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Plasma netrin-1 levels in Familial Mediterranean fever: A potential biomarker?

Ebru ATALAR [1,*], Kevser GÖK [1], Esra FIRAT OĞUZ [2], Ahmet KOR [1], Yüksel MARAŞ [1]

[1]Department of Rheumatology, Ankara City Hospital, Ankara, Türkiye
[2]Department of Biochemistry Ankara City Hospital, Ankara, Türkiye

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Abstract

Netrins are primarily defined as being of guidance in axonal cell migration in the embryonic period. Cell migration has an important role in the occurrence of organogenesis and inflammation. In the etiopathogenesis of autoimmune diseases, there is said to be tissue destruction associated with uncontrolled activation of the immune system and uncontrolled migration of inflammatory cells to tissues. We aimed to determine whether netrin-1 has a role in the etiopathogenesis of the FMF disease. This study included 42 patients with Familial Mediterranean Fever (FMF) and 44 healthy control subjects. The plasma netrin 1 levels were measured, and relationships were examined between the netrin levels and demographic data, clinical findings and laboratory test results in both groups. No significant difference was determined between the groups in respect of the plasma netrin levels. The lack of difference between the two groups in this study in respect of plasma netrin levels could suggest that netrin-1 does not have an important role in the etiopathogenesis of FMF. There is a need for further in vivo and in vitro studies to examine netrin-1 and receptors in inflammation tissue samples and in normal tissue.

Keywords: amilial mediterranean fever, netrin 1 protein, human; inflammation; etiology

1. Introduction

Cellular networks play an important role in the formation of tissues in the human body and in their ability to perform specific functions. Netrins, which play an active role in the functioning of these networks, are one of the key molecules in glycoprotein structure. The word netrin is derived from the Sanskrit word meaning “one who guides” (1). Netrins are laminin-like proteins, which were first identified as axonal guiding clues during embryonic development. Two membrane-bound forms (glycosylphosphatidylinositol-linked netrins, netrin G1 and G2) and five secreted forms of netrin (Netrin1-5) have been reported in vertebrates (2). Receptors play a role in the functioning of netrins. The most well-known netrin receptors are Deleted in colorectal cancer (DCC), neogenin, Uncoordinated family member 5 (UNC5A), and the Down syndrome cell adhesion molecule (DSCAM-1) (3, 4). DCC and neogenin receptors attract while the UNC receptors have the effect of repulsion (5, 6). Mediated by the receptors, growing axons move towards or away from the region of intense netrin concentration.

Previous studies have shown that netrin-1 plays a role in several events such as angiogenesis, inflammation, osteoarthritis, osteoporosis, atherosclerosis, and tumour formation (2, 7). Netrin-1 also acts as a guide to important osteocytes in the formation of bone micro-architecture, and has been shown in vitro to have an active role in the differentiation of osteoclasts (8, 9). By taking a role in the formation of new vessels in some cancer types, Netrin-1 has been reported to cause a more aggressive course of the tumour (10, 11).

Initially found to be effective in axonal and neuronal migration, it was then shown that netrin-1 had a role in embryogenic angiogenesis, and was found in heavily vascularized organs and those with more vascular endothelium as well as in immune tissue (12, 14). However, the up-regulation and down-regulation mechanisms of the receptors that induce the effect of netrin-1 have not yet been fully understood (4).

Cell migration plays an important role in both organogenesis and the etiopathogenesis of diseases seen with inflammation, including autoimmune diseases (5). It has been reported that in synovial fibroblasts of rheumatoid arthritis patients, Netrin 1 and its potential inducer of IL-17 are higher than in osteoarthritis patients (2). Schubert et al examined joint synovial samples in osteoarthritis (OA), rheumatoid arthritis (RA) and a control group, and reported that there was greater expression of UNC5B and UNC6C receptors, which have repulsion properties, in the synovial samples of RA and OA patients. When recombinant netrin-1 was given to synovial fibroblast cells, the migration of synovial fibroblasts was seen to be reduced in the RA and OA patient groups and not to change in the control group (5).

As they are found in vascular endothelium and have a role in inflammation, netrin-1 and its receptors are thought to have

*Correspondence: atalarebrudr@yahoo.com

a role in the regulation of the immune system and in the etiopathogenesis of autoimmune diseases. Familial Mediterranean Fever (FMF) is a systemic inflammatory disease which is seen with autoimmune vasculitis (15). The aim of this study was to examine the plasma netrin-1 levels of FMF patients and a control group and to determine whether or not netrin-1 has a role in the etiopathogenesis of the disease. Thus, it was aimed to answer the question of whether netrin-1 could be used as a biomarker in diagnosis of the disease or as a potential treatment agent.

2. Materials and Methods

The study protocol was approved by the Local Research Ethics Committee (Number; E1-21-1589). The study was conducted in accordance with the ethical principles as described by the declaration of Helsinki. All participants provided their signed informed consent.

The study included 43 patients diagnosed with FMF, with a mean age of 37.04±11.27 years, who were in an attack-free period, and 44 healthy control subjects with a mean age of 36.20±10.54 years. The plasma netrin-1 levels were measured in both groups and compared. Correlations were examined between the netrin-1 levels and age, height, weight, body mass index, gender, smoking status (current smokers, non-smokers, ex-smokers; those who have not smoked for the past 3 months), and laboratory findings. In addition, correlations were examined in the patient group between netrin levels and family history, peritonitis, pleuritis, arthralgia, erysipelas, fever, attack frequency, age at diagnosis, fibrinogen level, colchicine dose, and duration of disease. Patients with malignancy, active infection and other concomitant inflammatory diseases such as Ankylosing spondylitis and Crohn's disease were not included in the study. Informed consent was obtained from all individual participants included in the study.

2.1. Determination of plasma netrin-1 amount by ELISA

Ten mL of venous blood samples were collected into vacutainer tubes and centrifuged at 1300 × g for 10 minutes. Separated sera were aliquoted into Eppendorf tubes and stored at –80 °C until analysis. Netrin-1 levels were measured with an ELISA kit (Elabscience, Texas, USA; catalog no: E-EL-H2328; lot no: GZWTKZ5SWK) using a quantitative sandwich enzyme immunoassay technique.

Netrin-1 standard and serum samples were added to the antibody-coated 96-well plate and incubated for 90 minutes at 37°C, followed by addition of biotinylated detection antibody specific for netrin-1 and incubation for an additional 1 hour at 37°C. The plate was then washed three times and incubated with avidin-horseradish peroxidase conjugate for 30 minutes at 37°C. After the five times washing step, in order to develop the color tetramethylbenzidine substrate was added and incubated for 15 minutes at 37°C, and the enzyme-substrate reaction was terminated by adding stop solution. The optical density (OD) is measured spectrophotometrically using a microplate reader at a wavelength of 450 nm. The OD value is proportional to the concentration of human Netrin-1. The detection range of the assay was 31.25–2000 pg/mL. Intra- and interassay precision were all <10% for low, medium and high levels of Netrin-1.

2.2. Statistical analysis

Data obtained in the study were analyzed statistically using SPSS vn. 25 software. In the comparisons between the patient and control groups, the t-test was applied to continuous data that met parametric conditions, and the Mann Whitney U-test or Kruskall Wallis variance analysis to data that was not parametric. The Chi-square test was used in the comparisons of categorical data. In the evaluation of the netrin level within the groups in respect of demographic and clinical data, the Spearman correlation test was used for continuous data and the Mann Whitney U-test or Kruskal Wallis variance analysis for categorical data. A value of $p<0.05$ was accepted as statistically significant.

3. Results

When the relationships were examined between the netrin level and clinical and demographic data within groups, a negative correlation was determined between height and netrin level in the patient group (Table 1, Fig. 1). No significant difference was determined between the patient group and the control group in respect of demographic data, clinical characteristics and laboratory findings (Table 2). A statistically significant difference was determined between the patient and control groups in respect of the CRP values ($p<0.01$).

Table 1. Relationships between the netrin level and variables in the patient group

Variables	p value
Age	0.73*
Height	**0.005***
Body weight	0.52*
BMI	0.37*
Gender	0.43**
Smoking	0.18***
Familial history	0.36**
Clinical findings	
Peritonitis	0.40**
Pleuritis	0.33**
Arthralgia	0.69**
Erysipelas	0.08**
Fever	0.11**
Attack frequency	0.15***
Laboratory tests	
CRP	0.90*
Sedimentation rate	0.73*
WBC	0.59*
Neutrophil	0.75*
Lymphocyte	0.97*
Neutrophil / Lymphocyte ratio	0.64*
Hgb	0.39*
Platelet	0.17*
Creatinin	0.79*
ALT	0.12*
AST	0.35*
GGT	0.34*
Fibrinogen	0.63*
Age of diagnosis	0.36*
Disease duration	0.74*
Colchicine dosage	0.49*

Table 2. The demographic data, clinical characteristics and laboratory findings of the patient group and control group

	Total (n=87)	Groups		p
		Patient	Control	
Number of subjects	87	43	44	
Age	36.6±10.8 (19-62)	37.04±11.27	36.20±10.54	0.72*
Height (cm)	169.01±10.09 (148-190)	168.1±8.9	169.8±11.1	0.44*
Body weight (kg)	75.03±12.76 (50-106)	74.3±14.2	75.6±11.3	0.63*
BMI	26.2±3.61 (19-34)	26.1±4	26.2±3.2	0.94*
Gender				
Male	42	21	21	0.91**
Female	45	22	23	
Smoking				
Non-smokers	57	27	30	0.37**
Current smokers	20	9	11	
Ex-smokers	10	7	3	
Laboratory tests				
Netrin-1 level (pg/ml)	256.56±88.80	241.9±76.3	270.8±98.2	0.13*
CRP [mg/l]	2.69±3.63	3.8±4.7	1.5±1.4	0.03*
Sedimentation rate [mm/h]	9.42±6.33	10.6±7.4	8.2±4.8	0.08*
WBC [10^9 / L]	7.154±1.608	7.284±1.624	7.027±1.600	0.45*
Neutrophil [10^9 / L]	4.294±1.516	4.538±1.551	4.055±1.460	0.13*
Lymphocyte [10^9 / L]	2.170±0.614	2.087±0.489	2.252±0.711	0.21*
Neutrophil/lymphocyte ratio	2.15±0.99	2.3±1	1.9±0.8	0.12*
Hgb [gr/dl]	14.2±1.5	13.9±1.2	14.5±1.8	0.07*
Platelet [1/mm3]	265942±51133	273186±49372	258863±52389	0.19*
Creatinine [mg/dL]	0.78±0.13	0.78±0.13	0.78±0.13	0.85*
ALT [U/l]	27.7±14.3	27.6±13.9	27.8±14.9	0.95*
AST [U/l]	20.4±10.6	20.9±8.6	20±12.3	0.70*
GGT [U/l]	21.5±11.4	23.5±13.9	19.5±7.9	0.10*
Fibrinogen [gr/l]		2.92±0.75		
Age of diagnosis		25.4±11.9 (5-62)		
Disease duration (year)		11.6±6.5 (1-26)		
Colchicine dosage (mg)		1.45±0.44 (0.5-2)		

*t-test, ** Chi-square test, BMI: body mass index, Hgb: haemoglobin, AST: aspartate aminotransferase, ALT: alanine aminotransferase, CRP: c-reactive protein, ESR: erythrocyte sedimentation rate, WBC: white blood count

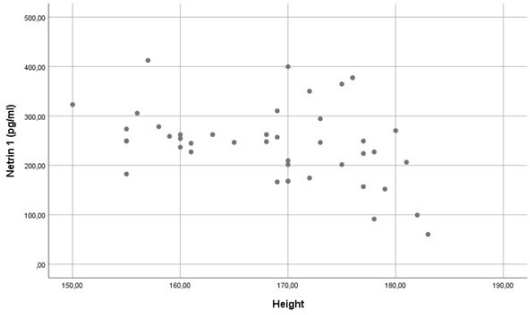

Fig. 1. The correlation between height (cm) and netrin level in the patient group

4. Discussion

That the Deleted in Colorectal Cancer (DCC) and Uncoordinated-5 (UNC5) receptors of netrin-1 have been identified in cells other than neurons strengthens the hypothesis that this protein could have roles other than in the central nervous system (1). The ability of the guidance molecule netrin-1 to repulse or eliminate the attraction of neuronal cells expressing the UNC5b receptor makes it an attractive candidate for the regulation of inflammatory cell migration.12 Cell migration is known to have a role in organogenesis in the embryonic stage, and then in inflammation and tissue homeostasis in subsequent periods (4, 16). Netrin-1 has been shown to be a regulator of leukocyte migration during inflammation (12). It is thought that diseases may start associated with inflammation when there is inappropriate migration to cells. In addition to stability of the vascular endothelium, the balance of attraction and repulsion signals between netrin-1 and its receptors has an effect on the inflammation process (12).

However, whether netrin-1 is protective against the formation of inflammation, or whether it triggers inflammation remains a matter of debate. The results of this study showed that the mean netrin level was lower in the patient group than in the control group, but the difference was not statistically significant. Miralkaj et al demonstrated that netrin-1 was reduced in pulmonary and peritoneal inflammation.17,18 In an experimental model, the application of netrin-1 was observed to reduce renal ischaemia reperfusion damage and associated inflammation (19).

Netrin-1 expressed from the vascular endothelium shows an anti-inflammatory effect by preventing migration to inflammatory cells (12). In addition, the anti-inflammatory effect of Netrin-1 as a potential treatment agent has been shown in experimental models (20, 21). With the application of netrin-1 in animal studies, neutrophil infiltration and the induction of proinflammatory cytokines have been shown to be reduced and thereby peritoneal inflammation was suppressed (18). In another animal study, netrin-1 application reduced inflammation associated with hypoxia (22). Netrin-1 prevents inflammatory cells penetrating the vascular endothelium. Inflammation becomes evident with leukocyte migration in conditions such as infection where the endothelial barrier is damaged. In such cases, the return of the netrin-1 level to normal prevents excessive tissue destruction by taking leukocyte migration under control (12).

In an experimental model, netrin-1 expression was shown to be suppressed by cytokines such as TNF and IFN, which play an active role in inflammation, and thus inflammation was facilitated (12). In that study, in inflammation associated with infection created with Staph. Aures in the lungs of mice, netrin-1 expression was rapidly down-regulated in the lungs, and the netrin-1 had an inhibitory effect on monocyte, leukocyte and granulocyte migration. Blocking the UNC5b receptor, which provided this effect, eliminated the effect of netrin-1 suppressing the migration of inflammatory cells (12).

There are also studies reporting that netrin-1 could have an inflammation triggering effect. In samples taken from patients applied with revision surgery due to prosthesis loosening after arthroplasty, the netrin-1 expression was compared with netrin-1 expression in samples taken during primary arthroplasty operations. The netrin-1 expression was determined to be significantly greater in the samples taken from the revision operations. Taking the effect of osteoclastic activity into consideration, it was reported that netrin-1 could have a key role in inflammatory osteolysis that develops following arthroplasty operations (23). The same authors reported that in an animal model, the blockage of netrin-1 reduced wear particle-induced inflammation seen after arthroplasty operations. Consistent with that study, there are studies that have reported that by inhibiting macrophage movement netrin-1 increased inflammatory events as a result of atherosclerosis and insulin resistance (3,24). Similarly, it has been reported that when netrin-1 was removed from cells obtained from bone marrow in mice, the dimensions of atherosclerotic lesions reduced significantly, and netrin-1 had an active role in the development of atherosclerosis by preventing the migration of cholesterol-loaded macrophages to the lymphatic system (24,25). In another animal model study of diet induced obesity, it was shown that the reduced migratory capacity of macrophages could be restored by blocking netrin-1. It was demonstrated that hematopoietic deletion of netrin-1 reduced inflammation and improved insulin sensitivity, so it was concluded that netrin-1 promotes chronic inflammation and insulin resistance (26). Paradisi et al determined increased netrin-1 levels in the mucosa of patients with inflammatory intestinal disease (Crohn's disease and ulcerative colitis), especially in the inflammation region, compared to normal colonic mucosa (27). According to that study, netrin-1 up-regulation in inflammatory bowel disease is necessary for progression to colorectal cancer, although a change in netrin-1 levels does not affect inflammation (27). In addition, netrin-1 may show a dose-dependent effect. From an in vitro study it was reported that while the application of netrin-1 at a low concentration stimulated angiogenesis on the CD-146 adhesion molecule, a high concentration of netrin-1 inhibited angiogenesis on the UNC5B receptor (28).

The above-mentioned studies were conducted on tissue samples. Due to the difficulties of obtaining tissue samples from the patients and control group in the current study, and as netrin is expressed by a protein and therefore found in the peripheral circulation, the plasma levels of netrin-1 were investigated in this study (4). The conflicting results related to netrin-1 that have been reported in literature can be considered to be the result of interaction of different receptors with chemoattractive and chemorepulsant mediators of netrin-1.

As a result of the current study, a significant inverse relationship was determined between the plasma netrin level in the patient group and height. No similar correlation was found in the control group. The mechanism of this cannot be explained in the scope of this study, but there may be a similar relationship in other autoinflammatory diseases. The reliability of this finding should be questioned because the number of patients is relatively small and it is a cross-sectional study. There is a need for prospective long-term follow-up studies on this subject with a greater number of patients and including other autoinflammatory diseases. That the CRP level of the patient group was higher than that of the control group was an expected finding. However, no correlation was found between CRP and the netrin level.

The most important limitations of this study were that netrin-1 and receptors were not examined in tissues, and that plasma netrin levels were not examined during attack periods. Another limitation of our study is that we did not examine the plasma netrin-1 levels in patients in the attack period.

The results of this study demonstrated no difference between the plasma netrin-1 levels of the patient and control groups. There was also no difference found between the clinical and laboratory findings and the netrin levels of the patient group. These findings suggest that netrin-1 does not have a significant role in the etiopathogenesis of FMF disease. It would be useful for further studies to examine the netrin-1 and receptor levels in tissue samples obtained from patients and healthy control subjects, and to evaluate the effects on inflammation of the application of recombinant netrin-1 to cell cultures of FMF patients to be able to establish a relationship between netrin-1 and FMF.

Conflict of interest
The authors declared no conflict of interest.

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Authors' contributions
Concept: E.A., Design: Y.M., Data Collection or Processing: A.K., Analysis or Interpretation: E.F.O., Literature Search: K.G., Writing: E.A., Y.M.

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