

**Original Article**

Matrix Metalloproteinase-9 Level and Gene Polymorphism in Sleep Disordered Breathing Patients with or without Cardiovascular Disorders

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Objective: Obstructive sleep apnea syndrome (OSAS) is associated with increased cardiovascular morbidity and mortality. We aimed to investigate the matrix metalloproteinase-9 (MMP-9) level and MMP-9 gene polymorphism in sleep apnea patients with or without cardiovascular disease.

Study Design: Case-control study.

Material and Methods: Two hundred nine patients [Mean age (\pm SD), 47 (\pm 12) yrs; M/F, 170/39] diagnosed with sleep-disordered breathing were included in the study. Serum MMP-9 level was performed using enzyme-linked immunosorbant assay (ELISA) and MMP-9 gene polymorphism with polymerase chain reaction-restriction fragment length polymorphism. We divided the patient group into two subgroups: (1) patients with confirmed cardiovascular disease, i.e. CV-P Group and (2) patients without cardiovascular disease, CV-N Group. We compared all parameters between the two groups.

Results: There were 56 OSAS patients with cardiovascular disorder (CV-positive group) and 153 OSAS patients without cardiovascular disorder (CV-negative group). CC, CT and TT genotype distributions between groups were similar [31 (55%), 25 (45%), 0 (0%) vs 88 (57%), 61 (40%), 4 (3%); respectively, $p>0.05$]. MMP-9 level was significantly higher in CV-P patients (442.7 ± 139.3 pg/mL) than in CV-N patients (364.4 ± 165.0 pg/mL; $p=0.0018$).

Conclusion: Our results showed that the presence of MMP-9 polymorphism was not associated with cardiovascular disease. MMP-9 level was higher in OSAS patients with cardiovascular disorders than without cardiovascular disorders. Finally, MMP-9 genotype was not associated with serum MMP-9 levels.

Key Words: Cardiovascular disease, MMP-9, polymorphism, sleep apnea, hypertension

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Introduction

Increased cardiovascular morbidity and mortality are well-known consequences of obstructive sleep apnea syndrome (OSAS) (1, 2). Epidemiological data demonstrated definite associations between OSAS and cardiovascular disorders such as hypertension, ischemic heart disease, congestive heart failure, and stroke (3). Clinical and experimental studies yielded convincing evidence that various acute (negative intrathoracic pressure, hypoxia and arousals) and chronic (sympathetic nervous system activity, hormonal alterations) factors resulting from repetitive abnormal respiratory events during sleep may play key roles in the development of cardiovascular disorders during the course of OSAS. However, the pathophysiological link between sleep apnea syndrome and cardiovascular disorders is not clear. Recently, matrix metalloproteinases have become an important focus of research in the pathogenesis of atherosclerotic cardiovascular diseases and hypertension (4).

Matrix metalloproteinase-9 (MMP-9) is a proteolytic enzyme which contributes to extracellular matrix degradation and vascular remodeling (5). MMP-9 shows proteolytic activity on type IV collagen which forms a basement membrane under the endothelium

of all blood vessels. Studies have shown that certain cardiovascular disorders such as coronary artery disease, arterial stiffness, atherosclerosis and hypertension may be associated with MMP-9 polymorphism and serum levels (4, 6, 7). There is also an association between increased serum levels and activity of MMP-9 and severity of OSAS (8, 9). Systemic inflammatory markers such as C-reactive protein, interleukin-6 (IL-6) and TNF- α were found to be positively correlated with MMP-9 activity in patients with OSAS (8, 9). However, there are no published studies reporting MMP-9 levels in OSAS patients with or without cardiovascular disorders who carry different alleles of MMP-9 gene polymorphism. In addition, although it has been shown that genetic polymorphism of MMP-9 does not necessarily affect the plasma activity in healthy subjects (10), the relationship of MMP-9 polymorphism and serum MMP-9 level was not assessed in sleep apnea patients.

In this study, we aimed to investigate MMP-9 polymorphism and level in OSAS patients with or without cardiovascular (CV) disorders. We also aimed to yield further evidence for the lack of association between MMP-9 polymorphism and serum MMP-9 level. We hypothesized that MMP-9 level is higher in CV-positive (CV-P) OSAS patients with respect to CV-negative (CV-N) OSAS patients.



Material and Methods

Patient Population

A total of 209 OSAS patients followed at the Sleep Disorders Unit, Süreyyapaşa Chest Diseases and Thoracic Surgery Teaching Hospital, Istanbul were included in the study. All patients underwent a standard battery of examinations, including medical history, physical and cardiological examination, laboratory screening tests. Written informed consent was obtained from all patients. The study was approved by the Local Ethics Committee, Marmara University, School of Medicine.

The study population consisted of 209 (39 female, 170 male) patients with OSAS who were examined using computerized polysomnography. All patients were free from diabetes mellitus type 2 and other known metabolic disorders, respiratory infection, and other respiratory disorders at the time of polysomnography. However, patients with coronary artery disease, hypertension and cardiac arrhythmia were included in the study. They were asked to complete the Epworth Sleepiness Scale and a questionnaire on sleep symptoms and medical history. Diagnosis of OSAS was established on the basis of clinical and polysomnographic criteria (11). The average number of episodes of apnea and hypopnea per hour of sleep was calculated and expressed as apnea-hypopnea index (AHI). In addition to clinical symptoms, an AHI of >15 was also used as an inclusion criterion. Liver function tests, renal function tests, and thyroid function tests were performed to determine co-morbid disorders. Circulating total cholesterol, triglycerid (TG), high density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C) measurements were also performed by enzymatic methods using commercial kits as standard evaluations.

Polysomnography

Overnight polysomnography (Somnologica, Flaga, Iceland) was performed between 11 PM and 7 AM. Polysomnography consisted of simultaneous recordings of two channels EEG (C_3A_2 and C_4A_1), left and right electro-oculography, and chin electromyography from surface leads for sleep staging. In addition, air flow from a nasal cannula, thoracic and abdominal strain gauges for respiratory effort, tracheal microphone for snoring, pulse oximetry for oxyhemoglobin level, and sensor for body position during sleep were used. Sleep staging was performed manually according to standard criteria and as described previously (12, 13). Respiratory data including AHI, minimum and mean oxyhemoglobin desaturations, were produced automatically by a computer program (Somnologica version 2.0.1, Flaga, Iceland).

Blood samples were taken in the morning after PSG. Whole blood samples were used for DNA extraction and genomic polymerase chain reaction (PCR) -restriction fragment length polymorphism (RFLP) analysis and serum samples were used for MMP9 level analysis.

PCR-RFLP determination

Genomic DNA was extracted from peripheral blood leukocytes by a high pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany). Genotypes for

polymorphism in MMP9 C-1562T mutation were detected using the PCR-RFLP technique (14). 50 μ l final volume of PCR mixture for these genes consisted of 10 ng genomic DNA, 0.2 mM of each dNTP, 1 x PCR buffer [75mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20], 1.5 mM MgCl₂, 1 U Taq polymerase and 10 pmol of each primer. The mixture was subjected to PCR with a Techne TC 312 thermal cycler (Barloworld Scientific, Cambridge, UK). The PCR profile for this gene consisted of an initial melting step of 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 1 min, and a final elongation step of 72°C for 10 min. The PCR product of MMP9 gene were checked on a 1% agarose gel and digested with Pael (Fermentas, Latvia) restriction endonuclease enzyme at 37°C for 3 hours. If there was a C>T transition, the 436 bp PCR fragment generated two sub fragments of 244 and 192 bp.

MMP9 Assay

Serum levels of MMP9 were quantified according to the manufacturer's instructions and guidelines using an enzyme-linked immunosorbant assay (ELISA) kit specific for humans (Invitrogen Corp., Camarillo, CA, USA).

Statistical analysis

Differences in genotype distributions and allele frequencies in OSAS patients with CV-P versus CV-N patients were compared for statistical significance using the chi-square test. Statistical significance for deviations from Hardy-Weinberg equilibrium was determined using the Pearson chi-square test. Odds ratios (ORs) were calculated and given with 95% confidence intervals. Differences were considered significant if the value of probability $p<0.05$. The differences of age at illness onset were evaluated using Mann-Whitney U test. Data are given as mean \pm SD. All statistical analyses were performed with GraphPad InStat for Windows (San Diego, CA, USA).

Results

There were 56 OSAS patients with cardiovascular disorder (CV-positive group) and 153 OSAS patients with no cardiovascular disorder (CV-negative group). Comparisons of demographic, anthropometric, polysomnographic and biochemical parameters between CV-P and CV-N patients with obstructive sleep apnea were shown in Table 1. Patients in the CV-P group consisted of 34 (60.7%) patients with coronary artery disease and 40 (71.4%) patients with hypertension and cardiac arrhythmia. The mean duration of cardiovascular disorder was 6.8 ± 5.3 years. The numbers of current smokers were 37 (66.1%) in CV-P and 55 (35.3%) in CV-N groups. CV-P and CV-N groups were similar in terms of mean age and gender distribution. Although height and weight comparisons failed to reach statistically significance level, the mean body mass index was slightly higher in CV-P patients than in CV-N patients (33.5 ± 6.2 vs 31.1 ± 5.0 respectively, $p<0.01$). The severity of OSAS, which was demonstrated by apnea hypopnea index, was significantly higher in CV-P patients than in CV-N patients (Table 1). Biochemistry profiles were similar in both

study groups except MMP-9. The mean MMP-9 level was significantly higher in CV-P patients (442.7 ± 139.3 pg/mL) than in CV-N patients (364.4 ± 165.0 pg/mL; $p=0.0018$).

For MMP9 C-1562T polymorphism, patients with CC, CT and TT allele frequencies were 119 (57%), 86 (41%) and 4 (2%) in the whole study group and C and T allele frequencies were 77.1% and 22.9% in CV-P patients vs 55.4% and 44.6% in CV-N patients. There was no patients with TT genotype in CV-P group. C-1562T polymorphism was significantly differentiated between cardiovascular disorder-positive and-negative obstructive sleep apnea patients (95% CI, 0.67 to 0.76; $p<0.0001$). Comparisons of polysomnographic parameters in different genotypes with respect to cardiovascular disorders were presented in Table 2. Due to the low frequency of TT genotype, we combined TT and CT genotypes (as CT+TT group) and compared the presence of cardiovascular disorders between CC group and CT+TT group, which also failed to show any statistically significant difference (Chi-square test, $p>0.05$). Interestingly, despite the high level of serum MMP-9 in the CV-P group, the presence of T allele seemed to be ineffective in determining the MMP-9 level (Figure 1). The highest MMP-9 level was found in CT genotype subgroup of CV-P patients. Patients with CT genotype in CV-N group had MMP-9 levels lower than patients with CC genotype in CV-P group. MMP-9 did not predict the MMP-9 level in OSAS patients.

The MMP-9 level in CV-P patients with CC or CT genotypes were 402.6 ± 131.6 pg/ml, and 492.3 ± 134.7 pg/mL, respectively ($p<0.05$). On the other hand, the MMP-9 level in CV-N patients with CC, CT or TT genotypes were 358.4 ± 155.7 pg/mL, 375.0 ± 180.1 pg/mL and 332.1 ± 142.1 pg/mL, respectively ($p<0.01$, Figure 1).

Discussion

We found that the mean serum level of MMP-9 was significantly higher in obstructive sleep apnea (OSA) patients with cardiovascular disorders with respect to OSA patients without cardiovascular disorders and the MMP-9 level was not associated with different MMP-9 genotypes, i.e. CC, CT and TT in OSA patients. MMP-9, also known as type IV collagenase, shows proteolytic activity on type IV collagen, elastin and fibronectin. During atherogenesis, both vascular smooth muscle cell migration and macrophage infiltration requires degradation of type IV collagen by MMP-9 (6). Blankenberg demonstrated a strong association between baseline MMP-9 levels and the future risk of cardiovascular death (15). As the relationship of obstructive sleep apneas and cardiovascular disorders has been demonstrated in a large number of epidemiological and animal studies, several recent studies assessed MMP-9 levels and activity in patients with OSAS. Tazaki et al. (8) compared 44 obese OSAS patients free from other diseases with 18 obese controls and found that MMP-9 serum levels and activity were significantly higher in patients with OSAS. They also reported that 1 month nasal continuous positive airway pressure treatment decreased both serum levels and activity of MMP-9. After the initial study of Tazaki et al. (8), in another study Ye et al. (9) reported that serum concentrations of C-reactive protein (CRP) and MMP-9 were significantly

Table 1. Comparison of demographic, anthropometric, polysomnographic and biochemical parameters between cardiovascular disorder-positive (CV-P) and -negative (CV-N) obstructive sleep apnea patients

	CV-P n=56	CV-N n=153	p value
Demographic			
Mean age, year	50.3 ± 9.6	48.4 ± 11.3	$p>0.05$
Male/Female, n	42/14	128/25	$p>0.05^*$
Anthropometric			
Height, cm	167 ± 10	169 ± 15	$p>0.05$
Weight, kg	92.7 ± 15.2	90.5 ± 14.4	$p>0.05$
BMI, kg/m ²	33.5 ± 6.2	31.1 ± 5.0	$p=0.0061$
Sleep Tests			
AHI, n/h	43.9 ± 23.1	28.1 ± 23.8	$p<0.0001$
MinO ₂	74.3 ± 10.5	76.1 ± 11.1	$p>0.05$
MeanO ₂	90.4 ± 5.4	91.4 ± 3.9	$p>0.05$
ODI	41.5 ± 24.2	37.0 ± 24.3	$p>0.05$
Biochemistry			
Triglyceride, mg/dL	206.6 ± 119.4	178.9 ± 99.0	$p>0.05$
Total C, mg/dL	199.6 ± 35.3	205.8 ± 46.9	$p>0.05$
HDL-C, mg/dL	48.8 ± 46.1	44.7 ± 13.9	$p>0.05$
LDL-C, mg/dL	126.2 ± 56.1	132.0 ± 35.4	$p>0.05$
MMP-9 Act, pg/mL	442.7 ± 139.3	364.4 ± 165.0	$p=0.0018$

BMI: body mass index; AHI: apnea-hypopnea index; HDL-C: high density lipoprotein-cholesterol; LDL-C: low density lipoprotein-cholesterol; ODI: oxyhemoglobin desaturation index; MMP: matrix metalloproteinase

All comparisons were performed by using Student's t-test unless otherwise stated. *Fisher's exact test.

higher in 51 OSAS patients with respect to 25 controls. This study also reported a positive correlation between the levels of CRP and MMP-9 in patients with sleep apnea. And finally, Kaditis et al. (16) studied morning plasma CRP and MMP-9 levels in a group of pediatric OSAS patients. They also provided further evidence that plasma levels of MMP-9 is affected by low-grade systemic inflammation as assessed by CRP (16). Apparently, all of these four studies were performed on adult or pediatric OSAS patients free from other chronic diseases and any of the cardiovascular disorders. To our knowledge, this is the first study that compared MMP-9 levels in adult OSAS patients with and without cardiovascular disorders, and found a significant difference in favour of CV-P patients.

Studies on the influence of genetic polymorphism in the promoter region (C-1562T) of the MMP-9 gene (rs3918242) have shown that "T" allele carriers had increased MMP-9 expression (17). Blankenberg also found similar results in their research group for about 1127 coronary artery disease patients (15). However, another study demonstrated that plasma pro-MMP-9 and MMP-9 activities were not affected by the C-1562T polymorphism in healthy subjects (10). In a large patient group of stable coronary artery disease (n=1001), there was no association between C-1562T polymorphism and coronary

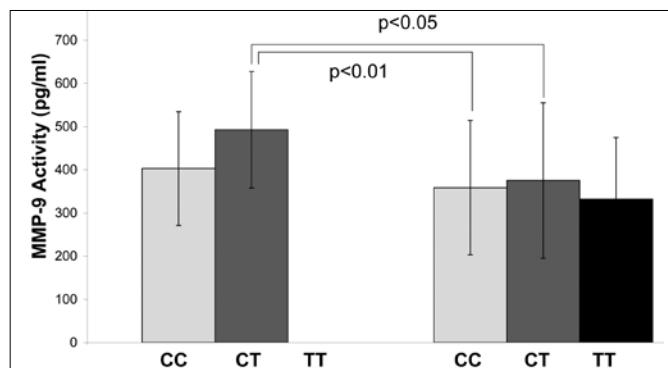
Table 2. Matrix metalloproteinase-9 polymorphism in obstructive sleep apnea patients with or without cardiovascular disorders

	CV-P			CV-N		
	CC	CT	TT	CC	CT	TT
M/F	23/8	19/6	-	70/18	54/7	4/0
AI, n/h	30.7±24.8	38.9±23.4	-	24.9±25.2	25.5±23.2	17.4±4.3
HI, n/h	10.3±11.4	9.5±10.2	-	7.4±8.5	8.2±10.2	17.6±11.8
AHI	41.6±22.5	46.7±24.0	-	25.1±24.1*,+	32.5±23.3	23.3±14.9
MinO ₂ , %	75.0±1.9	73.4±2.0	-	75.8±1.2	76.6±1.4	74.8±1.7
ODI, n/h	42.6±4.4	40.2±5.2	-	36.6±2.5	37.3±2.5	40.0±6.5

CV-P: cardiovascular disorder-positive; CV-N: cardiovascular disorder-negative; M/F: Male/Female; ODI: oxyhemoglobin desaturation index; AI: apnea index; HI: hypopnea index.

All comparisons showed that these groups were similar in terms of T-allele distribution.

*p<0.01 with respect to CV-P CC genotype, + p<0.01 with respect to CV-P CT genotype

**Figure 1.** Comparison of MMP-9 level in obstructive sleep apnea patients with or without cardiovascular disorders grouped according to MMP-9 gene polymorphism

artery disease (18). The "T" allele was associated with lower mRNA level in an experimental ex-vivo lipopolysaccharide stimulated model, and "T" allele carriers have shown a higher concentration of MMP 9 (18). Similarly, our results suggested that the presence of a cardiovascular disorder rather than "T" allele is a predictor of higher serum MMP-9 level in OSAS. The distribution of different allele frequencies were similar in CV-P and CV-N patients with OSAS. In addition, the mean MMP-9 level of CC patients with cardiovascular disorders was higher than that of CT patients without cardiovascular disorders. A recent study in a Turkish coronary artery disease population reported lack of association between MMP-9 gene polymorphism and presence of coronary artery disease (19). In an autopsy study, the C-1562T promoter polymorphism of MMP-9 gene in aged men ≥53 years was significantly associated with the mean area of complicated lesions in three coronaries. This study confirmed the MMP-9 polymorphism as an independent predictor of complicated lesion area after adjustment for age, body mass index, hypertension, diabetes and smoking (20).

The reason for increased activity or elevated levels of circulating MMP-9 in patients with OSAS is obscure. OSAS is characterized by repetitive cessation episodes of breath-

ing during sleep. These abnormal respiratory events lead to hypoxia and reoxygenation cycles which eventually results in oxidative stress. Oxidative stress gives rise to the production of reactive oxygen species which may regulate MMP levels in various cells and in vitro systems (21). MMP-9 is suggested to be a strong predictor of oxidative stress in OSA patients (21). Thus, reactive oxygen species produced during oxidative stress may contribute to elevated levels and activity of MMP-9 in patients with OSAS. Another possibility is that the levels of inflammatory cytokines such as IL-6 and TNF α in OSAS are elevated and these inflammatory cytokines may stimulate MMP-9 release from neutrophils and monocytes (22, 23).

One potential limitation of the current study is lack of a non-apneic control group. However, comparisons between OSAS patients and non-apneic controls in previous studies showed that MMP-9 levels and activity are higher in OSAS patients than non-apneic controls (8, 9). Such an evaluation would be useless for the purpose of this study, as our primary aim was to determine MMP-9 level in relation with cardiovascular disorders in OSA population. Another limitation is the relatively small number of patients with "TT" genotype. There were only four patients with "TT" genotype and all these patients were in the CV-N group. The numbers of "T" allele carriers in the CV-P and CV-N groups were 25 (all CT) and 65 (CT=61 and TT=4) respectively. These figures suggest that "T" allele carriers among OSAS patients are not under higher risk of cardiovascular disease than "C" allele carriers.

The present study represents the first attempt to assess the potential association between MMP-9 polymorphism and level in sleep apnea patients with or without cardiovascular disorders. MMP-9 level was significantly higher in OSAS patients with cardiovascular disorders independent of MMP-9 genotype. Thus, the MMP-9 level may be a marker for the development of cardiovascular disease in patients with obstructive sleep apnea.

Ethics Committee Approval: Ethics committee approval was received for this study.

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - M.Y., H.K.O.; Design - M.Y., H.K.O.; Supervision - M.Y., Z.P.; Resource - M.Y.; Materials - M.Y., H.K.O., Z.P.; Data Collection&/or Processing - M.Y., H.K.O., A.V.Ö.; Analysis&/or Interpretation - M.Y., H.K.O., Z.P.; Literature Search - M.Y., Z.P.; Writing - M.Y., Z.P.; Critical Reviews - Z.P., A.V.Ö.

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