Endothelial nitric oxide gene polymorphism and risk of systemic sclerosis: predisposition effect of T-786C promoter and protective effect of 27 bp repeats in intron 4

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Abstract

Objective

An impaired availability of nitric oxide (NO), related to polymorphisms in endothelial nitric oxide synthase (eNOS) gene, may influence the microvasculature in systemic Sclerosis (SSc). Three potential eNOS gene polymorphisms [tandem 27-bp repeats (VNTR) in intron 4, T786C in promoter region and G894T in exon 7] were investigated to affect the susceptibility to and the clinical course of SSc.

Methods

Fifty-nine patients with SSc (mean age 47.1±12.1 years) and 83 control subjects (mean age 41.1±12.8 years) were studied. Genotypes were determined through PCR with or without RFLP.

Results

Genotype distribution was significantly different between SSc patients and controls for intron 4aa (alleles for four repeats), genotype frequency being 3.4% and 17.1%, respectively (odds ratio for dominant effect, 0.35; 95% CI, 0.17 to 0.78; p=0.004). The CC genotype of the promoter was significantly high in frequency in the SSc patients (16.9%) compared to controls (7.3%) (odds ratio for dominant effect, 2.26; 95% CI: 1.14 to 4.48; p=0.020).

Conclusion

Intron 4 aa genotype of eNOS gene is protective and homozygosity (CC) of T-786C promoter region is a risk factor for SSc in Turkish population. Our results highlight a possible mechanism by which a potential reduced availability of NO, related to VNTR in intron 4 and T-786C promoter polymorphism, may influence the predisposition to SSc.

Key words

Systemic sclerosis, eNOS gene, polymorphism, intron 4, promoter

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Introduction

Systemic sclerosis (SSc) is a connective tissue disease characterised by generalised microangiopathy and excessive deposition of extracellular matrix both in the skin and the internal organs. The main pathologic hallmark of the disease is endothelial activation and damage, which is the main cause of vascular features, such as Raynaud's phenomenon and pulmonary hypertension (1). The nature of the factors that induce endothelial dysfunction are still unclear, however endothelin, selectin, anti-endothelial antibodies and nitric oxide (NO) are known markers that reflect the vasculopathy of the disease (2,3). NO is biosynthesised from L-arginine by NO synthase (NOS). The endothelial NO (eNOS) is one of the three isoforms of NOS (4). The eNOS is expressed consititutively by the vascular endothelium, where it is regulated by shear stress forces, endothelin, neuropeptides and certain cytokines. The physiological and pathological function of NO are diverse and often contradictory (3). The protective role of NO produced by eNOS includes control of vascular tone, antithrombotic and cytoprotective effects. On the other hand, the harmful role of NO produced by inducible NOS is related with cytotoxic effects. In fact, there is a NO paradox in SSc (3). The NO production in SSc is confusing as both increased and decreased circulating total nitrate levels have been reported (5-8). NO levels are markedly elevated in patients with early-stage diffuse cutaneous SSc, particularly when the SSc is accompanied by active alveolitis, however serum levels of NO are low in late stage limited cutaneous SSc (9).

eNOS is encoded by a gene located within chromosome 7q35–36, expressed in endothelium. eNOS gene contains 26 exons which are spanning 21 kb (10-11). Many polymorphisms of eNOS gene have been identified. Among them the three polymorphisms have received more interest as role players in microvascular function (12-13). A variable number of tandem 27-bp repeats (VNTR) in intron 4 and T-786C polymorphism in promoter are assumed to be associated with alterations in promoter activity (14). The polymorphism in exon 7 (G894T) encodes an amino acid change from Glu298Asp which is believed to render the enzyme more susceptible to proteolytic cleavage (15). The effects of these polymorphisms on in vivo NO generation cannot be measured directly as most endogenous NO rapidly oxidises to nitrite (NO^{2-}) and is eventually converted to nitrate (NO^{3-}), the predominant stable form of NO. In addition, measuring plasma or urinary nitrite/nitrate in humans requires strict control of confounding variables such as diet, medication, smoking, concurrent infection (16). Although recent studies have shown that plasma nitrite can reflect endogenous NO formation (17, 18), the factors outlined above cause difficulties in directly assessing the NO. Yet it is quite important to evaluate the genetic polymorphisms of eNOS in relation to SSc.

The objective of this study was to investigate the influence of the three eNOS polymorphisms (VNTR in intron 4, T-786C in the promoter region and G894T in exon 7) on the susceptibility of patients to and clinical expression of SSc.

Patients and methods

Study population

Fifty-nine patients with SSc who have referred to Hacettepe University Rheumatology Department were enrolled in this study. A detailed interview addressing personal and family history was performed in the context of a physical examination by expert physicians. Patients were classified as limited or diffuse SSc regarding to clinical involvement. Raynaud's phenomenon, musculoskeletal involvement and digital ulcers were assessed and circulating auto antibodies characteristic of SSc (anti-topoisomerase I [anti-Scl-70], anticentromere and antinuclear with a nucleolar pattern) were tested. Pulmonary involvement was evaluated by high resolution computerised tomography (for interstitial lung disease), pulmonary function test (FEV1: Forced expiratory volume) and diffusion capacity (DLCO: Diffusing capacity for carbon monoxide, DLCO/VA: Diffusing capacity for carbon monoxide corrected for alveolar volume).

Pulmonary arterial hypertension (PAH) was evaluated by echocardiography with measuring of peak right ventricular systolic pressure and greater than or equal to 40 mmHg was accepted as PAH. If peak right ventricular systolic pressure and greater than or equal to 40 mmHg, right heart catheterisation was performed to document definitively the presence of PAH for 7 patients. Cardiac involvement was defined as pericardial effusion and/or myocarditis at the echocardiography or cardiac enzymes. The treatments of patients were steroid, cyclophosphamide, azathioprine, and vasodilatators. Exclusion criteria were coronary heart disease, chronic renal failure, chronic liver disease and nerologic diseases. Eighty-three controls with no history of cardiovascular disease/thromboembolism were recruited from the Hacettepe University staff. The study was approved by the local research ethics committee and written informed consent was obtained from all patients and controls.

Genotyping

Genomic DNA was isolated from the whole blood by using Nucleospin Blood Quick Pure Kit (Machenery-Nagel, Germany). eNOS gene polymorphisms were determined by polymerase chain reaction (PCR) and restriction endonuclease digestions using specific oligonucleotide primers and restriction enzymes in specific conditions, respectively.

Tandem 27-bp repeats (VNTR) in intron 4: Sense primer 5'- AGG CCC TAT GGT AGT GCC TTT-3' and antisense primer 5'- TCT CTT AGT GCT GTG GTC AC-3' that flank the VNTR in intron 4 region were used for PCR. Reactions were performed in a total of 40 µl volume containing 400 ng of genomic DNA, 10 pmol/µl of each primer, 0.2 mM of each dNTP, 4 µl of 10 x PCR buffer, 2.5 mM of $MgCl_2$ and 3 U of Taq DNA Polymerase (Fermentas Life Sciences, Germany). Thermocyclying procedure consisted of initial denaturation at 94° for 5 min, 35 cycles of denaturation at 94° for 30 s, annealing at 63° for 30 s, extension at 72° for 1 min and final extension at 72° for 5 min by using ICycler, BioRad. The VNTR was determined by separating intron 4aa (four repeats of 27 bp) (393 bp) and intron 4bb (five repeats of 27 bp) (420 bp) DNA fragments on a 3 % NuSieve agarose gel (19). Intron 4 ab indicates four repeats of 27 bp in one allele while five repeats of 27 bp in the other allele.

T-786C polymorphism in promoter region: The sense 5'- TGG AGA GTG CTG GTG TAC CCC A -3' and antisense 5'- GCC TCC ACC CCC ACC CTG TC-3' primers were used in a total volume of 50 µl, containing 400 ng of template DNA, 6.25 pmol/µl of each primer, 0.25 mM of each dNTP, 5 µl of 10xPCR buffer, 1.5 mM of $MgCl_2$ and 3 U of Taq DNA Polymerase (Fermentas Life Sciences, Germany). The reaction conditions were initial denaturation at 94° for 5 min followed by 40 cycles comprising denaturation at 94°C for 1 min, annealing at 61°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 5 min using ICycler, BioRad. PCR products were visualised on 2% agarose gels staining with ethidium bromide. Amplified products were digested with *Msp*I (Fermentas Life Sciences, Germany) for 3 h at 37°C. The presence of C at nucleotide 786 allows cleavage of the 140 bp product into 90 bp and 50 bp fragments. Resulting fragments of 140 bp and 40 bp for the wild type allele (TT) or 90 bp, 50 bp and 40 bp in case of polymorphic allele (CC) were determined by separating them on a 2% agarose gel (19).

G894T polymorphism in exon 7: DNA fragments covering the region of the G894T polymorphic site of the eNOS gene was amplified by PCR, as described above, however in a final volume of 50 µl, containing 400 ng of template DNA, 6.25 pmol/µl of each primer, 0.25 mM of each dNTP, 10 µl of 10xPCR buffer, 1.5 mM of $MgCl_2$ and 3 U of Taq DNA Polymerase (Fermentas Life Sciences, Germany) with the following sense and antisense primers, 5'- AAG GCA GGA GAC AGT GGA TGG A-3' and 5'- CCC AGT CAA TCC CTT TGG TGC TCA-3', respectively. The resulting 268 bp fragment was digested with *Mbo*I (Fermentas Life Sciences, Germany) for 3 h at 37°C, producing 178 bp and 90 bp fragments (polymorphic allele) (TT) or no digestion (wild type) (GG). Restriction sites were confirmed on 2% agarose gel (19).

Statistical analysis

Statistical analyses were carried out with SPSS for Windows version 15.0 statistical software (SPSS Inc., Chicago, IL, USA). Continuous variables are presented as mean±standard deviation, median and categorical variables as percentages. Continuous variables were compared by using student *t*-test or analysis of variance (ANOVA). Genotype distributions and allele frequencies were compared by Chi square test. Multiple logistic regression was used to determine the influence of eNOS polymorphisms on disease risk, controlling for age and gender. To assess different models at each locus, genotypes at each position were coded: 1 (894GG, intron 4bb, -786TT), 2 (894GT, intron 4ab, -786TC) and 3 (894TT, intron 4aa, -786CC). An additive model compared genotype 3 versus 2 versus 1. A dominant model compared genotypes 3 and 2 versus 1. A recessive model compared genotype 3 versus 2 and 1. Hardy Weinberg equilibrium for genotype frequencies was assessed by Chi square test. *p*-value 0,05 or less was considered significant. The haplotypes between the two groups were assessed using chi-squared tests.

Results

Subject characteristics

The mean (±SD) age was 41.1±12.8 for controls and 47.1±12.1 for patients with SSc (p=0.008). The mean disease duration was 11.4±8.1 years. Clinical characteristics of the patients were detailed in Table I. Twenty seven patients (46%) had limited SSc. The presence of interstitial lung disease, anti-topoisomerase I, low FEV1 were seen high in frequency and PAH, anticentromere antibody were observed less in patients with diffuse SSc regarding to limited SSc (Table I). The steroid, cyclophosphamide, azathioprine, iliomedine usage were 91%, 52%, 17% and 11%, respectively.

Genotype distribution

For each polymorphism genotyping was successful in 100% of the cases.

Table I. Characteristics of patients with SSc.

	Limited SSc n=27	Diffuse SSc n=32	*p*-value
Age (years)	50.4 ± 13.2	43.6 ± 10.7	>0.05
Sex (female, %)	88	97	>0.05
Disease duration (years)	10.4 ± 6.8	12.2 ± 9.1	>0.05
Raynaud's Phenomenon (%)	100	100	>0.05
History of digital ulcers (%)	72	62	>0.05
Musculoskeletal involvement (%)	20	38	>0.05
Esophageal involvement (%)	64	84	>0.05
Cardiac involvement (%)	16	23	>0.05
Interstitial lung disease (%)	41	77	0.011
PAH (%)	24	4	0.037
Anti-topoisomerase I antibody (%)	36	84	<0.001
Anti-centromere antibody (%)	32	0	0.002
FEV1	94.4 ± 14.8	78.5 ± 15.6	0.001
DLCO	65.4 ± 19.9	64.8 ± 17.9	>0.05
DLCO/VA	75.5 ± 16.8	82.3 ± 22.4	>0.05

PAH: pulmonary arterial hypertension; FEV1: Forced expiratory volume; DLCO: Diffusing capacity for carbon monoxide; DLCO/VA: Diffusing capacity for carbon monoxide corrected for alveolar volume.

The electrophoretic results for the three eNOS gene polymorphisms; T-786C in the promoter region, G894T in exon 7, tandem 27-bp repeats in intron 4, are shown in Fig. 1 as examples.

Genotype distribution and allele frequencies of the three eNOS gene polymorphisms in SSc and controls are shown in Table II. Genotype frequencies at all loci were in Hardy-Weinberg equilibrium for controls and patients. Significant differences were observed in the distribution of intron 4 VNTR and promoter T-786C polymorphisms between SSc patients and controls. The intron 4aa genotype were significantly high (p=0.005) in frequency (17.1 %) in controls when compared to patients (3.4%). The a allele of intron 4 was observed more common in controls compared to patients (p=001). The CC genotype of the promoter was observed significantly higher (p=0.038) in frequency in the SSc patients (16.9%) compared to controls (7.3%). The C allele for promoter was significantly higher in controls (p=0.008). The genotype and allele distributions of the exon 7 polymorphism did not differ significantly between the patients and controls.

The univariate odds ratios (ORs) associated with the exon 7 G894T, intron 4 VNTR and promoter T-786C alleles were shown in Table III. The intron 4 aa genotype was associated about three times decreased in the risk of SSc (OR=0.35; 95% CI: 0.17 to 0.71; p=0,004). In recessive model (aa *vs.* ab+bb) the risk was significantly higher (OR=0.17; 95% CI: 0.37 to 0.78; p=0.023). The additive model (aa vs. ab vs. bb) also revealed that the intron 4 aa genotype is a protective factor for SSc (OR=0.40; 95% CI: 0.23 to 0.71; p=0.002). Univariate analyses also revealed that the dominant model for CC genotype of the promoter region polymorphism was a risk factor for the disease (OR: 2.26, *i.e.* two times increased risk; 95% CI: 1.14 to 4.48; p=0.020). The risk was also observed for the CC genotype of the promoter polymorphism in additive model (OR=1.92; 95% CI: 1.15 to 3.19; p=0.012).

Multiple logistic regression analysis controlling for age and gender showed that the intron 4 aa allele (dominant model: OR=0.36; 95% CI: 0.15 to 0.87; additive model: OR=0.41; 95% CI: 0.20 to 0.85) and promoter CC allele (dominant model: OR=2.99; 95% CI: 1.28 to 6.99; additive model: OR=2.01; 95% CI: 1.08 to 4.75), remained independently associated with SSc.

Regarding the three loci, all 8 potential haplotypes (combinations of T/C for promoter, a/b for intron 4 and G/T for exon 7 regions) were represented in Table IV. There was a significant difference in haplotype distribution in SSc (p=0.007), where the G-a-T haplotype was less frequently seen in SSc patients. G-b-C and T-b-C haplotypes were observed high in SSc patients.

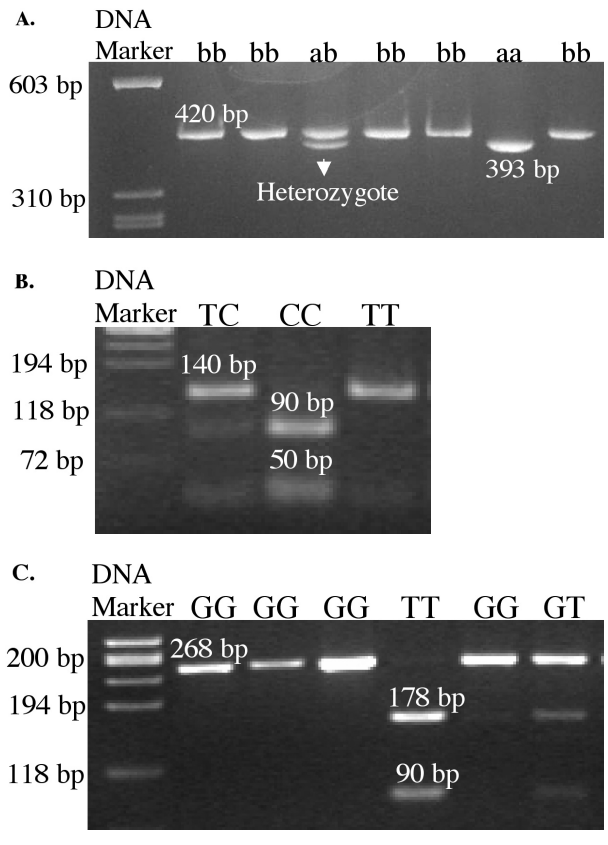

Fig. 1. Genotyping of eNOS gene polymorphisms. DNA marker was ØX174 DNA/BsuRI9(HaeIII) (Fermentas Life Sciences, Germany) **A.** Tandem 27-bp repeats in intron 4. Lane 1: DNA marker; lane 2, 3, 5, 6, 8: patients with five repeats of 27 bp (intron 4bb); lane 4: heterozygote patient (intron 4ab); lane 7: patient with four repeats of 27 bp (intron 4aa). **B.** T-786C in promoter region. Lane 1: DNA marker; lane 2: heterozygote patient carrying both the polymorphic and wild type alleles; lane 3: homozygote patient carrying a polymorphic allele; lane 4: a control carrying wild type alleles. **C.** G894T in exon 7. Lane 1, DNA marker; lane 2, 3, 4, 6: controls carrying wild type alleles; lane 5: a homozygote patient carrying the polymorphic alleles; lane 7: a heterozygote patient carrying both the polymorphic and wild type alleles.

Table II. Genotype distribution of eNOS polymorphisms.

Locus	Genotype n (%)	Controls n (%)	Patients	*p*-value
Exon 7 G894T	GG	38 (46.3)	22 (37.3)	0.527
	GT	37 (45.1)	30 (50.8)	
	TT	7 (8.5)	7 (11.9)	
	G allele	0.69 0.63	0.278	
Intron 4 VNTR	aa	14 (17.1)	2 (3.4)	0.005**
	ab	30 (36.6)	15 (25.4)	
	bb	38 (46.3)	42 (71.2)	
	a allele	0.35 0.16	0.001**	
Promoter T-786C	TT	47 (57.3)	22 (37.3)	0.038*
	TC	29 (35.4)	27 (45.8)	
	CC	6 (7.3)	10 (16.9)	
	C allele	0.25 0.40	0.008**	

*p<0.05, **p<0.001

Table III. Odds ratios associated with polymorphisms.

		Univariate OR (95% CI)	*p*-value
Exon 7 G894T	Additive	1.34 (0.79-2.26)	0.264
	Dominant	1.45 (0.73-2.88)	0.284
	Recessive	1.20 (0.69-2.09)	0.516
Intron 4 VNTR	Additive	0.40 (0.23-0.71)**	0.002
	Dominant	0.35 (0.17-0.71)**	0.004
	Recessive	0.17 (0.37-0.78)*	0.023
Promoter T786C	Additive	1.92 (1.15-3.19)*	0.012
	Dominant	2.26 (1.14-4.48)*	0.020
	Recessive	1.61 (0.94-2.75)	0.083

*p<0.05, **p<0.001

Discussion

Our results highlight a possible mechanism by which a potentially reduced availability of NO, related to VNTR in intron 4 and T-786C promoter polymorphism, might influence the predisposition to SSc. This is the first report, describing the protective role of intron 4 aa genotype for VNTR polymorphism of eNOS gene, to date. The homozygosity (CC) for T-786C polymorphism in the promoter region is demonstrated as a susceptibility genetic factor for SSc patients. We could not find any evidence of an association between the G894T exon 7 polymorphism of the eNOS gene and SSc.

A variety of vascular diseases that include coronary artery disease, hypertension, stroke, and renal disease have been associated with the eNOS gene polymorphism (20-23). In a meta-analysis, which involves 23.028 subjects, it has been shown that the -894T and intron 4a alleles of eNOS were at moderately increased risk of ischemic heart diseases (24). In fact, the association of eNOS gene polymorphism with several rheumatological diseases such as giant cell arteritis, Behçet's disease and systemic lupus erythematosus (SLE) has been previously found (25-29). Gonzales-Gay *et al*. demonstrated an increased risk for cardiovascular events in rheumatoid arthritis patients who were homozygous for the T-786 genotype (30). Since endothelial activation and damage are primary events throughout the course of SSc, eNOS polymorphism have already been evaluated in limited number of studies in different ethnic populations such as black South Africans, Frenchs, Italians and Americans (Table V) (31-36). All 6 studies evaluated the G894T polymorphism. Our results are concordant with 5 of them demonstrating no relationship between G894T polymorphism with SSc (31-34, 36). Two reports of Fatini et al evaluated T-786C polymorphism in the promoter region (35, 36). One of them includes 113 SSc patients and 113 controls, and they hypothesised that -786C allele might modulate the susceptibility to SSc by influencing the hemorheologic profile, in addition to their effect on vascular biology, supporting our results (36). On the contrary, the result of the previous study of Fatini *et al*. did not demonstrate an association between T-786C polymorphism and SSc (35). Our control group (Table V) is differing in frequency of alleles of T-786C polymorphism from Italians, thus explaining these conflicting findings might be a result of the interethnic differences in the distribution of genetic polymorphisms. The present study showed that intron 4 aa genotype was a protective factor for SSc. Nevertheless, this result should be assessed carefully. Previously, the only report evaluating the frequency of intron 4 polymorphism in SSc patients was by Fantini *et al*. (36). In fact, its results in SSc patients were similar with our results in aspect of SSc patients (Table V). Yet, similar to the T-786C polymorphism in the promoter region, differences exist in our control group as more frequently 4aa allele and less frequently 4bb allele do exist. Therefore, protective effect of intron 4 aa polymorphism may come from our ethnic baselines and these results should be confirmed in different countries with large studies. On the other hand, it is also important that the subjects evaluated are in Hardy-Weinberg equilibrium. Genotype frequencies at all loci in our study were in this context for controls and patients.

Very limited studies have attempted to determine the mechanisms by which these three genetic variations might affect eNOS enzyme activity. Among

Table IV. Haplotype frequencies.

	Proportion	
	Controls (n=164)	Cases (n=118)
T-a-T	0.03	0.017
T-a-C	0.03	0.008
T-b-T	0.128	0.144
T-b-C	0.122	0.203
G-a-T	0.257	0.11
G-a-C	0.037	0.025
G-b-T	0.336	0.332
G-b-C	0.06	0.161

p=0,007

Table V. eNOS polymorphisms studied in SSc.

Authors, Country		G894T			T-786C			Intron 4		
		GG	GT	TT	CC	TC	TT	4aa	4ab	4bb
Assassi, *et al*. (31)	SSc n (%)	86 (57)	54 (35)	11 (7)		–			–	
America	Controls n (%)	110 (60)	58 (31)	16 (9)						
Tikly, *et al*. (32)	SSc n (%)	41 (84)	8 (16)	0 (0)		–			–	
South Africa	Controls n (%)	50 (81)	12 (19)	0 (0)						
Biondi, *et al*. (33)	SSc n (%)	19 (39)	19 (39)	10 (22)		–			–	
Italy	Controls n (%)	36 (29)	73 (59)	14 (12)						
Allanore, *et al*. (34)	SSc n (%)	29 (37)	35 (46)	13 (17)		–			–	
French	Controls n (%)	22 (45)	22 (45)	5 (10)						
Fatini, *et al*. (35)	SSc n (%)	26 (36)	36 (49)	11 (15)	17 (23)	34 (47)	22 (30)		–	
Italy	Controls n (%)	57 (51)	49 (44)	6 (5)	32 (29)	52 (46)	28 (25)			
Fatini, *et al*. (36)	SSc n (%)	40 (35.4)	55 (48.7)	18 (15.9)	22 (19.5)	57 (50.4)	34 (30.1)	5 (4.4)	33 (29.2)	75 (66.4)
Italy	Controls n (%)	62 (54.9)	38 (33.6)	13 (11.5)	18 (15.9)	44 (38.9)	51 (45.1)	4 (3.5)	30 (26.5)	79 (69.9)
Our study	SSc n (%)	22 (37.3)	30 (50.8)	7 (11.9)	10 (16.9)	27 (45.8)	22 (37.3)	2 (3.4)	15 (25.4)	42 (71.2)
Turkey	Controls n (%)	38 (46.3)	37 (45.1)	7 (8.5)	6 (7.3)	29 (35.4)	47 (57.3)	14 (17.1)	30 (36.6)	38 (46.3)
Total	SSc n (%)	263 (46)	237 (42)	70 (12)	49 (20)	118 (48)	34 (32)	7 (4)	48 (28)	117 (68)
	Controls n (%)	375 (52)	289 (40)	61 (8)	56 (18)	125 (41)	136 (41)	18 (9)	60 (31)	117 (60)

them, there is no evidence for any changes that are directly associated with the VNTR in intron 4 (37). Any association indicated in reports is due merely to the fact that this polymorphism is in linkage disequilibrium with a functional polymorphism. Wang *et al*. demonstrate that the intron 4 polymorphism affects the transcription efficiency in a haplotype-specific fashion in linkage disequilibrium with the T-786C polymorphism in the promoter region (38). The others found that while eNOS genotypes are not solely associated with changes in NO levels, the specific eNOS haplotype that includes the 'C', '4b', and 'G' alleles of promoter, intron 4, exon 7 respectively, is associated with lower circulating NO concentrations (39, 40). A recent report by Sandrim *et al*. (41) suggested that there is a genetic contribution of eNOS haplotype to the development of endothelial dysfunction in hypertensive patients; however this contribution is obscured when specific eNOS genotypes are considered alone. Thus, we tested the three polymorphisms of the eNOS gene (tandem 27-bp repeats in intron 4, T786C in the promoter region and G894T in exon 7), to overcome the limitations that are caused by using single polymorphisms in genetic association studies. We found a significant difference in haplotype distribution whereas the G-a-T haplotype was seen less frequently in SSc patients, which supports our single polymorphism results. An increased transmission of the T786/intron 4a suggests that the regulatory polymorphism may be located on this haplotype. The protective effect of the intron 4aa could be mediated through changes in eNOS promoter activity.

Fantini C *et al*. have shown that T-786C polymorphism in the promoter region was susceptibility genetic factor for SSc patients (36). Our results were in conjunction with it. To date, there is evidence for impaired NO production as a potential result of the T-786C polymorphism of the promoter, which reduces the promoter activity by ~ 50%, thereby lending experimental support for the notion of a physiologic role of this polymorphism (14, 42). Data from experimental studies demonstrated that homozygosity (CC) for the rare variant of the polymorphism in the promoter region of the eNOS gene is associated with a deficit of eNOS expression in human endothelial cells exposed to laminar shear stress, as well as with a reduced NO-mediated vasomotor function (42). Dosenko *et al*. found that the patients with the CC promoter genotype had 2.1 times lower NO-producing activity than in TT promoter genotypes and 2.9 times lower than in heterozygotes (43). In our study, an increased frequency of CC alleles in T-786C promoter region polymorphism in SSc patients consisted with the *in vitro* functional work, indicating this polymorphism contributes to endothelial injury in SSc.

This study has two limitations. First, larger sample sizes are needed to confirm the interactions between intron 4aa, T-786C promoter polymorphisms on SSc. The second limitation lays in the lack of serum NO level and so functional role of assessed polymorphisms. Our findings document that VNTR in intron 4 and T-786C polymorphism in promoter of eNOS gene influence the microcirculation in SSc. The final phenotype of SSc may result from the interaction of susceptibility genes with environmental factors, including known risk factors. Further investigations of the molecular mechanism are needed by which the eNOS gene is involved in the pathophysiological mechanism of SSc.

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