CONCISE REPORT

Lack of association between three vascular endothelial growth factor gene polymorphisms and systemic sclerosis: results from a multicenter EUSTAR study of European Caucasian patients

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Introduction: Systemic sclerosis (SSc) is characterised by disturbed vessel morphology and an overproduction of vascular endothelial growth factor (VEGF). The VEGF gene located on chromosome 6p21.3 has several polymorphisms.
Objective: To test the hypothesis that disturbed angiogenesis may be related to the genetic background of the VEGF gene.
Materials and methods: EUSTAR centres included European Caucasian patients with SSc and matched controls with osteoarthritis. The VEGF gene was genotyped by polymerase chain reaction, followed by restriction enzyme analysis. The 634 C/T and 936 C/G mutations and an 18-base pair insertion/deletion at −2549 of the VEGF promoter region were tested.
Results: 416 patients with SSc and 249 controls were included in the study population. Of the patients with SSc, 42% had a diffuse cutaneous subtype, 16% had increased pulmonary arterial pressure and 61% had decreased carbon monoxide diffusion capacity. The genotype frequencies in the patients with SSc and in controls were in Hardy–Weinberg equilibrium. The allele and genotype frequencies of the polymorphisms did not differ between patients with SSc and controls. No association was found between these polymorphisms and disease phenotypes.
Conclusion: This study shows that there is no association between the three selected functional VEGF polymorphisms and SSc.

Systemic sclerosis (SSc) is a connective tissue disease characterised by early generalised microangiopathy that culminates in systemic fibrosis. The key steps in the disease are endothelium injury, immune activation and collagen deposition by activated fibroblasts.

Vasculature has a marked effect on SSc prognosis, with the outcome depending on the extent and severity of the vascular lesions. Vascular changes are thought to occur at an early stage of the disease, and these changes may include endothelial cell apoptosis, endothelium activation with the expression of cell adhesion molecules, inflammatory cell recruitment, intimal proliferation and adventitial fibrosis.[1] Capillaroscopy can be used for microvascular investigation and may show disturbed angiogenesis, with changes in permeability and architecture, giant capillaries, branched capillaries and avascular areas.[2] Despite suggestions that patients with SSc have deficiencies in angiogenesis, we and others have reported high circulating levels of vascular endothelial growth factor (VEGF) in these patients,[3 4] and a chronic and uncontrolled upregulation of VEGF has been implicated in the disturbed morphology of skin vessels of patients with SSc.[5] VEGF is an endothelial cell-specific mitogen glycoprotein that promotes angiogenesis. It also acts as a proinflammatory cytokine by increasing endothelial permeability and inducing adhesion molecules that bind leucocytes to endothelial cells.[6]

The VEGF gene is located on chromosome 6p21.3 and consists of eight exons separated by seven introns, which undergo alternative splicing to form a family of proteins. VEGF gene expression is modulated by a variety of effectors, such as cytokines, lipopolysaccharide, hormones and hypoxia. There is also considerable variation in VEGF expression, with at least 25 different polymorphisms being reported.[7 8] Polymorphisms of the VEGF gene are associated with a susceptibility to disorders in which angiogenesis is important for development and progression, such as giant cell arteritis,[9] renal complications in Henoch–Schonlein purpura[10]. Diabetic retinopathy, cardiovascular diseases and cancer, and in several aspects of pregnancy.[7] The VEGF gene polymorphisms 634 C/G (rs2010963) in the 5′ untranslated regions, 936 C/T in the 3′ untranslated region and an 18-base pair (bp) insertion/deletion at position −2549 have been associated with variations in both VEGF plasma levels and production by mononuclear cells.[8 11 12]

PATIENTS AND METHODS

An association study was conducted to investigate VEGF polymorphisms in European Caucasian patients with SSc, defined as those having four European Caucasian grandparents. All EUSTAR centres able to work on DNA samples were contacted and asked to include patients with SSc together with controls matched for age, sex and origin. The DNA was extracted locally and then sent to the Paris centre for genotyping.

The following clinical data were collected: age, sex, duration of disease (date of first non-Raynaud symptom) and cutaneous SSc subtype according to the definition of LeRoy *et al*.[13] Involvement of the lungs was assessed according to international guidelines[14]: pulmonary fibrosis was investigated by a computed tomography scan and restrictive syndrome was defined as a forced vital capacity <75% of the predicted value, whereas vascular involvement with a measured pulmonary artery pressure by echocardiography of >40 mm Hg was considered to be pathological. The following immunological tests were carried out: anti-centromere antibodies (immunofluorescence on Hep2 cells) and anti-topoisomerase I antibodies (counter immunoelectrophoresis). All patients gave written informed consent and the ethics committee of each hospital approved the study.

Abbreviations: PCR, polymerase chain reaction; SSc, systemic sclerosis; VEGF, vascular endothelial growth factor

Table 1 Characteristics of patients with systemic sclerosis included in the study

Patients, n(%), unless otherwise stated	Patients with SSc (n = 416)
Mean (SD), age range (years)	53 (13), 20–78
Sex (F/M)	360 (87%)/56 (13%)
Diffuse/limited cutaneous form	176 (42%)/240 (58%)
Mean (SD) duration of disease (years)	8.1 (6.2)
Left ventricular ejection fraction <55%	29/416 (7%)
Increased pulmonary arterial pressure	68/416 (16%)
Pulmonary fibrosis on CT scan	241/416 (58%)
Positive for antinuclear antibodies	342/378 (90%)
Positive for anti-topoisomerase I antibodies	134/378 (35%)
Positive for anti-centromere antibodies	99/378 (26%)
Low FVC (<75% of predicted value)	132/416 (32%)
Low DLCO/AV (<75% of predicted value)	252/416 (61%)
Renal crisis	8/416 (2%)
Prostacyclin treatment	124/316 (40%)

CT,computed tomography; DLCO, diffusing capacity of the lung for carbon monoxide; F, female; FVC, forced vital capacity; M, male; SSc, systemic sclerosis; AV, alevolan volume.

VEGF was genotyped using polymerase chain reaction (PCR), followed by restriction analysis. The primers for the VEGF +634 polymorphism were 5′-ATTTATTTTTGCTTGCCATT-3′ and 5′-GTCTGTCTGTCTGTCCGTCA-3′; for the VEGF +936 polymorphism, 5′-AAGGAAGAGGAGACTCTGCG-3′ and 5′-TATGTGGG TGGGTGTGTCTA-3′; and for the 18-bp insertion/deletion polymorphism, 5′-CCTGGAGCGTTTTGGTTAAA-3′ and 5′-ATATAGGAAGCAGCTTGGAA-3′. PCR was carried out in a PE 9600 thermal cycler (Perkin Elmer Cetus, Norwalk, Connecticut, USA) in 50-µl reaction volumes containing 100 ng template DNA, 50 mM KCl, 10 mM TRIS-HCl, 0.1% Triton X-100, 200 mM each of dATP, dCTP, dGTP, dTTP (Amersham Pharmacia Biotech, Uppsala, Sweden), 2.5 mM $MgCl_2$, 0.5 mM each primer and 2.5 U Hot Star Taq DNA polymerase (Qiagen, Frankfort, Germany). After an initial denaturation step (2 min at 95℃), the samples were subjected to 35 cycles of 95℃ for 30 s, 58℃ for 30 s (for VEGF +634), 62℃ for 30 s (for VEGF +936), 55℃ for 30 s (for 18-bp insertion/deletion) and 72℃ for 30 s, with a final extension of 5 min at 72℃.

The PCR products for the VEGF +634 polymorphism were digested with restriction endonuclease *Bsm*FI (New England Biolabs, Beverly, Massachusetts, USA), and the restriction fragments were analysed on a 2% agarose gel. The 304-bp C allele remained uncut, whereas the G allele was cut into two fragments of 193 and 111 bp. The PCR products for the VEGF +936 polymorphism were digested with restriction endonuclease *Nla*III (New England Biolabs), and the restriction fragments were analysed on a 2% agarose gel. The 208-bp C allele remained uncut, whereas the T allele was cut into two fragments of 122 and 86 bp. The PCR products for the VEGF 18-bp insertion/deletion were analysed on a 2% agarose gel. The pattern showed two fragments: a 234-bp I allele with the 18-bp insertion and a 216-bp D allele with no insertion.

Statistical analysis

Statistical analysis was carried out using StatView software. Genotype distributions and allele frequencies were compared using the χ^2 test. The Hardy–Weinberg equilibrium for genotype distribution was estimated using software available at the Institute of Human Genetics, Munich (http://ihg.gsf.de). p Values <0.05 were considered significant.

RESULTS

We included 416 patients with SSc: 360 (87%) women and 56 (13%) men, with a mean (standard deviation (SD)) age of 53 (13) years (range 20–80 years). Control groups comprised 249 patients with osteoarthritis: 229 (92%) women and 20 (8%) men, with a mean (SD) age of 59 (12) years (range 35–75 years). The number of patients and controls from each participating centre were (patients with SSc /controls): Paris (Cochin Hospital; 75/68), Brescia (80/35), Florence (70/50), Pecs (62/20), Moscow (45/44), Berlin (30/14), Verona (25/18), Lublin (22/0), and Graz (7/0). Table 1 gives the characteristics of the patients with SSc included in the study.

We found no deviation from the Hardy–Weinberg equilibrium for the patient and control populations. The genotype and allele distributions of the three VEGF polymorphisms did not differ significantly between the patients and controls (table 2). We also found no difference when the populations were studied by centre. We found no significant differences in the genotype and allele frequencies in the patients with SSc according to disease phenotype, and, in particular, according to the cutaneous subtype and whether major vascular complications had occurred (for pulmonary arterial hypertension, 32 of 35 tested patients had pre-capillary hypertension on right heart catheterisation). Haplotypes were not associated with either SSc or its characteristics.

DISCUSSION

Systemic sclerosis is characterised by a marked vascular involvement with disturbed angiogenesis that suggests the involvement of VEGF. VEGF is a major angiogenic factor and a prime regulator of endothelial cell proliferation. It has a crucial role in physiological vasculogenesis and vascular permeability and is implicated in several pathological processes. Therefore, it is an attractive candidate gene for a role in SSc.

Several VEGF polymorphisms have been reported, although we believe that until now they have not been investigated in SSc. We chose functional polymorphisms that could account for

Table 2 Genotype and allele distributions of the three vascular endothelial growth factor polymorphisms for the patients with systemic sclerosis and controls

Patients, n (%)	Controls (n = 249)	Patients with SSc (n = 416)	χ^2 test
634C/G	GG: 97/249 (38.9)	GG: 168/416 (40.4)	NS
	GC: 115/249 (46.2)	GC: 181/416 (43.5)	NS
	CC: 37/249 (14.9)	CC: 67/416 (16.1)	NS
	Allele C: 189/498 (38.0)	Allele C: 315/832 (37.9)	NS
936 C/T	CC: 174/249 (69.9)	CC: 288/416 (69.2)	NS
	CT: 71/249 (28.5)	CT: 121/416 (29.1)	NS
	TT: 4/249 (1.6)	TT: 7/416 (1.7)	NS
	Allele T: 79/498 (15.9)	Allele T: 135/832 (16.2)	NS
18-bp insertion/deletion	II: 56/249 (22.5)	II: 85/416 (20.4)	NS
	ID: 111/249 (44.6)	ID: 202/416 (48.6)	NS
	DD: 82/249 (32.9)	DD: 129/416 (31.0)	NS
	Allele D: 275/498 (55.2)	Allele D: 460/832 (55.3)	NS

SSc, systemic sclerosis.

the disturbed angiogenesis seen in SSc. However, our results clearly show no association between the three tested VEGF polymorphisms and SSc. We found no association with certain major vascular complications, but a more detailed analysis of vascular phenotype and the effect of duration of disease might reveal a potential association. Nevertheless, our large sample size allows us to make a robust conclusion. Moreover, we have tried to limit the influence of ethnicity by the restriction of the study to Caucasian European patients and controls. However, the influence of different geographical factors was not taken into account in the selection of patients. Using healthy subjects as controls would have been more accurate, as VEGF may take part in progression of osteoarthritis, but our results are further validated by the finding of similar genotype frequencies in the healthy control population as that reported by other studies investigating European Caucasian patients.[10 15] Although our results may support the hypothesis that chronic and uncontrolled VEGF up regulation is mediated by an orchestrated expression of cytokines and not the genetic background, they do not rule out other polymophisms[7 11] or mutations of the VEGF gene or its receptors being associated with SSc.

CONCLUSION

This study from a large cohort of European Caucasian patients with SSc shows that there is no association between SSc and the −634C, −936C and −2549I functional VEGF polymorphisms.

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