under treatment of colchicine (1–2 mg). These patients had applied to

the hospital for routine control. The healthy subjects included in the control group were selected among the individuals who applied to CUFM Hospital Department of Internal Medicine-General Internal Medicine outpatient clinic for the purpose of check-up. They didn't have any active or chronic disease. Sixty patients with FMF and sixty healthy subjects who met the inclusion criteria were included in the study after reading the subject information form and after signing the informed consent form for those who agreed to participate in the study. The disease severity score defined by Pras was used. Parameters in PRAS scoring were as onset age of disease, number of attack at a month, arthritis, erysipelas like erythema, amyloidosis and dose of colchicine. According to PRAS scoring a score of 3 to 5 were reflected mild disease, a score of 6–8 mild-to-moderate disease, and a score of > 9 severe disease.

MAIN OUTCOME VARIABLES

Demographic characteristics of subjects with FMF and healthy controls as well as information on their diagnoses, diseases courses and treatments were obtained from the patient files and by one-to-one interviews with patients. In addition, laboratory parameters of the patients and information on the treatments they received were recorded. The blood samples were taken from a peripheral vein into suitable tubes, and centrifuged at 5000 G for 15 minutes and stored in an Eppendorf tube at -80°C . Thereafter, the samples were studied by ELISA method in CUFM Division of Biochemistry. SinoGeneClon® Prolidase ELISA Kit and Thermo Fisher® HIF-1 ALPHA ELISA Kit were used for measuring Serum Prolidase enzyme levels and HIF-1 α levels, respectively. The routine examination findings of patients, MEFV gene (a total of 12 mutations in exon 2 and exon 10 were examined by medical genetic department), creatinine, ALT, AST, Fibrinogen, C reactive protein (CRP), Hemogram parameters, Erythrocyte Sedimentation Rate (EsR) were recorded retrospectively.

PROCEDURES

For our study, ethics committee approval was obtained from CUFM Clinical Trials Ethics Committee with the decision dated 11.07.2017 and numbered 2017-07/28. The study was conducted in accordance with the principles of the Helsinki Declaration of World Medical Association.

STATISTICAL ANALYSIS

Statistical assessments were performed using SPSS 22 (Statistics Program for Social and Science) software. Whether the data showed normal distribution was evaluated using histogram, q-q plots and Shapiro-Wilk test. The homogeneity of variance was tested by the Levene's test. For two-group comparisons, Mann-Whitney U test and two independent sample t-tests were used for quantitative variables. For comparisons of more than two groups, one-way analysis of variance and Kruskal-Wallis tests were performed. Pearson χ^2 analysis was used to compare categorical data. Tukey's test and Dunn-Bonferroni test were performed for multiple comparisons. The correlation between quantitative data was evaluated via Spearman correlation analysis. ROC (Receiver operating characteristics) analysis was used to investigate the estimations of prolidase and HIF1- α variables on FMF status. The area under the ROC curve were calculated with a 95% confidence interval. The cut-off values for each marker were calculated with Youden's index. Sensitivity, specificity, positive predictive and negative predictive values for optimum cut-of values were in the 95% confidence interval. The significance level was considered as $p < 0.05$.

RESULTS

In our study, 60 patients with FMF and 60 healthy controls were included. Age and gender characteristics were similar in both groups (Table 1). There was no significant difference between the two groups in terms of serum creatinine, Platelet, WBC (White Blood Cell), Fibrinogen, Neutrophil, Lymphocyte and MPV (Mean Platelet Volume) values, and Neutrophil lymphocyte ratio (N/L). In the patient group ESR and CRP levels were significantly higher than healthy groups (Table 1).

In the MEFV gene mutation analysis; homozygous M694V mutation was detected in 12 (20%) people, heterozygous M694V in 27 (45%) people, Heterozygous E148Q in 7 (11.6%) people, Heterozygous V726A in 5 (8.3%) people, Heterozygous R202Q in 3 (5%) people, Heterozygous M680I in 3 (5%) people and V761H in 1 (1.66%) person. No mutation was detected in the MEFV gene in 2 (3.33%) patients. All of FMF patients were under treatment of colchicine (1–2 mg). Three patients were under anti-IL-1 treatment. Two patients had amyloidosis (Table 2).

Table 1

Demographic data and laboratory values

	FMF Patient Group (n = 60)	Healthy Control Group (n = 60)	p
Male	33 (55%)	30 (50%)	
Female	27 (45%)	30 (50%)	0.583
Age	33 years (16–60)	34 years (21–42)	0.231
Neutrophil (%) (6–8)	62.27 (52.2–100.3)	60.31 (42.2–85.3)	0.238
Lymphocyte (%) (2–3)	29.2 (20.–34.62)	31.49 (26.3–43.2)	0.127
N/L	2.2 (1.5–2.9)	1.9 (1.5–2.4)	0.091
Hemoglobin (g/dL) (13–17)	13.87 ± 1.80	14.92 ± 1.65	0.001*
Platelet (ul) (x10 ³) (200–400)	260.47 ± 71.30	273.82 ± 70.36	0.304
Log(WBC) (fl) x10 ³ (3.5–6)	3.8 (3.8–3.9)	3.9 (3.8–3.9)	0.431
Creatinine mg/dL (0.6–1)	0.7 (0.6–0.9)	0.8 (0.7–0.9)	0.121
MPV (8–10)	9.5 (8.9–10.4)	9.6 (8.8–10.5)	0.795
ESR mm/hr (0–20)	10.0 (5.0–26.5)	7.5 (4.0–10.0)	0.003*
CRP mg/dL (0–8)	3.9 (2.2–8.9)	3.0 (1.9–4.0)	0.011*
Fibrinogen mg/dL (200–400)	326.0 (266.3–412.3)	284.0 (265.0–336.3)	0.227

Values were presented as mean (± standard deviation), median (minimum–maximum), %n.

*: p < 0.05 Mann Whitney U, and Student-t tests were used.

Table 2

Distribution of MEFV genetic mutations and clinical features in FMF Patient Group

Features	Explanation
Genotype	Number of cases (%)
Homozygous M694V	12 (20%)
Heterozygous M694V	27 (45%)
Heterozygous E148Q	7 (11.6%)
Heterozygous M680I	3 (5%)
Heterozygous V726A	5 (8.3%)
Heterozygous R202Q	3 (5%)
Heterozygous R761H	1 (1.6%)
No mutation	2 (3.3%)
Age of diagnosis	Median 18 years (min:4 – max:40)
Number of attack	< 1/month n:56 > 2/month n:4
PRAS score	n:39 (65%) (mild) n:17 (29%) (mild-moderate) n:4 (6%) (severe)
Amyloidosis	n: 2 (3%)
Colchicine dose	n: 36 (60%) 1mg/day n: 15 (25%) 1,5mg/day n: 9 (15%) 2mg/day
Co-morbidities (hypertension, diabetes mellitus, etc)	n:0 (0%)
Biologic drug (anti-IL-1)	n:3 (5%)

SERUM PROLIDASE AND HIF-1 ALPHA LEVELS

The median serum prolidase level was 72.1 (25.1–114.9) ng/mL in FMF group and 30.7 (21.3–86.2) ng/mL in control group. There was a statistically significant difference between the two groups (p = 0.018) (Figure 1).

The mean serum HIF-1 α level was 482.0 (292.0–3967.0) pg/mL in the FMF group, and 632.0 (362.0–927.0) pg/mL in the control group.

There was no statistically significant difference between the two groups (p = 0.434).

When prolidase values were compared between both groups, ROC Curve analysis was performed because the difference was statistically significant (Figure 2). Since prolidase had distinctive characteristics, cut-off value was determined according to Youden's index. This value was determined to be 54.03 ng/mL (65% sensitivity and 68.3% specificity). Positive predictive value was calculated as 67.2% and negative predictive value was calculated as 66.1% (Table 3).

Figure 1. Serum levels of prolidase.

Figure 2. ROC curve analyses of serum prolidase levels.

Table 3

ROC analysis values

Marker	ROC statistics		Diagnostic statistics			
	AUC	p	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Prolidase > 54.3 ng/mL	0.625 (0.524–0.727)	0.015	65.0(51.6–76.9)	68.3(55.0–79.7)	67.2(53.8–78.6)	66.1(52.8–78.1)

AUC: Area Under Curve, ROC: Receiver Operating Characteristic. Diagnostic statistics were shown with 95% confidence intervals.

COMPARISON OF FMF PATIENT SUBGROUPS ACCORDING TO PRAS DISEASE SEVERITY SCORING

In the FMF group, according to the severity scoring system recommended by Pras *et al* [8]. 39 subjects with mild disease, 17 subjects with

mild-to-moderate disease and 4 subjects with severe disease were included in the study. There was no significant difference among the prolidase levels and disease severity groups. HIF-1 α levels were significantly higher only in individuals with severe disease activity compared to other groups ($p = 0.02$)

(Table 4 and Table 5). There was no significant difference between Prolidase and HIF-1 α values in terms of clinical features and gene mutations ($p > 0.05$).

There was no correlation between acute phase reactants (CRP, ESR) and prolidase or HIF-1 α levels ($r < 0.25$).

Table 4

Prolidase and HIF-1 α levels by PRAS score

	Mild disease (n = 39)	Mild-to-moderate disease (n = 17)	Severe disease (n = 4)	p
Prolidase (ng/mL)	73.03 (8,04–185,61)	56.7 (13.04–154.16)	57.28 (14.95–151.86)	0.844
HIF-1α(pg/mL)	477.0 (87.0–8587.0)	387.0 (157.0–8787.0)	7137.0 (6687.0–8087.0)	0.03

Table 5

Post hoc analyses

	Group (I)	Group (J)	Mean Difference (I–J)	Std. Error	p
HIF-1α	1	2	–543.81599	856.24020	1.00
		3	–5557.05128*	1546.78621	.002
	2	1	543.81599	856.24020	1.00
		3	–5013.23529*	1637.24455	.010
	3	1	5557.05128*	1546.78621	.002
		2	5013.23529*	1637.24455	.010
Prolidase	1	2	7.83778	15.14193	1.0
		3	9.63731	27.35368	1.0
	2	1	–7.83778	15.14193	1.0
		3	1.79954	28.95337	1.0
	3	1	–9.63731	27.35368	1.0
		2	–1.79954	28.95337	1.0

Group 1: Mild disease , Group 2: Mild-to-moderate disease, Group 3: Severe disease

* The mean difference is significant at the 0.05 level.

DISCUSSION

Prolidase is significant in many physiological and pathophysiological processes, such as wound healing, inflammation and angiogenesis, by regulating the collagen metabolism, and expression of growth factors and transcription factors. Prolidase activity increases when the collagen cycle accelerates [9]. Prolidase level has been studied in various diseases (Behçet's Disease (BD), Pulmonary Tuberculosis, various cancers, Rheumatoid Arthritis (RA), etc.). When literature is reviewed, Serum Prolidase Activity (SPA) was found to be higher in patients with BD compared to healthy controls in a study conducted by Bozkurt et al. In has been found that increased angiogenesis and inflammation, prolidase increase may be associated with an increase in HIF-1 α and Vascular Endothelial Growth Factor (VEGF) levels in the pathophysiology of BD [10]. In our study, serum prolidase level was higher in FMF group than in healthy control group. In other rheumatological diseases, there are also studies in which the exact opposite results were obtained. For example, in the study of Uçar et al. which included Ankylosing Spondylitis (AS), RA and healthy control groups, SPA was found to be lower in both patient groups compared to the control group [11]. It was stated that the low level of SPA in these diseases may be

related to irregularities in collagen turnovers and decreased physical activity levels of these patients [11]. There are studies suggesting that SPA is correlated with the evaluation of treatment response in disease follow-up and disease activity scores in patients with AS. In rheumatic diseases, it has been stated that low SPA may be due to ineffective collagen turnover and poor quality of bone and may be related to decreased levels of physical activity of these patients [12]. Çelik et al. determined that SPA was lower in patients with systemic sclerosis compared to controls [13]. In these diseases, particularly collagen and bone turnovers are impaired. In particular, this suggests that prolidase level is not a marker of chronic inflammation in these patients, but rather that prolidase has different effects on bone turnover. FMF is a disease in which the bone turnover is not actually much affected and that acute/chronic inflammation is more prominent. Therefore, our study showed different results compared to the above-mentioned RA and AS studies. In cases of acute infection, for example in patients with pulmonary tuberculosis (PTB), the SPA was higher than healthy controls and correlated with acute phase reactants [14]. Since PTB has both acute and chronic process of inflammation, it was stated that collagen degradation increased in response to inflammation. For this reason, it has been suggested that SPA may be

considered among the acute phase reactants [14]. In our study, the use of prolidase as an attack marker could not be evaluated since the number of patients with acute inflammation, i.e. number of patients in FMF episodes is low. This is one of the limitations of our study. However, the high levels of prolidase may indicate a persistent subclinical inflammation or chronic inflammation in patients with FMF. There was a paradoxical decrease in prolidase according to PRAS scoring. This was not statistically significant, but it was a remarkable feature, although it could not be fully evaluated because of the small number of patients.

Helper T cells, T-reg and dendritic cells are critical in the pathogenesis of autoimmune diseases such as Systemic Lupus Erythematosus (SLE) and Rheumatoid Arthritis (RA), HIF1 α is considered to have a role in these autoimmune disorders [15]. In RA, hypoxia effects were investigated in important pathophysiological events such as synovial inflammation, angiogenesis and cartilage destruction, and it was suggested that HIF inhibitors could provide successful results in the treatment of RA [16]. However, no clinical study has been conducted in rheumatic diseases. In our study, no statistically significant difference was determined between the FMF group and the healthy control group. However,

when we compare the patient groups according to the PRAS scoring, although HIF 1- α level was found to be significantly higher in the group with severe disease, the number of patients in this group was 4 and this caused study limitation. Nevertheless, this result suggests that the duration, severity, and complications of the disease may be related to the HIF-1 α level. One of the limitations of the study is the absence of serum amyloid A as comparator. The other limitation of study was the absence of patients with active attack period. If we had such a group, we would have the opportunity to show the level of these biomarkers in active inflammation.

In conclusion, our study is the first study comparing serum prolidase and HIF-1 α levels in FMF patients with healthy controls. Serum prolidase level was determined to be higher in FMF patients even in the attack-free period. This suggests that subclinical inflammation may persist in FMF patients even in the attack-free period. In order for serum prolidase levels to be used as a marker of subclinical inflammation and to better understand the role of HIF-1 α in the pathogenesis of FMF, there is a need for larger studies with more patients comparing the individuals in attack and attack-free periods with the healthy controls.

Introducere: *Febra Mediteraneană Familială (FMF) este o boală autoinflamatorie. Prolidaza este o imidodipeptidază specifică implicată în degradarea collagenului. Prolidaza joacă un rol important în inflamație și vindecarea rănilor. Factorul inductibil al hipoxiei 1 α (Hypoxia-inducible factor-1 α , HIF-1) este o proteină importantă în reglarea răspunsului imun, hemostază, vascularizație. Scopul studiului a fost compararea prolidazei serice și nivelului HIF-1 α la pacienții cu FMF în perioada de afebrilitate și în cazul unui grup de control de sănătoși.*

Metode: *Între august 2017 și decembrie 2017, 60 de pacienți diagnosticați cu FMF conform criteriilor Tel-hashomer internați în Departamentul de Reumatologie al Secției de Medicină Internă arondat Universității de Medicină Sivas Cumhurîvet precum și 60 de voluntari sănătoși au fost înrolați în studiu.*

Rezultate: *Mediana nivelului prolidazei serice a fost 72,1 (25,1–114,9) ng/ml în grupul FMF și 30,7 (21,3–86,2) ng/mL în grupul sănătos de control ($p = 0,018$). Analiza ROC a obiectivat o sensibilitate de 65% și o specificitate de 68.3% pentru o valoare serică a prolidazei de 54,03 ng/mL ($p < 0,05$). Mediana nivelului seric al HIF-1 α în grupul FMF a fost 482,0 (292,0–3967,0) pg/mL și 632,0 (362,0–927,0) pg/mL în grupul de control de oameni sănătoși ($p > 0,05$). Nu s-a stabilit o asocierie semnificativă statistic între rezultatele de laborator, gen, vârstă și prolidază ($p > 0,05$).*

Concluzii: *Nivelurile serice ale prolidazei în cazul pacienților FMF aflați în afara unui episod acut au fost semnificativ mai mari comparativ cu cele ale grupului de control de persoane sănătoase. Cu toate acestea, rolul prolidazei și al HIF-1 α în FMF rămâne să fie clarificat prin studii ulterioare extensive.*

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