Association Between Thr21Met and Ser89Asn Polymorphisms of the Urotensin II Gene and Systemic Sclerosis

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ABSTRACT. ***Objective.*** Systemic sclerosis (SSc) is an autoimmune chronic fibrotic disorder. Urotensin II (U-II) is predominantly a vasoactive peptide with fibrotic and prothrombotic features. Like endothelin-1 (ET-1), U-II could play an important role in SSc pathogenesis. We evaluated the possible role of the U-II gene polymorphisms (Thr21Met and Ser89Asn) in the genetic susceptibility to SSc in a Turkish population.

Methods. A total of 189 patients with SSc and 205 healthy controls were enrolled in our study. We analyzed the genotype and allele frequencies of the U-II (*UTS2*) gene polymorphisms Thr21Met and Ser89Asn in patients with SSc and in controls.

Results. We found that the Thr21Met polymorphism of the *UTS2* gene was markedly associated with the risk of developing SSc ($p < 0.0001$), but there was no relationship between the Ser89Asn polymorphism and SSc ($p > 0.05$). Two haplotypes (MS and TS) were markedly associated with SSc ($p < 0.05$). There were significant associations between the genotype and allele frequencies of *UTS2* gene Thr21Met polymorphism and cases with diffuse or limited SSc, systemic or lung involvement, finger flexion deformity, pitting scars at the fingertips, positive anticentromere, or positive antitopoisomerase 1 antibody groups.

Conclusion. Our study shows the association between Thr21Met, but not Ser89Asn, in the *UTS2* gene and SSc. The results strongly suggest that this single-nucleotide polymorphism may be an important risk factor in the development of SSc, and a powerful indicator of severe skin and lung involvement in patients with SSc. (First Release Nov 1 2011; J Rheumatol 2012;39:106–11; doi:10.3899/jrheum.110509)

Key Indexing Terms:
SYSTEMIC SCLEROSIS UROTENSIN II UROTENSIN II GENE

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Accepted for publication August 19, 2011.

Scleroderma, or systemic sclerosis (SSc), is a multifactorial, chronic fibrotic collagen tissue disorder that is mainly based on 3 pathogenetic features: accumulation of extracellular matrix, vasculopathy of the small vessels, and autoimmunity. Clinical and experimental data show that the pathogenesis of SSc is multifactorial, involving both genetic and environmental factors[1,2]. SSc may be a clinical feature of limited anatomic extent affecting only the skin and adjacent tissues, or it may be associated with systemic involvement. Although skin fibrosis is a hallmark feature of SSc, the systemic manifestations are diverse[3]. The most prominent manifestations are abnormalities of the circulation and involvement of multiple organ systems, including the musculoskeletal, renal, pulmonary, cardiac, and gastrointestinal systems, with fibrotic and/or vascular complications. The disease's main feature is an overproduction and accumulation of collagen and other extracellular matrix proteins, resulting in fibrosis and tissue dysfunction[4]. Although cytokines and immune mediators play a major role in the initiation and progression of disease, the importance of the genetic component in SSc pathogenesis has not been fully defined.

Urotensin II (U-II) is an 11-amino acid cyclic peptide, and

U-II and its receptor, UT, are widely expressed throughout the body. U-II is known to be the most potent endogenous vasoconstrictor discovered to date. Its physiological mechanisms are similar in some ways to other mediators, such as endothelin 1 (ET-1)[5]. Since serum ET-1 levels were significantly increased in patients with diffuse SSc or diffuse cutaneous SSc (dcSSc), ET-1 may be important in the pathogenesis of both the fibrotic and vascular manifestations in SSc[6]. U-II produces potent vasoconstriction in human arteries and veins. U-II was found to be 50 times more potent in arteries and about 10 times more potent in veins than ET-1[7]. Recent studies showed that U-II can play a fundamental role in cardiovascular remodeling and fibrosis[8], renal fibrosis[9], and hepatic fibrosis[10], and that it enhances collagen production[11]. We have recently demonstrated that U-II was significantly elevated in patients with SSc, and the plasma levels of U-II-like immunoreactivity were positively correlated with ET-1 level[12].

The gene for U-II (*UTS2*) is located in human chromosome 1p36-p32[13]. According to the US National Center for Biotechnology Information (NCBI) database, over 60 single-nucleotide polymorphisms (SNP) have been noted in the human *UTS2* gene. Three of these SNP show amino acid changes in the *UTS2* gene sequence. High allelic frequencies in Japanese populations were documented for the Thr21Met and Ser89Asn polymorphisms[13,14], and we selected these polymorphisms for our study. Although involvement of some genetically determined factors and polymorphisms in SSc has been suggested[1,2], to our knowledge there is no study investigating the role of U-II gene polymorphism in patients with SSc. Our purpose was to test a possible association between U-II gene polymorphisms (Thr21Met and Ser89Asn) and SSc in a Turkish population.

MATERIALS AND METHODS

Study populations. The study involved 189 patients with SSc according to the classification criteria of the American College of Rheumatology[15] (172 women and 17 men: mean age 47.78 ± 12.95 yrs) and 205 healthy volunteers matched by age and sex, between March 2008 and September 2010 in 4 rheumatology clinics. All patients and controls were recruited from the same racial, ethnic, geographic (southeastern Turkey), and environmental stratification. The ethics committee of the University of Gaziantep, Faculty of Medicine, gave its approval, and all the volunteers provided written informed consent. Patients were excluded if they had a diagnosis of myocardial infarction before the initiation of SSc, hypertension, hyperlipidemia, diabetes mellitus, renal dysfunction, or additional chronic or other autoimmune diseases.

Study protocol. All 189 patients' data related to anamnesis, physical examination, routine and serologic laboratory features, electrocardiography (ECG), echocardiography (ECO), and high-resolution computed tomography (HRCT) findings were recorded into the database. All patients were categorized as having either dcSSc or limited cutaneous SSc (lcSSc). Patients with proteinuria were evaluated with the Esbach method for estimating the quantity of urine albumin, and kidney functions were calculated by glomerular filtration rate in patients with proteinuria. Patients with possible pulmonary arterial hypertension (PAH) were evaluated with right cardiac catheterization. According to the European Society of Cardiology guidelines, PAH is defined by an increase in mean pulmonary arterial pressure ≥ 25 mm Hg and a pulmonary capillary wedge pressure ≤ 15 mm Hg, associated with a normal or reduced cardiac output[16]. We regarded patients with arrhythmia, pericarditis, pericardial effusion, heart failure, and/or PAH as having cardiac involvement. We used lung radiographs to evaluate the patients' respiratory systems. Patients with possible lung involvement were also evaluated with respiratory function tests and HRCT. We regarded patients with ground-glass opacification and honeycomb formation as having lung involvement. Esophageal involvement was evaluated through esophagography.

Blood samples and DNA isolation. Peripheral blood samples (5 ml) were collected by venipuncture into sterile siliconized vacuum tubes with 2 mg/ml disodium EDTA. Immediately after collection, whole blood was stored at –20˚C until use. Genomic DNA was extracted from whole blood using standard proteinase K digestion and the salting-out method[17], and stored at –20˚C.

Restriction fragment length polymorphism (RFLP). RFLP is a simple and reliable technique based on polymerase chain reaction (PCR), agarose gel electrophoresis, and restriction enzyme. Two primer sets were chosen, covering the exons 1 and 4 of the *UTS2* gene (sequence in the NCBI database: NT_021937.19 contig). PCR primers were designed using the NCBI Blast primer interactive program on the Internet. PCR was carried out with primers to both directions on sample DNA to PCR fragments. We used agarose gel electrophoresis to screen for PCR fragments.

Nucleotide sequences of the PCR primer sets, sizes of the amplicons, and annealing temperatures are listed in Table 1. PCR optimization for each primer set was validated by temperature gradient. The thermocycling procedure consists of initial denaturation at 95˚C for 4 min, 30 cycles of denaturation for Thr21Met (T21M, rs228648), and 32 cycles of denaturation for Ser89Asn (S89N, rs2890565; 95˚C for 30 s), annealing at temperatures given in Table 1 for 30 s, and extension (72˚C for 30 s) and final extension (72˚C for 4 min). Amplification was achieved with in-house GeneAmp 9700 (Applied Biosystems, Foster City, CA, USA). Amplicons were resolved as a single band by 2% agarose gel electrophoresis, to ensure that a specific single product was amplified.

To determine the 2 SNP on the *UTS2* gene, we used the PCR-RFLP technique. The first polymorphic region showed a G/A nucleotide transition in codon 62 of the *UTS2* gene. This nucleotide transition showed amino acid transition from Thr to Met at amino acid position 21. This transition was identified by *Hsp92II* restriction enzyme and restricted on this region. The second polymorphic region showed a C/T nucleotide transition in codon 266 of the *UTS2* gene. This nucleotide transition showed amino acid transition from Ser to Asn at amino acid position 89. This transition was identified by *AfaI* restriction enzyme and restricted on this region. Genotyping was conducted in a blinded fashion.

Statistical analysis. Results are expressed as the mean ± SD or percentage. Statistical analysis was performed using GraphPad Instat version 3.05 (GraphPad Software Inc., San Diego, CA, USA). For calculation of the significance of differences in genotype and allele frequencies, the chi-square test (with Yates' correction) or Fisher's exact test was used. For comparisons of the differences between mean values of 2 groups, the unpaired Student's t test was used. The effects of genetic polymorphisms on the risk of SSc were estimated with OR and 95% CI. The haplotype analysis was performed with SHEsis software (http://analysis.bio-x.cn/myAnalysis.php)[18]. All statistical tests and p values were 2-sided, and $p < 0.05$ was considered statistically significant.

RESULTS

In our study, 99 patients with dcSSc and 90 with lcSSc who were admitted to the Rheumatology Clinic took part. The mean age of patients was 47.78 ± 12.95 years, 91% of the patients were women, 25.8% had ever had spontaneous abortions, and 8.6% had rheumatologic disease history in their family.

Mean disease duration was 6.22 ± 7.78 years. Rheumatoid factor positivity was documented in 17.3% of the patients, and

Table 1. Nucleotide sequences of the polymerase chain reaction (PCR) primer sets designed for tracing the exons 1 and 4 regions of urotensin II, including the sizes of the amplicons.

Primers	Nucleotide Composition (5'–3')	Position	Expected Size of PCR Product	Annealing Temperature (°C)
UT21MFw	GGA AAC CAA CGT ATT TCA TC	EXON 1	141 bp	55
UT21MRw	GCA AAA GAG GCA ACT TAC AGC			
US89NFw	GTG CCT GTC TGT CTG CAT TCA	EXON 4	263 bp	57.7
US89NRw	GAG TCC TGT AAA ACC AGT ACA G			

94.7% of the patients' antinuclear antibody results were positive. Positive scores also were found in 38.3% of the patients' anticentromere antibody scores, 41.8% of antitopoisomerase-1, 12.4% of anti-Ro and anti-La, and 4.6% of anti-RNP. Six (3.2%) patients had renal involvement, 34 (18.0%) had PAH, 35 (18.5%) had cardiac involvement, 76 (50.2%) had esophagus involvement, 105 (55.9%) had lung involvement, 76 (40.2%) had finger flexion deformity, and 106 (56.1%) had pitting scars at the fingertips. Organ involvement in accord with the disease type and antibody positivity is shown in Table 2.

Genotype and allele frequencies of the *UTS2* gene Thr21Met and Ser89Asn polymorphisms in SSc and control groups are shown in Table 3. We found that Thr21Met, but not Ser89Asn, polymorphism of the *UTS2* gene was markedly associated with the risk of developing SSc. The T21T genotype was markedly high in the SSc group (26.5% in patients vs 9.8% in controls), but the M21M genotype was less frequent among patients with SSc (37.0% in patients vs 46.3% in controls; $p < 0.0001$). Thr21Met polymorphism was revealed to have a significant allele frequency difference between cases and controls. There was also an increase in the 21T allele (44.7% in patients vs 31.7% in controls) and a decrease in 21M allele frequencies (68.3% in controls vs 55.3% in patients) in the SSc groups ($p < 0.0002$). Three haplotypes (MS, TN, and TS) with high frequency were detected, but 2 of them (MS and TS) were markedly associated with SSc ($p < 0.05$; Table 3). While MS haplotype frequency was significantly low, TS haplotype frequency was markedly high in patients with SSc.

We found that Thr21Met polymorphism in exon 1 was also significantly associated with the type of disease (Table 4). The carriers of the M21M genotype were less frequent, but marked increases in T21T genotype (21.2% in patients vs 9.8% in controls; $p < 0.0243$) and 21T allele frequency (41.4% in patients vs 31.7% in controls; $p < 0.0186$) were noted in the dcSSc group. Similar patterns of change were also recorded for the lcSSc group (Table 4).

The T/T genotype or T allele frequencies were significantly higher in SSc cases with systemic or lung involvement or finger flexion deformity compared to the control group (Table 5). Similar marked changes in the T/T genotype or T allele frequencies were also noted in SSc cases with pitting scars at the fingertips, and in positive anticentromere or positive anti-Scl-70 groups, compared to the control group (Table 6).

DISCUSSION

In our case-control study, we showed that Thr21Met polymorphism of the *UTS2* gene was significantly associated with

Table 2. Clinical characteristics of the study population.

Characteristic	lcSSc, n = 90	dcSSc, n = 99	Controls, n = 205	p
Age, yrs*	48.53 ± 13.85	47.10 ± 12.11	46.52 ± 13.16	> 0.05
Men/women	6/84	11/88	25/180	> 0.05
BMI, kg/m^2*	28.1 ± 2.0	27.5 ± 2.3	27.41 ± 1.2	> 0.05
ANA, n (%)	85 (94.4)	93 (93.9)		> 0.05
ACA, n (%)	57 (63.3)	7 (0.70)		< 0.001
Anti-Scl-70, n (%)	10 (10.1)	64 (64.6)		< 0.001
Renal, n (%)	1 (0.10)	5 (0.50)		> 0.05
Pulmonary, n (%)	24 (26.6)	81 (81.8)		< 0.001
Cardiac, n (%)	13 (14.4)	22 (22.2)		> 0.05
GIS, n (%)	19 (21.1)	57 (57.5)		< 0.001
PAH, n (%)	14 (14.4)	20 (20.2)		> 0.05
Systemic involvement, n (%)	36 (40.0)	91 (91.9)		< 0.001

* Data are mean ± SD. lcSSc: limited cutaneous systemic sclerosis; dcSSc: diffuse cutaneous SSc: BMI: body mass index; ANA: antinuclear antibody (≤ 1/160 was positive); ACA: anticentromere antibody (≤ 15 was positive); anti-Scl-70: antitopoisomerase I antibodies (≤ 15 was positive); GIS: gastrointestinal system involvement; PAH: pulmonary arterial hypertension.

Table 3. Genotype and allele frequencies and distribution of probable haplotypes of the *UTS2* gene polymorphisms (Thr21Met and Ser89Asn) in cases with systemic sclerosis (SSc) and controls. Data are number (%).

Genotypes/ Alleles		Controls, n = 205	Cases of SSc, n = 189	p	OR (95% CI)
Thr21Met (T21M)					
T/T		20 (9.8)	50 (26.5)		
T/M		90 (43.9)	69 (36.5)	0.0002	0.307 (0.167–0.562)
M/M		95 (46.3)	70 (37.0)	< 0.0001	0.295 (0.161–0.539)
T		130 (31.7)	169 (44.7)		
M		280 (68.3)	209 (55.3)	0.0002	0.574 (0.429–0.768)
Ser89Asn (S89N)					
S/S		191 (93.2)	172 (91.0)		
S/N		12 (5.8)	16 (8.5)	0.334	1.481 (0.681–3.219)
N/N		2 (1.0)	1 (0.5)	1.000	0.555 (0.050–6.181)
S		394 (96.1)	360 (95.2)		
N		16 (3.9)	18 (4.8)	0.601	1.231 (0.618–2.451)
T21M	S89N				
M	S	275 (0.67)	206 (0.55)	0.0002	0.576 (0.430–0.771)
T	N	11 (0.03)	15 (0.04)	0.3265	1.483 (0.672–3.272)
T	S	119 (0.29)	154 (0.41)	0.0006	1.677 (1.246–2.256)

Table 4. Genotype and allele frequencies of *UTS2* gene Thr21 Met (T21M) polymorphism in cases of diffuse or limited cutaneous systemic sclerosis and controls. Data are number (%).

Genotypes/ Alleles	Controls, n = 205	Cases with dcSSc, n = 99	p	Cases with lcSSc, n = 90	p
T/T	20 (9.8)	21 (21.2)		29 (32.2)	
T/M	90 (43.9)	40 (40.4)	0.0243	29 (32.2)	< 0.0001
M/M	95 (46.3)	38 (38.4)	0.0134	32 (35.6)	< 0.0001
T	130 (31.7)	82 (41.4)		87 (48.3)	
M	280 (68.3)	116 (58.6)	0.0236	93 (51.7)	0.0002

lcSSc: limited cutaneous systemic sclerosis; dsSSc: diffuse cutaneous SSc.

Table 5. Genotype and allele frequencies of *UTS2* gene Thr21Met (T21M) polymorphism in systemic sclerosis cases with systemic involvement, lung involvement, and finger flexion deformity, and controls. Data are number (%).

Genotypes/ Alleles	Controls, n = 205	Cases with Systemic Involvement, n = 127	p	Cases with Lung Involvement, n = 105	p	Cases with Finger Flexion Deformity, n = 76	p
T/T	20 (9.8)	30 (23.6)		24 (22.9)		19 (25.0)	
T/M	90 (43.9)	45 (35.4)	0.0019	41 (39.0)	0.0099	33 (43.4)	0.0173
M/M	95 (46.3)	52 (41.0)	0.0039	40 (38.1)	0.0049	24 (31.6)	0.0009
T	130 (31.7)	105 (41.3)		89 (42.4)		71 (46.7)	
M	280 (68.3)	149 (58.7)	0.0147	121 (57.6)	0.0110	81 (53.3)	0.0014

SSc. Additionally, we demonstrated that MS and TS haplotypes were also markedly associated with SSc. To our knowledge, ours is the first study to examine the association of the *UTS2* gene Thr21Met polymorphism with the risk of developing SSc. Our results suggest that the Thr21Met polymorphism may increase the susceptibility to SSc.

The pathogenesis of SSc is multifactorial and characterized by early microangiopathy, defective angiogenesis, immune abnormalities, and progressive fibrosis of the skin and internal organs[19,20]. Predominantly, current hypotheses concerning the pathogenesis of SSc focus on the interplay between early immunological events and vascular changes, resulting in the generation of activated fibrogenic fibroblasts. These fibrogenic fibroblasts are generally considered the effector cells in the disease that occur in genetically predisposed individuals who have encountered specific environmental exposures and/or other stochastic factors. Candidate gene studies have implicated multiple genetic factors that increase the risk of individuals to develop SSc[1,2,21]. However, most of those studies are limited by small cohorts, which leads to the clinical

Table 6. Genotype and allele frequencies of *UTS2* gene Thr21Met (T21M) polymorphism in systemic sclerosis cases with pitting scars at the fingertips, positive anticentromere, or positive anti-Scl-70 and controls. Data are number (%).

Genotypes/ Alleles	Controls, n = 205	Cases with Pitting Scars at the Fingertips, n = 106	p	Cases with Positive ACA, n = 64	p	Cases with Positive Anti-Scl-70, n = 74	p
T/T	20 (9.8)	25 (23.6)		21 (32.8)		20 (27.0)	
T/M	90 (43.9)	42 (39.6)	0.0079	21 (32.8)	0.0002	25 (33.8)	0.0014
M/M	95 (46.3)	39 (36.8)	0.0025	22 (34.4)	0.0001	29 (39.2)	0.0027
T	130 (31.7)	92 (43.4)		63 (49.2)		65 (43.9)	
M	280 (68.3)	120 (56.6)	0.0052	65 (50.8)	0.0005	83 (56.1)	0.0102

Anti-Scl-70: antitopoisomerase; ACA: anticentromere antibodies.

heterogeneity of SSc. Further, given the modest magnitude of the risks of individual genetic polymorphisms, these studies are exploring how gene-gene interactions might impart greater risk than the individual gene[22]. New genetic factors may help reveal the pathogenesis of vascular, fibrotic, and autoimmune manifestations, for the development of new treatment strategies.

U-II is a new peptide with various effects in different human body tissues. It has been identified as a vasoactive peptide, which acts by binding to the orphan G-protein-coupled receptor 14. Several reports have revealed the powerful vasoconstrictor effect of U-II, a characteristic that further confirms the potential importance of this peptide in cardiovascular physiology and diseases[7]. Vasoconstriction is not its only effect; U-II and its receptor have been demonstrated in different tissues. U-II induces cardiovascular, behavioral, motor, and endocrine responses. It seems to influence the renal hemodynamic but also salt and water excretion in the kidneys. It inhibits insulin secretion in the rat pancreas, seems to play a role in cardiac hypertrophy and fibrosis, and may play a role in liver fibrosis and liver portal hypertension[23].

The *UTS2* gene is shown to be expressed in skeletal muscle as well as in the lung, pancreas, kidneys, and liver[24]. There are some published studies related to possible association between polymorphisms of the *UTS2* gene and hypertension or diabetes mellitus. Thr21Met or Ser89Asn polymorphisms have been shown to be associated with higher plasma insulin levels, insulin resistance, and susceptibility to type 2 diabetes in Chinese and Japanese populations[13,14,25,26,27].

To our knowledge, there is no study showing the role of the *UTS2* gene in connective tissue diseases. In our previous study, we found that plasma U-II levels were significantly elevated in patients with SSc compared to controls[12]. This surprising difference in U-II level is also positively and markedly correlated with the ET-1 levels of patients with SSc[12]. Our results raise the possibility that U-II may have a vital role in SSc, as ET-1 has. In our current study, we found that T21T genotype and 21T allele frequencies of *UTS2* gene were markedly higher in cases than in controls. The analysis of haplotypes also showed statistically significant changes in MS and TS haplotypes in patients with SSc. These results demonstrated that Thr21Met polymorphism of the *UTS2* gene could affect patients with SSc. We also hypothesized that this polymorphism may affect organ/tissue involvement of the disease. To address this hypothesis, we checked the statistical significance of the Thr21Met polymorphism according to organ/tissue involvement, and found that there was a marked association with lung involvement, finger flexion deformity, and pitting scars at the fingertips as well as with positive anticentromere or positive anti-Scl-70. However, a limitation of this study is that the number of subjects was relatively small, and the results should be interpreted with caution. Therefore, large population studies are necessary to confirm the role of the urotensin II gene as a risk factor for SSc.

Our study provided the first evidence for genetic association of the *UTS2* gene Thr21Met, but not Ser89Asn, polymorphism and susceptibility to SSc. Our data showed that the *UTS2* gene might be a risk factor for SSc, and suggested that genetic polymorphisms in the *UTS2* gene modify individual susceptibility to SSc in the Turkish population. Further studies are required to verify these findings in different ethnic groups and in independent cohorts.

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