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The relationship of promoter methylation of calcium voltage-gated channel alpha 1 and interleukin-16 to primary osteoarthritis

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Abstract. – OBJECTIVE: Osteoarthritis is the most prevalent joint disease worldwide and the primary cause of musculoskeletal dysfunction. Epigenetic changes in various genes, particularly methylation, have been implicated as possible underlying causes of primary osteoarthritis. The aim of our study was to investigate the promoter methylation status of the calcium voltage-gated channel alpha 1 subunit G *(CACNA1G)* and interleukin-16 *(IL-16)* genes, which are strongly associated with calcium channel activity and antigen presentation, respectively, in primary osteoarthritis patients.

PATIENTS AND METHODS: Twenty-one patients with primary osteoarthritis and 25 healthy controls were included in our study. The methylation status of *CACNA1G* and *IL-16* genes was analyzed with methylation-specific Polymerase Chain Reaction (PCR), and the serum levels of IL-16 were determined with Enzyme-Linked Immunosorbent Assay (ELISA).

RESULTS: The age of the patients was 63.95±8.41 years, and they were 15 females and 6 males. The promoter of the *CACNA1G* gene was found to be hypermethylated in primary osteoarthritis patients ($p<0.001$). In contrast, the promoter of the *IL-16* gene was found to be hypomethylated compared to the control ($p<0.001$). The serum levels of *IL-16* increased in parallel with the hypomethylated promoter status of IL-16 gene in primary osteoarthritis patients compared to the control ($p<0.001$).

CONCLUSIONS: Our study indicates that the methylation status of *CACNA1G* and *IL-16* gene promoters are epigenetically altered in patients with primary osteoarthritis. Moreover, increased serum *IL-16* levels in osteoarthritis patients may be associated with hypomethylation of the *IL-16* gene promoter.

Key Words:
Primary osteoarthritis, Methylation, Ion channel, Interleukin-16.

Introduction

Osteoarthritis is the most prevalent joint disease worldwide and the primary cause of musculoskeletal dysfunction, especially in the elderly[1]. It is characterized by excruciating pain in joints, stiffness, limited movement ability, and different degrees of inflammation resulting from progressive destruction and degeneration of articular tissue, loss of joint space, osteophyte formation, and sclerosis in subchondral tissue[2]. Osteoarthritis is seen as a whole-joint disease. It causes cartilage loss, severe pain, and, in the end, severe joint damage. There is no cure for osteoarthritis, and the treatments being applied are not sufficient to improve the symptoms. Eventually, the patients are compelled to joint replacement surgery[1]. Based on the osteoarthritis classification, knowledge about primary (idiopathic) osteoarthritis is limited[3]. Therefore, identification of mechanisms underlying disease progression has utmost importance for therapeutic interventions.

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Heritable changes in gene expression, due to environmental or lifestyle factors, are major factors in several pathologies[4]. Epigenetics, which refers to alterations in gene function but not the genetic sequence of Deoxyribonucleic acid (DNA), gained significant attention in different diseases that affect bone structure[5]. Although several epigenetic mechanisms were described during this period, promoter methylation of genes and acetylation of histone proteins have been extensively investigated[6]. Previous genome-wide and clinical studies[7] indicated that the methylation status of several genes was significantly different in osteoarthritis patients. Although many of these genes are investigated broadly in different osteoarthritis patient populations, there is still an unmet need for further investigation on these genes.

Additionally, recent genome-wide studies[8] investigating the methylation profile of osteoarthritis patients revealed that *interleukin-16 (IL-16)* and *the calcium voltage-gated channel alpha1 (CACNA1)* genes in osteoarthritis patients could be regulated by methylation. *IL-16* is a pleiotropic cytokine that has a role in antigen presentation and inflammation[9]. Scholars[10] have demonstrated that *rs11556218*, *rs4072111*, and *rs4778889* polymorphism in the *IL-16* gene has an essential role in primary osteoarthritis and increases the risk of osteoarthritis. CACNA1 is a voltage-gated calcium channel that modulates cellular calcium entry and is the primary target for calcium channel blockers used to treat hypertension[11]. CACNA1 channels also play a significant role in bone remodeling and inflammation in the bone structure[12]. Additionally, antagonism of these channels is shown to alter osteoarthritic joint pain via a different mechanism[13]. However, there is limited knowledge about these two possible suspects responsible for osteoarthritis progression. Therefore, the present study aimed to reveal the promoter methylation status of *IL-16* and *CACNA1G* genes, and serum IL-16 levels in primary osteoarthritis patients.

Patients and Methods

Twenty-one patients diagnosed with primary knee osteoarthritis and twenty-five healthy controls were consecutively selected from those who applied to the orthopedic clinic in our hospital. Blood samples were collected in October 2021. The Local Ethics Committee (Ethics approval number: KAEK 2021/437) was approved the study and written, and signed consent was collected from all volunteers. Patients were selected as type 4 according to the Kellgren Lawrence classification with a diagnosis of primary osteoarthritis[13]. Patients who had ankylosing spondylitis, rheumatoid arthritis, septic arthritis, and other arthritis-related diseases or autoimmune and inflammatory disorders were excluded from the study. Additionally, patients were also selected, excluding those who consume alcoholic beverages and smokers. Control patients were selected as mentioned and randomly selected from the same hospital that applied for routine physical exams. Sociodemographic characteristics of all volunteers were obtained by history taking. All experiments were performed and reported according to the STROBE guidelines[14].

Peripheral blood samples (~1.5 mL) were collected to EDTA-coated vials from all patients and stored at -80°C until the day of experiments. Before isolation of DNA samples, serums were separated and collected with centrifugation at 3000×g for 5 minutes. Subsequently, IL-16 levels were determined by Enzyme-Linked Immunosorbent Assay (ELISA) kit (Sunred, Wuhan, China). Three replicates from each sample were used. Genomic DNA was extracted from leukocytes with a commercially available DNA isolation kit (#GB300, Geneaid, New Taipei, Taiwan). The purity and concentrations of DNA samples were determined by a nanodrop spectrophotometer (Jenway Genova Nano, UK). Following DNA isolation, bisulfite modification in samples was carried out with Epi-JET Bisulfite Conversion Kit (ThermoFisher Scientific #K1461, Vilnius, Lithuania), strictly following the manufacturer's instructions. Subsequently, methylation-specific PCR (MSP) was performed with methylation and unmethylation-specific primer pairs for *IL-16* and *CACNA1G* promoter regions. The primers were designed *via* MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi)[15], and sequences and amplicon lengths were provided in **Supplementary Table I**. The methylation-specific PCR was carried out in a final volume of 25 µl containing Hibrigen *Taq* DNA Polymerase (Hibrigen #MG-KTAQ-01, Kocaeli, Turkey) enzyme (1.25 U), dNTPs (10 Mm), $MgCl_2$ (2 mM), methylation-specific or unmethylation-specific primers, and under the following cycling condition for *IL-16*: initial denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for the 40s; 57°C for 40s and 72°C for 70s and *CACNA1G*: initial denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 40s; 57°C for 40s and 72°C

Table I. Demographic characteristics of experimental groups. Data expressed as mean ± SD.

	Control	Osteoarthritis	*p*-value
Sex (Female/Male)	13/12	15/6	>0.05
Age (years)	57.05±11.43	63.95±8.41	>0.05
Weight (kg)	81.78±8.85	90.47±11.15	>0.05
CRP (mg/L)	6.07±4.96	6.58±6.76	>0.05
WBC# (10^3/μL)	8.46±3.61	7.58±2.46	>0.05
NEU# (10^3/μL)	5.80±2.99	4.85±2.05	>0.05
LYMPH# (10^3/μL)	2.29±0.79	2.83±3.77	>0.05

CRP: C-reactive protein, WBC: White blood cell, NEU: Neutrophil, LYMPH: Lymphocyte.

for 70s. Final extensions were carried out at 72°C for 7 min and then maintained at 4°C. Unmethylated and methylated gDNA Controls (Cells-to-CpG™, Applied Biosystems #4445555, Carlsbad, CA, USA) were used as positive controls. Methylated and unmethylated PCR products were separated on a 2% agarose gel by electrophoresis. Amplicons of *CACNA1G* and *IL-16* were distinguished by 100 bp and 200 bp bands, respectively.

Statistical Analysis

All data were analyzed by GraphPad Prism (version 9.0; La Jolla, CA, USA). Continuous variables were analyzed with Student's *t*-test, and categorical variables were analyzed by χ^2 test. Correlation analysis was performed with Spearman's analysis. *p*-values that were lower than 0.05 were considered significant.

Results

Demographic and laboratory data of patients are summarized in Table I. Our results demonstrated no significant difference between the control and osteoarthritis groups in terms of gender, age, and weight values ($p>0.05$). Additionally, C-reactive protein, white blood cell, neutrophil, and lymphocytes levels were similar between experimental groups ($p>0.05$).

The methylation status of the *CACNA1G* promoter was investigated by methylation-specific PCR. It was found that the *CACNA1G* promoter was found significantly methylated (35.8%±3.79) in the osteoarthritis group compared to control (12.8%±0.6, $p<0.001$, Figure 1).

Serum levels of IL-16 were determined by ELISA, and *IL-16* promoter status was evaluated

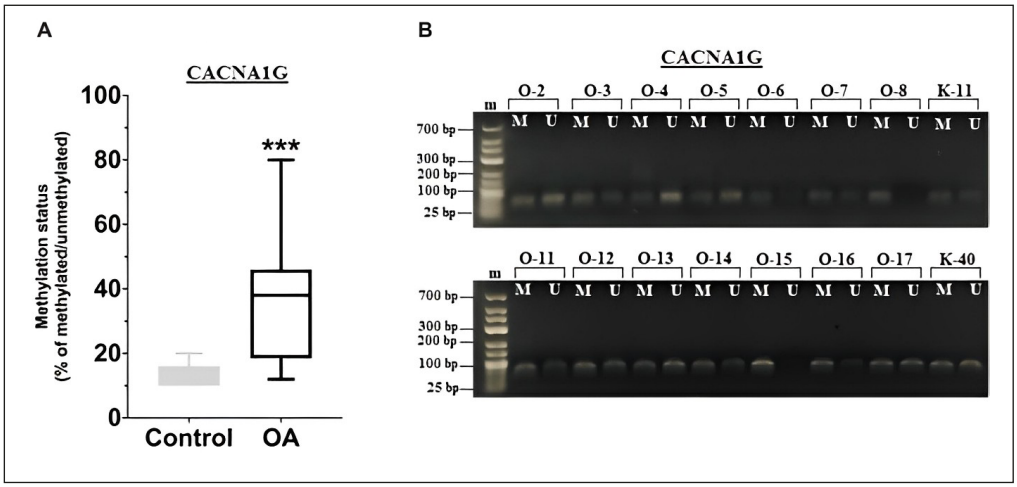

Figure 1. Methylation profile of CACNA1G promoter in healthy controls and primary osteoarthritis patients. Data were expressed as 5-95% in box and whiskers (**A**). Gel picture stands as representative of all groups (**B**). ***$p<0.001$ *vs*. the control group. bp, base pairs; M, methylated PCR product; U, unmethylated PCR product; m, size marker.

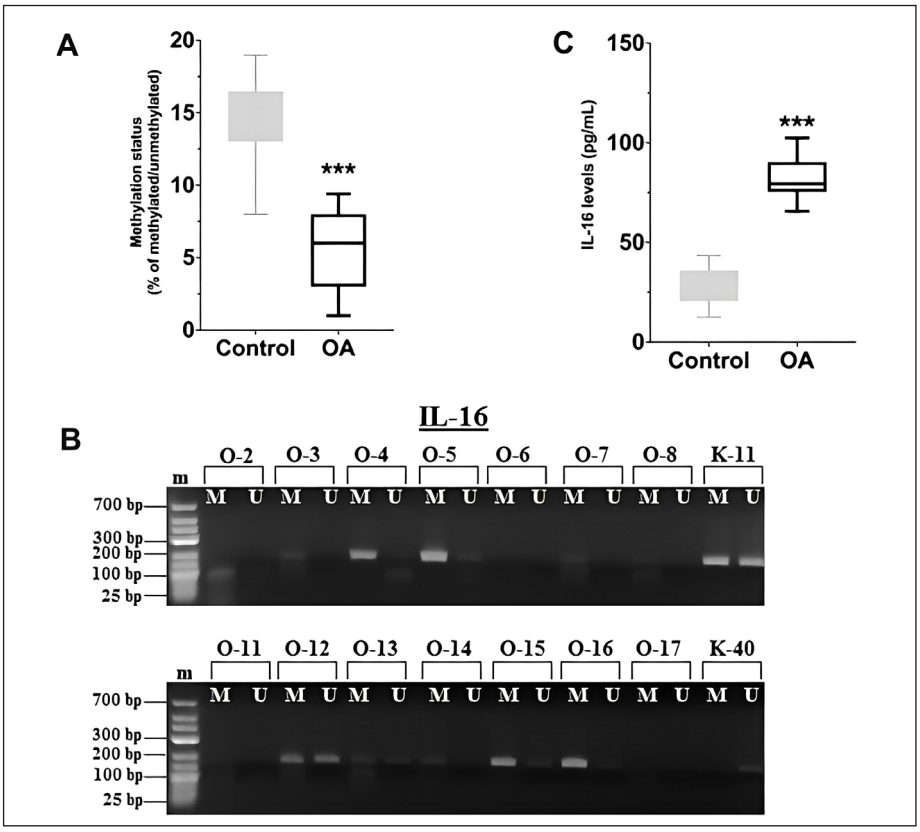

Figure 2. Methylation profile of IL-16 promoter in healthy and primary osteoarthritis patients, and gel picture stands as representative of all groups (**A**, **B**). Serum IL-16 levels were determined by ELISA (**C**). Data were expressed as 5-95% in box and whiskers. ***$p<0.001$ *vs.* the control group.

with methylation-specific PCR. Serum IL-16 levels were found to be significantly increased in the osteoarthritis group (81.9±2.13) compared to the control (28.7±1.81, $p<0.001$, Figure 2C). Furthermore, the methylation status of the *IL-16* promoter was found to be significantly unmethylated in the osteoarthritis group (14.5%±0.53) compared to the control (5.42%±0.6, $p<0.001$, Figure 2A, 2B).

Discussion

In the physiopathology of osteoarthritis, a sensitive balance between synthesis and degradation of chondrocytes is lost, resulting in cartilage destruction[2]. Several molecular and metabolic changes occur during this progressive degenerative process[16]. Therefore, unmasking possible differences in the methylation status of the genome during different stages is a significant aim for halting cartilage breakdown. DNA methylation, the most widely investigated epigenetic regulation, is found to strongly regulate several molecular processes that have a role in primary osteoarthritis[17]. Studies[18] investigated the role of DNA methylation in different genes or pathways related to osteoarthritis. Different groups identified these genes and found that many of the genes related to extracellular matrix regulation and bone morphogenesis have different methylation profiles in osteoarthritis patients[19]. Delgado et al[20] demonstrated that osteoarthritis patients and osteoporosis patients have differentially methylated regions in their genome. Fernandez et al[21] also showed that the methylation profile of articular chondrocytes in osteoarthritis patients showed many different methylated genes compared to the control groups. Furthermore, Jeffries et al[8] identified significant epigenomic changes mediated by methylation in osteoarthritis patients. However, current knowledge about the methylation of *IL-16* and *CACNA1G* genes is still limited. Therefore, we specifically selected these two genes in our study.

Here, in the current study, the promoter methylation profile of *IL-16* and *CACNA1G,* and serum levels of IL-16 were assessed in patients with pri-

mary osteoarthritis patients and healthy controls. The patient and control demonstrated a significant difference regarding the methylation profile of the *IL-16* and *CACNA1G* genes. The promoter of the *CACNA1G* gene was hypermethylated in primary osteoarthritis patients; however, the promoter of the *IL-16* gene was hypomethylated compared to the control. Moreover, the serum levels of IL-16 increased in parallel with the hypomethylated promoter status of *IL-16* gene in primary osteoarthritis patients.

IL-16 is strongly related to antigen presentation and CD_4-specific ligand for initiation of CD_4 bioactivity[22]. Additionally, several studies demonstrated that IL-16 also causes an increase in the production of inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-15 (IL-15)[23]. Thus, it led us to think that changes in the methylation status of *IL-16* promoter could be related to osteoarthritis. In parallel with previous studies, our results indicated that methylation of *IL-16* is strongly associated with osteoarthritis, as seen in serum levels and unmethylated status of *IL-16* promoter.

Calcium channel blockers and cellular calcium regulation are among the primary regulators of bone turnover. Scuolar[24] indicated that chronic use of calcium channel blockers increases osteoarthritis incidence in the long term. Additionally, different groups demonstrated that long-term use of calcium channel blockers might increase joint pain and osteoarthritis[25]. Therefore, we investigated the methylation status of the *CACNA1G* gene promoter which is the primary calcium channel activity regulator in bone tissue. Our results indicated that promoter methylation of calcium channels increased in osteoarthritis patients. Based on the current knowledge, due to the increased methylation of *CACNA1G* channels, it is rational to think long-term arthritis-related effects of calcium channel blockers could be related to this mechanism.

Conclusions

In conclusion, this study indicates that the methylation status of *CACNA1G* and *IL-16* gene promoters are epigenetically altered in patients with primary osteoarthritis. While the promoter methylation of *CACNA1G* increased in osteoarthritis patients, the *IL-16* promoter was significantly hypomethylated in osteoarthritis patients. Moreover, increased serum IL-16 levels in osteoarthritis patients may be associated with hypomethylation of the *IL-16* gene promoter. In this study, we investigated *CACNA1G*, and other subunits of *CACNA1* should be investigated, as *CACNA1G* could be related to long-term arthritis-like effects of calcium channel blockers.

Ethics Approval

This study was conducted in accordance with the ethical standards of the Samsun University Ethics Committee and the 1975 Declaration of Helsinki revised in 2013. Ethics committee approval was obtained (Decision No: KAEK 2021/437).

Informed Consent

Written and signed consent was collected from all volunteers.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' Contribution

LK: Conception and designing, drafting the article. ZBC: Data collection, analysis and interpretation of data. DYK: Preparing the article; critically reviewing the article for important intellectual content. İE: Data collection and analysis. CG: Data collection, analysis and interpretation of data. MA: Preparing the article; critically reviewing the article for important intellectual content.

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