Original Article

Evaluation of Serum Levels of Progranulin and Bone Morphogenetic Protein-4 in Female Patients with Knee Osteoarthritis

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ABSTRACT

Objective: To investigate serum bone morphogenetic protein-4 (BMP-4) and progranulin (PGRN) levels in patients with osteoarthritis (OA) and to present a new evidence of pathogenesis OA disease
Design: Prospective study
Setting: Dicle University Medical Faculty Hospital
Subjects: Thirty-eight female knee osteoarthritis patients and thirty-eight healthy female volunteers were enrolled from January 2016 to April 2016.
Intervention: Family histories, clinical histories and examinations, and radiological examinations were obtained from the hospital data system. Blood samples were obtained from the antecubital vein of all participants after overnight fasting.
Main outcome measures: Serum PGRN and BMP-4 concentrations were measured using enzyme-linked immunosorbent assay. Body mass index, erythrocyte sedimentation rate, white blood cells and neutrophil lymphocyte ratio were also assessed.
Results: Mean BMP-4 levels were significantly lower in OA women compared to controls ($p < 0.001$). Mean PGRN levels were found to be significantly lower in OA women compared to controls ($p < 0.001$). There was a significant positive correlation between BMP-4 and PGRN in patients with OA.
Conclusions: BMP-4 and PGRN levels may play a role in the pathogenesis of knee OA and could be a useful biomarker of knee OA, as well as a potential therapeutic target for the management of knee OA.

KEYWORDS: bone morphogenetic protein-4, osteoarthritis, progranulin

INTRODUCTION

Osteoarthritis (OA) is a chronic, slowly progressive disease of the joints and is the most common form of arthritis worldwide[1]. It is one of the most common causes of pain and disability in middle-aged and older people. The incidence of OA is increasing because of the aging population and the obesity epidemic[2]. It is characterized by articular cartilage degeneration, subchondral sclerosis, osteophyte formation, and inflammation of the synovial membrane. However, the etiology and pathogenesis underlying this disease are poorly understood[3].

Progranulin (PGRN), a secreted glycoprotein expressed in many cell types, has been linked to a wide variety of biological processes, including inflammation, infection, wound healing, angiogenesis, cell proliferation, neurodegeneration and tumorigenesis[4-7]. PGRN is also secreted in adipose tissue and contributes to the regulation of appetite and satiety, fat distribution, insulin secretion and sensitivity, energy expenditure and inflammation as adipokines[8]. It has a regulatory role in musculoskeletal diseases, autoimmune disorders, cardiac diseases, neurodegenerative diseases, metabolic disease and obesity pathogenesis[6,7,9,10]. Recently, studies revealed that it has a chondro-protective role in the cartilage degradative cascade, stimulates chondrocyte proliferation and is considered an essential regulator of cartilage metabolism[6,11,12].

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Bone morphogenetic protein-4 (BMP-4), a member of transforming growth factor-β superfamily of proteins, is involved in bone and cartilage metabolism, muscle development, and induction of adipogenesis[8,13]. BMP-4 regulates adipogenic precursor cell commitment and differentiation and is associated with obesity and inflammation as adipokines[8]. Studies showed that BMP-4 is expressed in normal synovial tissue, induces chondrogenesis and may be important in cartilage repair[14,15].

The aim of this study was to compare BMP-4, PGRN and other inflammatory parameters such as erythrocyte sedimentation rate (ESR), white blood cell (WBC) count, and Nod-like receptor (NLR) levels between knee OA patient group and the control group and to present new evidence of pathogenesis of OA disease.

SUBJECTS AND METHODS

Study population

This pilot, prospective, case control study was conducted at Dicle University, Medical Faculty Hospital, Diyarbakir, Turkey between January 2016 and April 2016. Thirty-eight female knee OA patients diagnosed according to the American College of Rheumatology were included in the study. To evaluate radiographic severity of knee OA, the Kellgren and Lawrence (KL) classification were used as follows: grade 1 - suspicious narrowed joint gap; grade 2 - definite cleared osteophytes and narrowed joint space; grade 3 - moderate multiple osteophytes, definite narrowing of joints space, some sclerosis, and possible deformity of bone contour; grade 4 - large osteophytes, marked narrowing of joint space, severe sclerosis, and definite deformity of bone contour.

Thirty-eight female grade 2 and grade 3 knee osteoarthritis patients aged between 39 - 59 years were included in the study. We included only female patients to homogenize the sample, as there are differences between women and men that could affect the adipokine profile[16]. Thirty-eight healthy female volunteers, with normal radiological examinations and clinical histories and examinations, who visited the hospital for routine physical examinations were enrolled as controls. Controls were matched in terms of age and body mass index (BMI) with the patients. The BMI is defined as the body mass (weight) divided by the square of the body height, and is universally expressed in units of kg/m^2. Clinical information such as age, course of disease, joint X-ray grades and biochemical analyses (whole blood count, ESR) were obtained from the hospital data system.

Participants were excluded on the base of having rheumatoid arthritis, post-traumatic arthritis, previous joint infection, crystal deposition arthritis, enteropathic arthritis, hemophilic arthropathy, previous knee injury, infectious- or endocrine-related arthropathy, clinically unstable medical illness, the use of any medication (such as non-steroidal anti-inflammatory analgesic, corticosteroids, *etc.*) within 4 weeks prior to initiation of the study, pregnant and lactating women and those who had severe cardiovascular disease, cerebrovascular disease, acute or chronic infectious diseases, systemic inflammatory, endocrine or autoimmune disorders, liver or renal insufficiency, immune system disease, and malignant diseases.

The study was approved by the Research Ethics Committee of Dicle University Medical School and was performed in accordance with the ethical standards stated in the 1964 Declaration of Helsinki. Written informed consent was obtained from all patients and healthy volunteers prior to their participation in this study.

Standard tubes with a constant amount of ethylenediaminetetraacetic acid (EDTA) were used for the whole blood count. All blood samples were studied within one hour of sampling. The whole blood count analyses, based on the technique of laser flow cytometry scattergrams, were performed in the central laboratory of our institution using the same analyzer (Medonic CA-620, Sweden) which is routinely checked every day. Whole blood count parameters of participants were recorded from the same computerized database. ESR was determined using an automated Westergren method (Sedimat 15, LP Italiana, Italy).

Measurement of PGRN and BMP-4

Blood samples were obtained from the antecubital vein of all participants after overnight fasting. After clotting, blood samples were centrifuged at $2000 \times g$ for 15 min, serum was separated, and aliquots stored at −80 °C until examination. Serum PGRN and BMP-4 concentrations were determined using a commercially available enzyme-linked immuosorbent assay kit (ELISA) (SunRedbio; Shangai, China) according to the manufacturer's instructions. The samples were processed as recommended by the kit and run in random for the ELISA. The colour intensities were measured by a plate reader (DAR 800 microplate reader, Chemtron Pte Ltd, Singapore) with a measuring filter of 450 nm. The results were expressed as nanograms per milliliter (ng/mL). The standard curve ranged from 10 ng/mL to 160 ng/mL of BMP-4 and the standard curve showed a direct relation between optical density and BMP-4 concentration. The standard curve of ELISA for PGRN ranged from 25 ng/mL to 400 ng/mL and the standard curve showed a direct relation between optical density and PGRN concentration. The sensitivity of the BMP-

4 and PGRN commercial kit were 0.927 ng/mL and 2.158 ng/mL, respectively. The intra-assay coefficients of variation of the assays were < 10%. Assay ranges were 1 - 300 ng/mL for BMP-4 and 2.5 - 720 ng/mL for PGRN.

Statistical analysis

All statistical analyses were performed using SPSS 22.0 software (Chicago, IL, USA). Data were tested for normal distribution using the Shapiro-Wilk test. Data are expressed as mean ± standard deviation. Student's t-test for independent samples was used to analyze and in cases of normal distribution, Mann-Whitney's U test was used to compare the groups. The correlations between the variable pairs were analyzed using Spearman's correlation test. Differences between groups were significant when $p < 0.05$.

RESULTS

A total of 76 female patients (38 knee OA and 38 controls) were included in the study. Baseline clinical characteristics are shown in Table 1. There were no significant differences between patient group with OA and healthy controls in terms of age and BMI ($p = 0.522$, $p = 0.452$, respectively). WBC values were 7.25 ± 1.55 x10^3/mm^3 in patients with OA vs. 7.39 ± 1.44 x10^3/mm^3 in the control group. Mean NLR levels were 2.16 ± 0.49 in patients with OA vs. 2.19 ± 0.47 in the control group. No difference was found between the two groups in terms of WBC and NLR values ($p = 0.763$, $p = 0.925$, respectively). ESR values were significantly higher in patients group than controls group ($p = 0.022$) (Table 1).

Table 1: Baseline clinical characteristics of osteoarthritis patients and the controls

Characteristics	Osteoarthritis patients (n = 38)	Control Group (n = 38)	p-value
Age	49 ± 10	47 ± 5	0.522
BMI (kg/m^2)	23.57 ± 1.47	23.68 ± 2.74	0.452
WBC (x10^3/mm^3)	7.25 ± 1.55	7.39 ± 1.44	0.763
NLR	2.16 ± 0.49	2.19 ± 0.47	0.925
ESR (mm/h)	15.2 ± 8.6	10 ± 3.4	0.022*

BMI: Body mass index; WBC: White blood cell; NLR: Neutrophil to lymphocyte ratio; ESR: Erythrocyte sedimentation rate; *: statistically significant

Mean BMP-4 values were 29.66 ± 13.61 ng/mL in patients with OA vs. 72.81 ± 44.06 ng/mL in the control group. BMP-4 values were found to be significantly lower in OA group ($p = 0.001$). Mean PGRN values were 71.93 ± 33.83 ng/mL in patients with OA vs. 268.33 ± 180.45 ng/mL in the control group. PGRN values were found to be significantly lower in OA group ($p = 0.001$) (Table 2).

Table 2: Serum levels BMP-4 and PGRN in osteoarthritis patients and the controls

Characteristics	Osteoarthritis patients (n = 38)	Control Group (n = 38)	p-value
BMP-4 (ng/mL)	29.66 ± 13.61	72.81 ± 44.06	0.001*
PGRN (ng/mL)	71.93 ± 33.83	268.33 ± 180.45	0.001*

BMP-4: Bone morphogenetic protein 4; PGRN: Progranulin; *: statistically significant

Patients with OA had a positive correlation between serum BMP-4 levels and serum PGRN levels (Table 3). There was no correlation between serum BMP-4 levels and WBC, NLR and ESR levels (Table 3). There was also no correlation between serum PGRN levels and WBC, NLR and ESR levels (Table 4).

Table 3: Relationship between BMP-4 and PGRN, WBC, NLR and ESR in osteoarthritis patients

		PGRN	WBC	NLR	ESR
BMP-4	r-value	0.898	-0.148	0.222	0.107
	p-value	0.001*	0.376	0.181	0.522

ESR: Erythrocyte sedimentation rate; WBC: White blood cell; NLR: Neutrophil to lymphocyte ratio; BMP-4: Bone morphogenetic protein 4; PGRN: Progranulin; *: statistically significant

Table 4: Relationship between PGRN and BMP-4, WBC, NLR and ESR in osteoarthritis patients

		PGRN	WBC	NLR	ESR
PGRN	r-value	0.898	-0.210	0.154	0.106
	p-value	0.001*	0.206	0.354	0.527

ESR: Erythrocyte sedimentation rate; WBC: White blood cell; NLR: Neutrophil to lymphocyte ratio; BMP-4: Bone morphogenetic protein 4; PGRN: Progranulin; *: statistically significant

DISCUSSION

OA is an age-related, chronic, progressive, degenerative joint disease, but its inductive factors and underlying mechanisms are still largely unknown. It can be the result of a complex interplay of metabolic, genetic, biomechanical and biochemical factors[2]. To our knowledge, this is the first study to show serum BMP-4 and PRGN levels in OA patients and we demonstrated that PGRN and BMP-4 levels were significantly decreased in sera of knee OA patients.

Progranulin, a secreted multifunctional growth factor, is widely expressed in epithelial, neurons, chondrocyte and immune cells and regulates

cell proliferation, migration and survival[17,18]. It plays a critical role in various diseases and conditions including wound repair, the regulation of inflammation, host defense, angiogenesis, early embryogenesis, bone regeneration, tumorigenesis and neurodegenerative diseases[17,19-22]. PGRN is a key regulatory factor in the resolution of inflammation. PGRN blocks the production of neutrophil attracting chemokines and cleavage of PGRN is promoted by enzymes including elastase and proteinase 3, which is secreted by neutrophils[9]. Kessenbrock *et al* showed that mice lacking both elastase and proteinase 3 were directly linked to the accumulation of anti-inflammatory activity of PGRN, and they concluded that proteinase 3 and elastase enhance neutrophil-dependent inflammation by eliminating the local anti-inflammatory activity of PGRN[23]. PGRN functions as an endogenous modulator of innate immune responses and promotes the upregulation of Th2 cytokines such as IL-4, IL-10 and IL-15[24]. It plays an important role in bone metabolism, especially inflammatory conditions, and is a critical mediator of the bone healing process modulating BMP-2 and TNF-α signaling[25].

PGRN is also expressed in human articular cartilage tissue and plays a crucial role in chondrocyte proliferation, differentiation and endochondral ossification during development[26]. PGRN is upregulated in the synovium of both OA and rheumatoid arthritis (RA)[27]. Studies revealed that cartilage oligomeric protein (COMP), a prominent non-collagenous component of cartilage, directly binds PGRN and enhances PGRN mediated chondrocyte proliferation[11]. Guo *et al* showed that PGRN is a novel specific inhibitor of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) 7/12 mediated COMP degradation, and may play a significant role in preventing the destruction of joint cartilage in arthritis[12]. They also showed that expression of PGRN is increased in cartilage of OA patients and suggested that PGRN may play a role in the inflammatory component of arthritis pathogenesis, and supports the concept that arthritic chondrocytes may exhibit increased anabolic activity, including the release of growth factors. Tang *et al* showed that PGRN is a ligand of tumor necrosis factor receptors (TNFR), an antagonist of TNF-α signaling and plays a critical role in the pathogenesis of inflammatory arthritis in mice models[21]. They suggested new potential therapeutic interventions for various TNF-α mediated pathologies and conditions and showed that the administration of recombinant human PGRN or a recombinant PGRN derivative named Atsttrin, had strong anti-inflammatory effects comparable to the administration of Etanercept. Therefore, PGRN is also a potential target for the treatment of inflammatory diseases and autoimmune diseases including OA and RA. Zhao *et al* found that deficiency of PGRN led to spontaneous OA-like phenotype in 'aged' mice[26]. They suggested that PGRN protects the progression of OA through multiple pathways in which first, PGRN activates the ERK1/2 signalling pathway and elevates levels of anabolic biomarkers in a TNFR2-dependent manner. Second, PGRN interacts with TNF-α and prevents the activation of the NF-κ B pathway, which upregulate the levels of various matrix metalloproteinases (MMPs) and ADAMTS, thus inhibiting cartilage degradation in OA. They also suggested that PGRN inhibits β-catenin signalling, which is also known to play a critical role in the development of OA. These findings were consistent with our study. In our study, we showed decreased serum PGRN levels in OA patients. Our results indicate that PGRN is related to the pathogenesis of osteoarthritis.

BMPs are signaling molecules, which are a member of transforming growth factor-β superfamily of proteins and were identified for their ability to induce bone formation[28]. BMP-4, a member of BMPs family, promotes bone formation by inducing endochondral ossification and promotes cartilage formation by inducing mesenchymal stem cells to become chondroprogenitors and chondrocyte maturation[15]. BMP-4 enhances the production of articular cartilage matrix by stimulating the synthesis of collagen type II and aggrecan and prevents chondrogenic hypertrophy by suppressing the production of collagen type X[29,30]. BMP-4 also accelerates chondrocyte maturation[31]. Miljkovic *et al* suggested with these findings that BMP-4 can be a promising agent for promoting cartilage repair in the future and modulation of BMP signaling may also become an important therapeutic approach in chronic joint diseases including RA and OA[15]. These findings were consistent with our study. To our knowledge, there is no study to show serum BMP-4 in OA patients and we showed decreased serum BMP-4 levels in OA patients. Our results indicate that serum BMP-4 levels can be of use to the osteoarthritis patients as a marker.

Given that PGRN acts as a strong anti-inflammatory mediator by antagonist of TNF-α signaling and BMP-4 contributes to inflammation, we further investigated the relationship between serum PGRN and BMP-4 levels and inflammatory markers including NLR and ESR in patients with osteoarthritis. We did not find any correlations between BMP-4 and PGRN levels and inflammatory markers. This may be because we could not use specific inflammatory markers. Furthermore, our results showed that ESR levels were higher in OA

patients than controls, but our ESR level results were in normal range.

BMP-4 and PGRN are members of adipokines. Adipokines are cytokines, predominantly produced in adipose tissue and are involved in many metabolism and disease pathogenesis. In previous studies, relationship between adipokines and osteoarthritis has been widely investigated[32]. Adipokines are produced in knee OA joints by infrapatellar fat pads, chondrocytes, synovium, osteoblasts and osteoclasts and secreted to circulation[33,34]. Adipokines could affect cartilage remodeling such as chondrocyte proliferation, proteoglycan synthesis, collagen synthesis and matrix mineralization in cartilage[32,33]. Adipokines also affect bone remodeling in OA[32]. Serum and synovial levels of adipokines have been shown in many studies and they suggested that adipokines may be used for monitoring disease progression including bone erosions and osteophyte formation and following the efficiency of therapeutic interventions as biomarkers[32,33]. For future clinical applications in OA disease, adipokine-targeted therapeutic strategies are considered to prevent cartilage and bone alteration as well as inflammation[32]. Our results support the hypothesis that adipokines are involved in the pathogenesis of osteoarthritis.

In our study, patient and control groups consisted of same gender (female), age and body mass index. They also had similar ethnicity, eating patterns and lifestyles. These factors may cause interferences to analyze BMP-4 and PGRN. BMI levels in patients group was normal range and there were no differences in BMI levels between the two groups. Previous studies have shown that serum BMP-4 and PGRN levels changed in obesity patients[8,35,36]. Obesity also contributes to OA pathogenesis[37]. Therefore, we included patients with normal BMI levels in our study.

We must acknowledge some limitations of this study. The sample size of our study was small. Statistical tests usually require a larger sample size to justify that the effect did not happen by chance alone. Moreover, in our study, patient and control groups consist of same gender (female). Thus, the subjects are not representative of the general population, but instead representative of the female gender. In future studies, the levels of BMP-4 and PGRN should be analyzed in the general population. Despite these limitations, these data do form a basis for future studies examining the relationship between OA patients and serum BMP-4 and serum PGRN levels.

CONCLUSIONS

This pilot study demonstrates that serum PGRN and serum BMP-4 levels decrease in female knee osteoarthritis patients group. Serum BMP-4 and PGRN levels could be useful biomarkers of knee OA. The level of PGRN and BMP-4 in patients with OA should be paid high attention and these markers could be a potential therapeutic target for the management of OA. Further studies using larger populations and more detailed investigation will be needed to confirm our observations and to validate the current findings.

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