CLINICS

CLINICAL SCIENCE

Is there a relationship between endothelial nitric oxide synthase gene polymorphisms and ankylosing spondylitis?

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OBJECTIVE: Nitric oxide is produced by endothelial nitric oxide synthase, and its production can be influenced by polymorphisms of the endothelial nitric oxide synthase gene. Because candidate genes responsible for susceptibility to ankylosing spondylitis are mostly unknown and available data suggest that there may be problems related to the nitric oxide pathway, such as endothelial dysfunction and increased asymmetric dimethylarginine, this study aimed to assess the association of common endothelial nitric oxide synthase gene polymorphisms with ankylosing spondylitis.

METHODS: One hundred ninety-four unrelated Turkish ankylosing spondylitis patients and 113 healthy without apparent cardiovascular disease, hypertension or diabetes mellitus were included. All individuals were genotyped by PCR-RFLP for two single-nucleotide polymorphisms, namely 786T>C (rs2070744, promoter region) and 786 Glu298Asp (rs1799983, exon 7). Variable numbers of tandem repeat polymorphisms in intron 4 were also studied and investigated by direct electrophoresis on agarose gel following polymerase chain reaction analysis. The Bath ankylosing spondylitis metrology index of the patients was calculated, and human leukocyte antigen B27 was studied.

RESULTS: All studied polymorphisms satisfied Hardy-Weinberg equilibrium. Sex distributions were similar between the patient and control groups. No significant differences were found in the distributions of allele and genotype frequencies of the studied endothelial nitric oxide synthase polymorphisms between patients and controls. There were no correlations between endothelial nitric oxide synthase polymorphisms, disease duration, Bath ankylosing spondylitis metrology index or human leukocyte antigen B27.

CONCLUSION: The results presented in this study do not support a major role of common endothelial nitric oxide synthase polymorphisms in Turkish ankylosing spondylitis patients.

KEYWORDS: Ankylosing Spondylitis; Endothelial Nitric Oxide Synthase; Nitric Oxide; Inflammation; Atherosclerosis.

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INTRODUCTION

Ankylosing spondylitis (AS) is a chronic inflammatory disease of the spine and sacroiliac joints. Although the pathogenesis of AS is not known, genetic factors play an important role in determining susceptibility and disease severity. HLA–B27 is the major gene associated with AS. Several non-MHC genes have also been linked to the disease (1). In recent years, there has been considerable interest regarding early atherosclerosis and AS (2-4). In this respect, several studies have suggested an increased prevalence of endothelial dysfunction, an early step in the pathogenesis of atherosclerosis (2,3,5,6), in AS. It is well-known that nitric oxide (NO) is essential in the maintenance of vascular tonus (7) and that the presence of endothelial impairment (reduced vascular relaxation) may suggest a problem regarding the NO pathway. In addition, a considerable number of studies have reported that asymmetric dimethylarginine (ADMA),

No potential conflict of interest was reported.

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an endogenous NO inhibitor, is significantly increased in the blood of AS patients (8-10). Both endothelial impairment and increased ADMA concentrations suggest the possibility of a problem related to the NO pathway in AS. NO is produced by endothelial NO synthase (eNOS), and its production can be influenced by polymorphisms of the eNOS gene (7). Because candidate genes responsible for susceptibility to AS are mostly unknown and available data suggest the possibility of a problem related with the NO pathway, this study aimed to assess the association of common eNOS gene polymorphisms with AS.

METHODS

Sample size, patients and controls

Sample size was calculated using the results of previous studies that investigated eNOS gene polymorphisms in Turkish patients with inflammatory rheumatic diseases based on $\alpha = 0.05$ and a power of 80% (11,12). The minimum minor allele frequencies required for this estimation for each group were as follows: Exon 7 (G-894T), 96 subjects; promoter (T-786C), 94 subjects; and intron 4 (variable number of tandem repeat polymorphisms, VNTR), 58 subjects.

We included 194 unrelated AS patients diagnosed according to the modified New York criteria (13). Patients without a history of hypertension (current anti-hypertensive treatment and/or observation of blood pressure levels >140/90 mmHg), diabetes mellitus (participants who reported having ever been told by a physician that they have diabetes mellitus or who reported taking insulin or pills to lower blood glucose levels) or coronary artery disease (history of myocardial infarction, angina pectoris or coronary artery angioplasty) were recruited consecutively from the rheumatology outpatient clinics of the Dokuz Eylul University and three training hospitals (Ataturk, Bozyaka and Tepecik) located in Izmir city. A total of 113 healthy controls subject undergoing the same exclusion criteria as the patients and who did not have any first-degree relatives with diagnoses of AS or related spondyloarthropathies were recruited from the relatives of health professionals and blood donors. Spinal mobility was assessed by the Bath Ankylosing Spondylitis Metrology Index (BASMI) (14). Patients were also evaluated with the Bath Ankylosing Spondylitis Functional Index (BASFI) (15) and the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) (16). The Ministry of Health of Turkey Ethics Committee approved the study, and all individuals provided informed consent prior to blood collection in compliance with the principles of the Declaration of Helsinki.

Genotyping

For all subjects, peripheral blood samples were collected into sterile tubes with EDTA. Genomic DNA was extracted from whole blood using standard proteinase K digestion and the salt-chloroform method. The PCR-RFLP analysis method was used to evaluate the association between three polymorphisms in the eNOS gene and AS. Three PCR primer sets were used for amplification of each polymorphic region, including the Glu298Asp (rs1799983, exon 7), −786T>C (rs2070744, promoter region) and 4b4a (intron 4, 27 bp repeat) regions. The primers were designed using the Primer3 algorithm via the primer-BLAST interface on the NCBI BLAST web site. PCR primer sequences, annealing temperatures and product sizes were as follows: Glu298Asp: (forward) 5′-GTCACGGAGACCCAGCCAATG-3′ and (reverse) 5′-GCCCTTCTTGAGAGGCTCAGGGAT-3′, 61.4 ℃, 325 bp; -786T>C: (forward) 5′-AGCTAGTGGCCTTTCTCCAGCCC-3′ and (reverse) 5′-CCCAGCCCCAATTTCCTGGAACC-3′, 61.4 ℃, 335 bp; and VNTR repeat region: (forward) 5′-GCCTTGGCTGGAGGAGGGGA-3′ and (reverse) 5′-TGGGGGAGAAGCAGCAGCCA-3′, 57.1 ℃, 242 bp. For the Glu298Asp and −786T>C regions, PCR products were digested using MboI (Fermentas, Vilnius, Lithuania) and HpaII (New England Biolab, Hitchin, UK) restriction endonuclease enzymes, respectively. Restriction fragments were separated by electrophoresis on a gel composed of 3% agarose. For the Glu298Asp region, the T allele resulted in 195 and 130 bp bands, while the G allele resulted in single band of 225 bp. For the −786T>C region, the C allele resulted in 167, 46 and 122 bp bands, while the T allele resulted in 122 and 213 bp bands. The 27 bp VNTR repeat region in intron 4 was analyzed by direct electrophoresis on 3% agarose gel after PCR amplification. The 4b allele amplicon size was 242 bp, whereas the 4a allele amplicon size was 215 bp. HLA B27 analysis for the patient group was performed using a commercially available SSP-typing kit (Olerup; QIAGEN Vetriebs GmbH, Wien, Austria) according to the manufacturer's recommendations. SSP-typing results were visualized using 2% agarose gel electrophoresis.

Statistical analysis

MedCalc software, version 12.3.0.0 (MedCAlc, Mariakerke, Belgium), was used to estimate sample size. The rest of the statistical analysis was performed on SPSS v. 16.0 software (SPSS Inc, Chicago, IL). Data are expressed as the means ± SDs for continuous variables or as percentages of the total for categorical variables. Pearson $\chi 2$ or Fisher's exact tests were used to assess intergroup significance, and a Student's t test was used to determine differences in means. The distribution of the control genotypes was checked for the Hardy–Weinberg equilibrium. The overall distributions of alleles and genotypes for each polymorphism were compared between cases and controls using $\chi 2$ analyses. Associations between alleles and genotypes and other variables were examined by $\chi 2$ tests. An analysis of covariance (ANCOVA) was used to control for confounding variables. A double-tailed p-value of < 0.05 was considered statistically significant.

RESULTS

There were 194 AS patients (139 male [M], 55 female [F]; 41.5 ± 10.8 years) and 113 healthy controls (69 M, 44 F; 38.6 ± 11.1 years). Sex distributions were similar between the patient and control groups ($p = 0.07$); however, age was significantly lower in the control group compared with the patients ($p = 0.03$). Disease duration for the patients was 14.9 ± 9.5 years. HLA-B27 was positive in 72% of patients. The mean BASMI (0-10), BASFI (0-10) and BASDAI (0-10) values were 2.8 ± 2.4, 3.2 ± 2.3 and 3.2 ± 2.5, respectively. The clinical and demographical features of the patient and control groups are given in Table 1.

All studied polymorphisms satisfied the Hardy-Weinberg equilibrium in the controls (eNOS T-786C: X2(1) = 1.96, $p = 0.16$; eNOS 4b4a VNTR: X2(1) = 2.74, $p = 0.09$; eNOS Glu298Asp: X2(1) = 1.21, $p = 0.27$).

Table 1 - Clinical and demographic characteristics of the patients and controls.

	AS patients (n = 194)	Healthy controls (n = 113)	*p*-value
Age (years)	41.5 ± 10.8	38.6 ± 11.1	0.03
Sex, M/F	139/55	69/44	0.07
HLAB27 positivity, %	72		
Disease duration (years)	14.9 ± 9.5		
BASMI (0-10)	2.8 ± 2.4		
BASFI (0-10)	3.2 ± 2.3		
BASDAI (0-10)	3.2 ± 2.5		

Continuous data are presented as the means ± standard deviations. BASMI = Bath Ankylosing Spondylitis Metrology Index, BASFI = Bath Ankylosing Spondylitis Functional Index, BASDAI = Bath Ankylosing Spondylitis Disease Activity Index.

Genotype distributions

The frequencies of the T/C polymorphisms at position -786 in the promoter region (patients *vs.* controls) were as follows: TT (46.3% *vs.* 34.8%), TC (46.3% *vs.* 53.6%) and CC (7.4% *vs.* 11.6%) ($p = 0.12$). The frequencies of polymorphisms in exon 7 (Glu298Asp; patients *vs.* controls) were as follows: GG (57.5% *vs.* 50.9%), GT (35.8% *vs.* 43.8%) and TT (6.7% *vs.* 5.4%) ($p = 0.36$). The frequencies of 4b4a VNTR (intron 4) polymorphisms (patients *vs.* controls) were as follows: bb (71.7% *vs.* 65.2%), ba (26.7% *vs.* 33.9%) and aa (1.6% *vs.* 0.9%) ($p = 0.3$). After controlling for age and sex, the distributions of the genotypes (T-786C, Glu298Asp and 4b4a VNTR) were still not significantly different between the patients and controls ($p = 0.06$, 0.43 and 0.38, respectively).

Allelic distributions

eNOS T-786C (patients *vs.* controls) was distributed as T (69.4% *vs.* 61.6%) and C (30.6% *vs.* 38.4%) ($p = 0.07$). Glu298Asp (patients *vs.* controls) was distributed as G (75.4% *vs.* 72.8%) and T (24.6% *vs.* 27.2%) ($p = 0.5$). 4b4a VNTR (patients *vs.* controls) was distributed as b (85.1% *vs.* 82.1%) and a (14.9% *vs.* 17.9%) ($p = 0.36$). There were still no significant differences between the groups regarding T-786C, Glu298Asp and 4b4a VNTR alleles after controlling for age and sex ($p = 0.08$, 0.44 and 0.48, respectively). A summary of the allele and genotype frequencies of the three investigated eNOS gene polymorphisms in AS patients and healthy controls is given in Table 2.

Correlation analysis

The correlation analysis showed that eNOS gene polymorphism T-786C was not correlated with the variables of disease duration, BASMI, BASFI, BASDAI or HLAB27 ($p = 0.2$, 0.1, 0.4, 0.4 and 0.8, respectively). These variables were also not correlated with the Glu298Asp ($p = 0.3$, 0.3, 0.2, 0.4 and 0.6, respectively) or 4b4a VNTR ($p = 0.9$, 0.6, 0.4, 0.4 and 0.2, respectively) polymorphisms.

DISCUSSION

In this study, we showed that the frequencies of common eNOS gene polymorphisms were not different between AS patients and controls. We also observed that these polymorphisms were not associated with disease duration, activity, function, severity or HLAB27.

NO is a molecule that plays an important role in a variety of physiologic functions, including the regulation of blood vessel tone, inflammation, mitochondrial functions and apoptosis (17). In mammals, NO can be generated by three different isoforms of the enzyme NO synthase, including the neuronal, inducible and endothelial forms (18). All three NOS isozymes have regulatory functions in the cardiovascular (CV) system (18). The most important isoform is eNOS, which keeps blood vessels dilated, controls blood pressure and has numerous other vasoprotective and antiatherosclerotic effects (18). Endothelium-derived NO synthase is encoded by the eNOS gene on chromosome 7 (7). In recent years, several polymorphisms of the eNOS gene and their associations with various diseases have been

Table 2 - Allele and genotype frequencies of the eNOS gene polymorphisms in patients with AS and healthy controls.

	Alleles % (n)		Genotypes % (n)		
eNOS -786	**T**	**C**	**TT**	**TC**	**CC**
Patients (n = 188)	69.4 (261)	30.6 (115)	46.3 (87)	46.3 (87)	7.4 (14)
Controls (n = 112)	61.6 (138)	38.4 (86)	34.8 (39)	53.6 (60)	11.6 (13)
p-value	0.07			0.12	
eNOS 27 bp VNTR	**b**	**a**	**bb**	**ba**	**aa**
Patients (n = 191)	85.1 (325)	14.9 (57)	71.7 (137)	26.7 (51)	1.6 (3)
Controls (n = 112)	82.1 (184)	17.9 (40)	65.2 (73)	33.9 (38)	0.9 (1)
p-value	0.36			0.3	
eNOS+894 (Glu298Asp)	**G**	**T**	**GG**	**GT**	**TT**
Patients (n = 193)	75.4 (291)	24.6 (95)	57.5 (111)	35.8 (69)	6.7 (13)
Controls (n = 112)	72.8 (163)	27.2 (61)	50.9 (57)	43.8 (49)	5.4 (6)
p-value	0.5			0.36	

Note that no significant differences were found in the distributions of allele and genotype frequencies of the studied eNOS gene polymorphisms between patients and controls.

studied. In particular, a single nucleotide polymorphism in the promoter region (T-786C), a 894G >T polymorphism leading to amino acid substitution at position 298 (Glu298Asp, rs1799983) in exon 7, and the 4b4a polymorphism (a VNTR) located in intron 4 of the eNOS have received the most attention due to their functional relevance to eNOS activity (19). It has been shown that the T-786C polymorphism reduces eNOS gene promoter activity and affects eNOS protein expression, while the Glu298Asp polymorphism causes a structural change in the eNOS protein associated with impaired eNOS activity (20,21). In addition, it has also been shown that the 27bp-VNTR polymorphism reduces the plasma concentration of NO (22). The dysregulation of eNOS caused by these gene polymorphisms leads to decreased NO production and is thought to contribute to the pathogenesis of several diseases, including inflammatory disorders. In this study, we investigated three common eNOS gene polymorphisms (T-786C, Glu298Asp = G894T and 4b4a VNTR) that have been shown to be associated with eNOS activity. It has been reported that these polymorphisms are associated with CV risk factors and CV disease (7). In the current study, to avoid the confounding effects of these variables on our parameters, we excluded subjects with hypertension, diabetes mellitus and CVD. We also performed statistical adjustments for age and sex to minimize the effects of demographics on our results.

Because of its relationship with inflammation, several studies have investigated the association of eNOS gene polymorphisms with inflammatory rheumatic diseases, including rheumatoid arthritis, systemic lupus erythematosus, systemic sclerosis and Behcet's disease (11,12,17,23,24). Some of these studies have yielded evidence of an association of the eNOS polymorphisms with either the pathogenesis of rheumatic diseases or extraarticular manifestations (12,17,23,24). It is well known that there is a strong genetic basis for AS, and some genes, particularly HLAB27, have already been implicated in disease pathogenesis. In recent years, several other non-MHC genes, including genes involved in intracellular antigen processing and cytokine production (especially genes in the IL–17–IL–23 pathway), have also been shown to be related to AS (1). However, it is not known whether AS is associated with eNOS gene polymorphisms.

In the current study, we investigated eNOS gene polymorphisms in AS patients for the following reasons. (1) Previous studies have reported an increased frequency of impaired vascular relaxation (which is known to be mediated by NO) (2-4,6) and higher levels of ADMA (8-10). Although ADMA impairs post-NO production (unrelated to eNOS polymorphisms), it is responsible for the decreased NO activity (8-10). Based on these findings, we suggest that the NO pathway may be impaired and that this might be associated with genetic polymorphisms in the eNOS gene (2). Some reports have revealed an association between eNOS gene polymorphisms and inflammatory diseases (11,12,17,23,24). Because the pathogenesis of the genes is not fully understood in such inflammatory diseases, we wondered whether there was a link between eNOS gene polymorphisms and AS. We did not find any differences between patients and controls regarding common eNOS gene polymorphisms. We also did not find any relationship between eNOS gene polymorphisms, HLAB27, disease severity, function or activity.

We acknowledge that there were some limitations in our study. (1) We did not study the surrogate markers of atherosclerosis or biomarkers such as carotid intima-media thickness, flow-mediated dilatation and ADMA in this study. Upon consideration, the use of these parameters may be more appropriate for drawing conclusions about eNOS polymorphisms in AS. (2) The inclusion of AS patients with CV risk factors may be more useful for understanding the significance of these polymorphisms in patients with or without CV conditions. In conclusion, despite these limitations, the results presented in this study do not provide support for a major role of common eNOS polymorphisms in Turkish AS patients. Further replication studies in different populations with larger numbers of patients are needed to confirm our results.

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■ AUTHOR CONTRIBUTIONS

Sari I wrote the manuscript, designed the study and performed the statistical analysis of results. Igci YZ, Gogebakan B and Eslik Z carried out the laboratory analyses. Akar S and Akkoc N helped with the general design of the manuscript and contributed to the discussion section. Taylan A, Can G and Bozkaya G collected the patient data. Solmaz D contributed to the statistical analysis and collected the patient data.

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