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Association of *Matrix Metalloproteinase-3 (MMP-3)* Gene Methylation Status with the Risk of Developing Achilles Tendonitis: A Preliminary Study

Matriks Metalloproteinaz-3 (MMP-3) Geni Metilasyon Durumunun Aşil Tendinit Gelişmi Riski ile İlişkisi: Bir Ön Çalışma

Ercan Tural[1], Z. Betul Celik[2], Esra Tekcan[3], Sengul Tural[3]

[1]Ondokuz Mayıs University, Faculty of Health Sciences, Department of Physiotherapy and Rehabilitation, Samsun, Turkey
[2]Samsun University, Faculty of Medicine, Department of Medical Biology, Samsun, Turkey
[3]Ondokuz Mayıs University, Faculty of Medicine, Department of Medical Biology, Samsun, Turkey

Abstract

Introduction: In this study, we aimed to examine the relationship between the *MMP-3* gene promoter methylation status and the risk of developing achilles tendinitis in endurance athletes.

Materials and Methods: A total of 20 endurance athletes with achille tendonitis and 20 sedantary controls were involved in the study. DNA isolation and bisulfite modification were performed from peripheral blood samples of all volunteers. Using methylation-specific primers designed for *MMP-3* gene promoters, methylation profiles were analyzed by the methylation specific polymerase chain reaction method.

Results: In the *MMP-3* gene promoter methylation analysis, the methylation frequency was found to be 75% in athletes with Achilles tendinitis, while it was found to be 100% in the control group. *MMP-3* promoter region methylation status in the patient group was found to be hypomethylated compared to the control group.

Discussion and Conclusion: The hypomethylated status of the *MMP-3* gene promoter may have a role in developing achilles tendonitis in athletes. Furher and larges studies are needed to confirm our results.

Keywords: Epigenomics; tendonopathy; methylation.

Özet

Giriş: Bu çalışmada, *MMP-3* geni promotör metilasyon durumu ile dayanıklılık sporu ile uğraşan bireylerde aşil tendinit gelişimi riski arasındaki ilişkiyi incelemeyi amaçladık.

Gereç ve Yöntem: Çalışmaya aşil tendinitli toplam 20 sporcu ve 20 sedanter kontrol dahil edildi. Tüm gönüllülerin periferik kan örneklerinden DNA izolasyonu ve bisülfit modifikasyonu yapıldı. Daha sonra *MMP-3* gen promotörleri için tasarlanmış metilasyona özgü primerler kullanılarak metilasyon profilleri, metilasyona özgü polimeraz zincir reaksiyonu yöntemiyle analiz edildi.

Bulgular: MMP-3 gen promotörü metiasyon analizinde, aşil tendiniti olan sporcularda metilasyon sıklığı %75 olarak saptanırken, kontrol grubunda %100 olarak bulundu ($p<0.05$). Hasta grubunda *MMP-3* promotör bölge metilasyon durumu kontrol grubuna göre hipometile olarak saptandı.

Sonuç: Sonuçlarımıza göre *MMP-3* gen promotörünün hipometile durumu, sporcularda aşil tendinit gelişiminde rol oynuyor olabilir. Sonuçlarımızı doğrulamak için daha fazla ve daha büyük çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: Epigenomik; tendonopati; metilasyon.

Introduction

Tendinopathy is used to describe clinical conditions that include tendon overuse, pain, pathology and it constitutes a significant part of sports injuries and occupational disorders. The most common tendinopathy due to overuse is the achilles, patellar, posterior tibialis, wrist extensors and long head of the biceps brachia (1-3). Tendinosis, a degenerative pathology without inflammatory changes and tendinitis refer to the inflammatory process (4). As a result of inflammation, a series of immune events develop to restore normal function and structure. Inflammation is defined by locally proliferated inflammatory cells and increased cytokine levels (5). Mechanical and physiological stress on the rotator cuff tendons is thought to cause microtrauma that turns into inflammation. Ongoing inflammation in the tendons of the shoulder delays the healing process (5). Therefore, new treatment options are needed to relieve inflammation and pain. Matrix metalloproteinases (MMPs) are endopeptidases containing zinc and are involved in the remodeling of through the

***Corresponding Author:** Şengül Tural, Ondokuz Mayıs University, Faculty of Medicine, Department of Medical Biology, Samsun, Turkey, **e-mail:** stural@omu.edu.tr Orcid: Ercan Tural 0000-0003-3894-7761, Zülfinaz Betül Çelik 0000-0003-1390-7309, Esra Tekcan 0000-0002-6953-9202, Şengül Tural 0000-0002-8946-8165

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degradation of the proteins. These enzymes are classified as stromelysins, collagenases, and gelatinases according to their substrate preferences (6). MMP-3 known as stromelysin-1 is a member of the MMP's, is encoded by the *MMP-3* gene located on chromosome 11q22.3. MMP-3 substrates include several types of collagen (II, IV, V, IX, X), fibronectin, proteoglycans aggrecan, laminin, and decorin (7,8). It has been observed in studies that genetic variation in the code of enzymes that regulate extracellular matrix hemostasis in genes such as *MMP* affects the risk of Achilles tendinopathy. MMP-3 is required to maintain the extracellular matrix hemostasis. It contributes to material integrity as well as mechanical properties of tendons. Increased expression of the *MMP-3* gene is related to increased matrix degeneration (9). It is known that genetic variation can cause tendinopathy, but there are contradictions in some of the genome-wide association studies performed to determine risk variants. However studies suggest that genetic risk assessment only is insufficient without evaluating epigenetic factors. The most widely studied epigenetic modification is DNA methylation. Adding a methyl group to a cytosine nucleotide creates 5-methylcytosine. Changes in DNA methylation, which can be reversible, can affect the structure and homeostasis of tendons (6). In the light of these data, we aimed to examine the relationship between promoter methylation status of *MMP-3* and tendinitis in Turkish athletes.

Material and Method

Subjects: This prospective study was performed on 40 individuals (20 endurance athletes and 20 controls). Power analysis was made with the Open Epi Info Software program, the minimum number of individuals required in each group was calculated as n=14 in the power analysis performed by taking power=80% and it was decided to include at least n=20 individuals in each group. All volunteers were informed regarding the study, and their signed approvals were obtained. A total of 40 volunteers, including 20 athletes(75% female, 25% male) with a diagnosis of tendinitis (mean age 26.5± 38.2 years) and 20 healthy controls (75% female, 25% male) without a history of tendinitis or any other disease (mean age 26.4 ± 25.2 years) were included in the study.

Table 1: MSP primer sequences, hybridization temperatures (Tm) and expected amplicon lengths for *MMP-3* gene promoter.

Primer Name	Primer Sequence (5'-3')	Base Number	Tm (°C)	Amplicon length (bp)
MMP-3 MSP-F	TAAGGATAGAGAGAATTTTAGTTCGG	26	58	100
MMP-3 MSP-R	ATTTATTAATAAATAATAAACCCACGTA	28	53	100
MMP-3 USP-F	GATAAGGATAGAGAGAATTTTAGTTTGG	28	59	100
MMP-3 USP-R	TTATTAATAAATAATAAACCCACATA	26	51	100

(MSP-F: methylation-specific forward primer, MSP-R: methylation-specific reverse primer, USP-F: forward primer not specific to methylation, USP-R: reverse primer not specific to methylation, bp: base pair)

DNA Isolation from peripheral blood: Peripheral blood samples of athletes and controls were taken into EDTA tubes. Genomic DNA from peripheral blood mononuclear cells, "Pure Link® Genomic DNA was isolated exactly following the manufacturer's protocol using "Mini Kit (Invitrogen, USA)." DNA concentrations and purities were determined spectrophotometrically using NanoDrop (Jenway Genova Nano, UK).

Bisulfite modification of DNA and methylation specific PCR (MSP): The bisulfite conversion of the DNA was applied with the "EpiJET Bisulfite Conversion Kit (Thermo Fisher Scientific, Lithuania)" using 500 ng / 20 mL DNA, in accordance with the kit protocol. For methylation specific PCR (MSP), methylated and unmethylated primers specific to the *MMP-3* gene promoter region were used. MSP primers were designed using, *MMP-3* promoter sequence and the "http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi" database. Methylated and unmethylated specific primer sequences are given in Table 1. The methylation-specific PCR reaction was carried out in a final

volume of 25 µl containing *Taq* ™ DNA Polymerase enzyme (1.25U) (Thermo Fisher Scientific, Lithuania), 10X Taq Buffer (2.5µl), dNTP mix (200µM), $MgCl_2$ (2mM) and methylation-specific or unmethylation-specific *MMP-3* primers (20pmol/µl) and (150ng/3µl) bisulfite-modified DNA, and under the following cycling condition in Thermal Cycler Gene Amp® PCR System 9700 (Applied Biosystems, USA); for methylation-specific *MMP-3* primers: initial denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for the 40s; 58 °C for 40s and 72 °C for 70s and for unmethylation-specific *MMP-3* primers: initial denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 40s; 53 °C for 40s and 72 °C for 70s. Final extensions were carried out at 72°C for 7 min and then maintained at 4 °C. Then, the PCR products were separated on a 2% agarose gel by electrophoresis and visualized with a UV transilluminator. As a result of the analysis, when the product was detected in the reaction with only methylated or both methylated and unmethylated specific primers, the methylation status of gene was evaluated as methylated. When the PCR product was observed in the reaction with only the unmethyleted specific primer, the methylation status was evaluated as unmethylated.

Ethical approval: The ethics committee of Ondokuz Mayıs University Clinical Research approved this study (Approval Number: OMU KAEK 2021/198) and all procedures applied in the study were carried out according to the 2013 Principles of the Declaration of Helsinki.
Statistical analysis: The obtained data in the study were analyzed with SPSS V.22. Kolmogorov-Smirnov test was used to test the normal data distribution. Chi-square ($\chi 2$) test was performed to compare the methylation frequencies of the patient and control groups. Pearson and Spearman, in evaluating the relationships between variables, the correlation coefficient was used. Independent groups test was used to compare normally distributed data. As descriptive statistics, normally distributed data are mean ± standard deviation, non-normally distributed data are median (minimum-maximum), categorical variables are sample numbers and given as a percentage. When the P-value was less than 0.05 it was considered as statistically significant.

Results

The study included 20 athletes with achilles tendinitis and 20 controls. Mean age of athletes were 20.24 ± 2.78 (range of age 18–27) and

Table 2: The methylation frequencies of *MMP-3* promoter region in patient and control groups

	Methylated *MMP-3* n(%)	Unmethylated *MMP-3* n(%)	*P*-Value
Tendonitis (n=20)	15 (75%)	5 (25%)	0.0236*
Control (n=20)	20 (100%)	0 (0%)	

(*P<0.05, n: number of individuals)

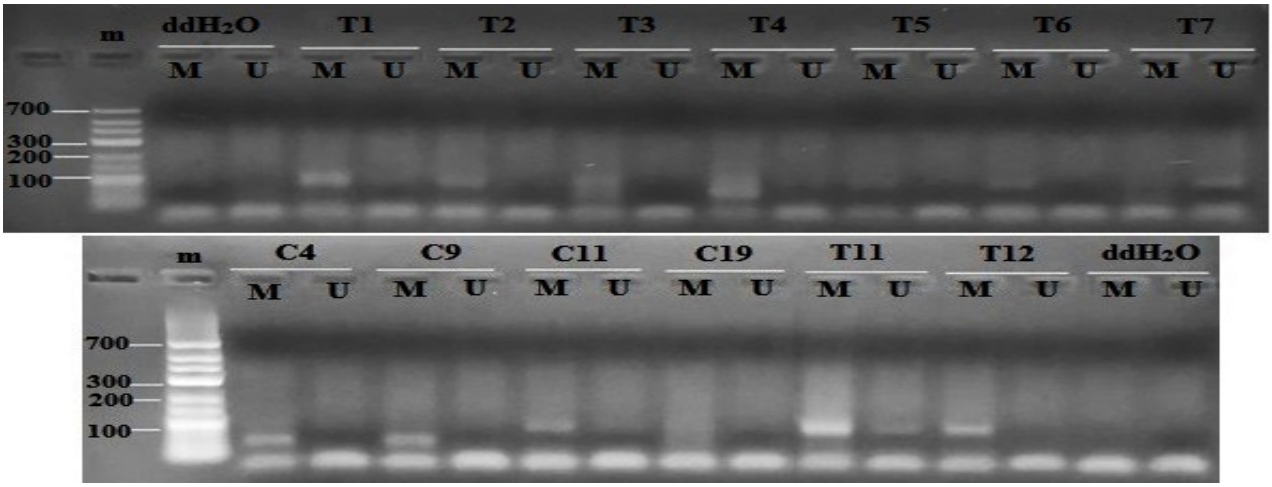

Figure 1. Representative agarose gel images for the promoter methylation status of *MMP-3* gene in the tendinitis group and control group (m: marker (base pair), M: methylated, U: unmethylated, T: tendinitis group, C: healthy control group).

control mean age were 24.52 ± 4.52 (range of age18–34). The promoter methylation analysis of the *MMP-3* gene was carried out in peripheral blood samples of 20 athletes with tendinitis and 20 controls. The frequency of methylation for *MMP-3* in the athlete group was found to be 75% (15/20) and 100% (20/20) in the control group (Table 2, Figure 1). When the athlete and control groups were compared in terms of *MMP-3* promoter methylation status, a statistically significant difference was observed between the athlete groups ($p = 0.0236$). *MMP-3* gene is hypomethylated in the athlete group with achilles tendinitis.

Discussion

MMP-3 is a proteolytic enzyme, it plays a key role in the destruction of extracellular matrix components and the inflammation that occurs as a result of tendinitis plays an important role. In the present study, we examined the relationship between the *MMP-3* gene promoter methylation status and the risk of developing achilles tendinitis and our results showed that the frequency of methylation of athlete group was 75% (15/20) for the *MMP-3* gene and it was 100% (20/20) in the control group. MMP-3 degrades some other collagenous and non-collagen extracellular matrix proteins and activates pro-MMPs into their functional form. MMP-3 provides activation of Pro-MMP-1 to MMP-1 (7-11). It appears how variation in the *MMP-3* gene may have a downside effect on collagen turnover and tendon structure (12). Thus, if the gene is overexpressed and the resulting MMP-3 protein production is increased, collagen conversion can also be seen to increase. As a result of homeostatic balance deterioration, the tendon structure will deteriorate, and this will cause pathology. There is no research in the literature regarding *MMP-3* gene methylation profile analysis in Achilles tendon tendinopathy. In previous studies, there were some gene polymorphism studies regarding the *MMP-3* gene. In a study, Briski et al. examined *MMP-3* gene polymorphisms in Croatian high-level athletes and they reported that the *MMP-3 rs650108* GG and *rs679620* AA genotypes were over-represented in athletes compared to controls. Their results highlight an association among the risk of tendinopathies and functional variants within the *MMP-3* gene in high-level athletes (13). Nie at al. found in their study that the *MMP-3* gene *rs679620* variant was associated with chronic Achille tendon risk in the Chinese study group (14). Khoury et al. estimated a significant association between the *MMP-3 rs679620* variant and achilles tendon rupture (15). Foster et al. examined the *MMP-3* gene (*rs679620*, *rs591058,* and *rs650108*) in Caucasians, but they did not find any relation with tendinopahy (16). In a recent meta-analysis; Association between *MMP-3* gene polymorphisms and tendon-ligament injurie analysed and it is reported that *rs679620* polymorphism is associated with a reduced achilles tendon rupture risk, and *rs3025058* polymorphism contributes to an increased tendon-ligament injuries risk in Caucasians and Brazilians. However, *rs591058* and *rs650108* polymorphisms do not show any association with tendon-ligament injuries (1). In another recent study, the methylation frequency of the *MMP-3* gene has been found to significantly decrease in Turkish rheumatoid arthritis patients compared to control (17).

Study limitations: Study sample size was limitation of our study. Although our sample number was higher than that determined in the power analysis, we could have obtained more meaningful results if we had included more samples.

Conclusion

The hypomethylated status of the *MMP-3* gene promoter have a role in developing achille tendonitis in athletes. Furher and larges studies are needed to confirm the results.

Conflict of interest: The authors declare that they have no conflict of interest for this study.

Ethical approval: The ethics committee of Ondokuz Mayıs University Clinical Research approved this study (Approval Number: OMU KAEK 2021/198)

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