Association between an endoglin gene polymorphism and systemic sclerosis-related pulmonary arterial hypertension

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Systemic sclerosis (SSc) is a connective tissue disorder characterized by early generalized microangiopathy with disturbed angiogenesis. Endoglin gene (ENG) encodes a transmembrane glycoprotein which acts as an accessory receptor for the transforming growth factor-β (TGF-β) superfamily, and is crucial for maintaining vascular integrity. A 6-base insertion in intron 7 (6bINS) of ENG has been reported to be associated with microvascular disturbance.

Objectives. Our objective was to investigate the relationship between 6bINS and the vascular complication pulmonary arterial hypertension (PAH) in SSc in a French Caucasian population.

Methods. Two hundred eighty SSc cases containing 29/280 having PAH diagnosed by catheterism were compared with 140 patients with osteoarthritis. Genotyping was performed by polymerase-chain-reaction-based fluorescence and direct sequencing of genomic DNA.

Results. The polymorphism was in Hardy–Weinberg equilibrium. We observed a significant lower frequency of 6bINS allele in SSc patients with associated PAH compared with controls [10.3 vs 23.9%, P = 0.01; odds ratio (OR) 0.37, 95% confidence interval (CI) 0.15–0.89], and a trend in comparison with SSc patients without PAH (10.3 vs 20.3%, P = 0.05; OR: 0.45, 95% CI: 0.19–1.08). Genotypes carrying allele 6bINS were also less frequent in SSc patients with PAH than in controls (20.7 vs 42.9%, P = 0.02).

Conclusions. Thus the frequency of 6bINS differs between SSc patients with or without PAH, suggesting the implication of ENG in this devastating vascular complication of SSc.

KEY WORDS: Systemic sclerosis, Pulmonary arterial hypertension, Endoglin, Gene, TGF-β.

Systemic sclerosis (SSc) is a connective tissue disease characterized by early generalized microangiopathy, culminating in systemic fibrosis. The key steps in the disease are endothelial cell apoptosis, endothelium activation, inflammatory cell recruitment, intimal proliferation and adventitial fibrosis, which may lead to vessel obliteration [2]. Among the vascular complication, pulmonary arterial hypertension (PAH), as the pulmonary vascular form of the disease, has emerged as a leading cause of death. Survival analysis suggests that outcome in SSc-associated PAH is worse than in haemodynamically equivalent primary PAH, although the reasons are not clear [3]. Systematic screening clinical assessment, annual Doppler echocardiography and pulmonary function tests are recommended to try detecting patients early in the disease, but these have some limitations [4]. Cardiac catheterization remains the diagnostic gold standard [5], but this technique is invasive and has morbidity and mortality risk. Thus, markers which would enable a high risk subgroup to be isolated and followed-up serially over time would allow earlier diagnosis which is necessary for improving the outcome.

Although SSc pathogenesis remains unclear, it is believed that both genetic and environmental factors contribute to disease susceptibility and clinical expression [6]. The genetic contribution is indeed emphasized by the fact that the disease occurs significantly more frequently within families with SSc cases than in the general population [7]. Among the factors that maintain vascular integrity, endoglin is a component of the transforming growth factor-β (TGF-β) receptor complex and is predominantly expressed on cell surfaces of endothelial cells. Endoglin, also called CD105, is a homodimeric membrane glycoprotein primarily associated with human vascular endothelium [8]. The endoglin gene (ENG) is located at 9q34.1 and ENG mutations are responsible for one of the two types of Hereditary Haemorrhagic Telangiectasia (or Osler–Weber–Rendu syndrome; HHT type 1; MIM# 131195), a Mendelian autosomal vascular disorder [9]. Protein expression studies have proposed haploinsufficiency as the most likely model explaining HHT1 [10, 11]. Moreover, an insertion polymorphism in intron 7 of the ENG gene (6bINS) (5‘-TCCCCC-3‘, starting

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23 bp distal from the 3' end of exon 7) was found to be associated with the occurrence of cerebral aneurysms in a Japanese population reflecting microvascular abnormalities [12]. However, the association was not replicated in a population of white Caucasian subjects consistent with ethnic-related differences of allele frequencies [13].

Our aim was to investigate whether the 6bINS allele of the ENG gene is associated with the major microvascular complication (i.e. PAH) of SSc in a large multicentric cohort of European Caucasian patients and controls.

Patients and methods

Patients

We included 420 consecutive unrelated subjects comprising 280 French SSc patients, classified according to LeRoy's cutaneous subtypes [14] and 140 controls. The control subjects were osteoarthritis patients fulfilling the American College of Rheumatology (ACR) criteria [15] free of any systemic disease and matched for age and sex. All 420 subjects were of European Caucasian origin, defined by the four grandparents being European Caucasian, and were recruited from three Rheumatology and three Internal Medicine departments. The Ethics Committee of Cochin Hospital approved the study, and all patients gave written informed consent for all procedures.

Pulmonary vascular involvement was considered pathological if patients had on echocardiography a systolic pulmonary artery pressure if sPAP > 40 mmHg with unexplained dyspnoea, patients then underwent right heart catheterism to confirm PAH according to international guidelines: it was haemodynamically defined as a resting mean pulmonary arterial pressure PAP > 25 mmHg with a normal pulmonary capillary or left atrial pressure (<15 mmHg) [16]. The following clinical data were also collected: age, sex, disease duration (date of first non-Raynaud symptom), cutaneous SSC subtype according to the definition of LeRoy et al. [14]. Lung involvement was assessed according to international guidelines [17]: pulmonary fibrosis was investigated by CT scan, and a restrictive syndrome was defined as a forced vital capacity <75% of the predicted value. The following immunological tests were carried out: anti-centromere antibodies (immunofluorescence on Hep2 cells) and anti-topoisomerase I (counterimmunoelectrophoresis).

Methods

DNA extraction. Genomic DNA was purified from fresh peripheral blood leukocytes using saline standard methods. DNA concentration was evaluated for each sample by biphotonic absorbometry. DNA samples were stored until use at -80°C.

Genotyping of 6bINS of ENG gene. Polymerase chain reaction (PCR) amplification was performed for genotyping of the 6bINS polymorphism in intron 7 of ENG with the following primers: 5'-GCTCAGAGGCTGATGT-3' and Hex labelled 5'-Hex-GAGGCGCTGACATACCT-3'. 150 ng genomic DNA was amplified in a total of 50 µl reaction mixture containing 5 µl of 10× PCR Buffer containing 100 mM Tris–HCl, 500 mM KCl, 15 mM MgCl2 (Sigma), 140 ng of each primer, 10 mM dNTP mix, 2.5 U Taq DNA Polymerase (Sigma). PCR conditions were as follows: initial denaturation was at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, and was completed by a final extension at 72°C for 5 min with a thermal cycler (GeneAmp PCR System, model 9700, Applied Biosystems). After amplification, the PCR products were genotyped on an ABIPRISM 3100, Applied Biosystem and analysed with Genotyper software. Genotypes were read independently by two experienced molecular geneticists who were blinded to phenotype.

Verification and genotyping. For verification of genotypes, direct sequencing was performed in an unselected group of 50 subjects with a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems) on PCR-amplified segments.

Statistical analysis

The sample of 280 patients and 140 controls allowed yielding 83% power at the 5% level (two-sided) to detect an absolute difference of 10% between the two groups, on the basis of an expected frequency of 28% of 6bINS allele.

Allele frequencies were determined by allele counting in each group. Hardy–Weinberg equilibrium was tested using Arlequin software (L. Excoffier; http://anthro.unige.ch/arlequin) with a significance threshold of 0.01. We compared the allelic frequencies of marker between SSc patients and controls with Cocaphase software (F. Dudbridge, www.rfe.gr.mrc.ac.uk/fudbrid/software). P-values < 0.05 were considered significant. Results were confirmed using a Fisher’s exact test if significant. Odds ratios (OR), their 95% confidence interval (CI) and the exact P-values of 2 x 2 tables were calculated with the Statexact software v7 (Cytel corp, Cambridge, MA, USA).

Results

Population characteristics

Among the 280 French SSc patients, 254 were women (91%), with a mean age of 57±13 yrs (range 25–87) and a mean disease duration of 8±7 yrs. Twenty-nine SSc patients had PAH; their mean age was 62±12 yrs, their mean disease duration was 8±7 yrs and 48% had Lof (14/29) the diffuse cutaneous form. The other characteristics of the SSc patients with and without PAH are detailed in Table 1. The control group contained 140 osteoarthritis patients: 116 (83%) women, with a mean age of 59±12 yrs (range: 35–75 yrs).

Genotype and allele frequencies

The polymorphic marker was in Hardy–Weinberg equilibrium in both the patient and control groups. The verification using sequencing confirmed in 100% cases the results of fluorescent PCR (Fig. 1).

The evaluation of the potential implication of allele 6bINS of ENG in PAH revealed that there was a significant difference between the frequency of allele 6bINS in SSc patients with PAH compared with controls (10.3±23.9%, P = 0.01; OR, 0.37, 95% CI: 0.15–0.89). This result was confirmed with the frequency of genotypes carrying the allele 6bINS analysis: in SSc patients with PAH this frequency was significantly lower than in controls (20.7 vs 42.9%, P = 0.02; OR: 0.35, 95% CI: 0.13–0.90). In addition, we

Table 1. Characteristics of the patients with systemic sclerosis with or without PAH

<table>
<thead>
<tr>
<th></th>
<th>SSc patients without PAH (n = 251)</th>
<th>SSc patients with PAH (n = 29)</th>
</tr>
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<tbody>
<tr>
<td>Diffuse/limited cutaneous form</td>
<td>85 (34)/</td>
<td>14 (48)/</td>
</tr>
<tr>
<td></td>
<td>166 (66)</td>
<td>15 (52)</td>
</tr>
<tr>
<td>Digital ulcers (current or past)</td>
<td>75 (30)</td>
<td>6 (21)</td>
</tr>
<tr>
<td>Low DLCO/VA (&lt;75% of predicted value)</td>
<td>80 (32)</td>
<td>19 (66)</td>
</tr>
<tr>
<td>Pulmonary fibrosis on CT scan</td>
<td>115 (62)</td>
<td>20 (69)</td>
</tr>
<tr>
<td>Positive for anti-nuclear antibodies</td>
<td>214 (85)</td>
<td>26 (90)</td>
</tr>
<tr>
<td>Positive for anti-topoisomerase I antibodies</td>
<td>65 (26)</td>
<td>7 (24)</td>
</tr>
<tr>
<td>Positive for anti-centromere antibodies</td>
<td>82 (33)</td>
<td>4 (14)</td>
</tr>
</tbody>
</table>
observed a trend toward a lower frequency of this allele when SSc patients with PAH were compared with SSc patients without this complication (10.3% \( P = 0.05 \); OR: 0.45, 95% CI: 0.19–1.08).

Among the 29 SSc patients with PAH, six had significant pulmonary fibrosis with decreased forced vital capacity <75% of normal value (Table 1); their genotypes did not differ from that of SSc patients with PAH, but with forced vital capacity >75% of normal value (Table 2).

No significant difference in allele frequencies was detected between the SSc patients and the controls: 19.3% vs. 23.9%, respectively (\( P = 0.12 \)). The frequency of genotypes carrying the 6bINS allele was also similar in SSc patients (34.6%) and in controls (42.9%) (\( P = 0.1 \)) (Table 2). The frequency of the 6bINS allele or of genotypes carrying the allele was not different in other subsamples of SSc patients.

### Discussion

Our results show that among a large cohort of SSc patients the frequency of intron 7 6bINS of the ENG gene differs between patients with related PAH and those free of this complication or controls. The 6bINS allele is negatively associated with SSc-related PAH.

PAH is a progressive arteriopathy of the pulmonary circulation that can affect about 12% of patients with SSc [18]; the prevalence of PAH in our cohort (10.3%) is consistent with this frequency. Natural history of PAH is linked to progressive right ventricular failure, and this complication has emerged as a leading cause of death despite recent improvement in therapeutic strategies [19]. The prognosis appears to be related to early recognition, and the determination of susceptibility markers is a major issue for this devastating condition. The rigorous definition of PAH based on widely recognized international criteria together with the large sample size of our series of patients strengthens our results. They suggest that allele 6bINS may partly protect against PAH. However, the disease duration may account for the occurrence of PAH and may influence with time our data. Therefore, a prospective follow-up is ongoing.

In recent years, genetic studies have significantly increased the understanding of the molecular basis of PAH, demonstrating that the most frequent cause of familial PAH is mutation of receptor members of the TGF-β superfamily. More than 140 disease-causing defects in the gene encoding the type II receptor BMPR2 are currently reported; many have been identified in patients with no known family history of PAH because of the low penetrance of these mutations, and haploinsufficiency is the predominant molecular mechanism underlying disease predisposition [20]. Investigation of PAH subjects in families exhibiting clinical features characteristic of HHT has identified mutations of the type I receptor ALK-1 as a rare cause of PAH [21, 22]. Recently, mutations were identified in BMPR2, ALK-1 and ENG in childhood PAH [23]. These data emphasize a critical role for receptor members of the TGF-β superfamily in the development and maintenance of the pulmonary vasculature.

A limitation of our study is the lack of evaluation of functional consequences of the finding, and this will require further specific investigations in order to determine if the 6bINS polymorphism in intron 7 may particularly affect splicing of the mRNA or folding of the protein. Moreover, we used no corrected statistical tests for the evaluation of the association of the 6bINS polymorphism with SSc-related PAH, as this objective was our primary and sole goal regarding the previous data suggesting the implication of 6bINS in microvascular disturbance [12]. If multiple tests were used for the evaluation of all SSc characteristics, the significance would be no more achieved partly because of the low prevalence of PAH; thus, the association will need to be confirmed in larger population of SSc-related PAH and also in primary PAH. Finally, we focused on European Caucasian SSc patients, but ethnic-related differences of allele 6bINS frequencies between Caucasian and Japanese patients with intracranial aneurysms or controls were reported [12, 13], and thus our results need replication in another ethnic group.

### Table 2. Frequency of genotypes in the whole SSc population and in SSc patients without and with PAH and controls

<table>
<thead>
<tr>
<th>Intron 7 6bINS allele and genotype</th>
<th>All SSc patients ( n = 280 ) [n(%)]</th>
<th>SSc patients free of PAH ( n = 251 ) [n(%)]</th>
<th>Whole group ( n = 29 ) [n(%)]</th>
<th>PAH without PF ( n = 23 ) [n(%)]</th>
<th>PAH with PF ( n = 6 ) [n(%)]</th>
<th>Controls ( n = 140 ) [n(%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele 6bINS</td>
<td>108 (19.3%)</td>
<td>102 (20.3%)</td>
<td>6 (10.3%)</td>
<td>5 (10.9%)</td>
<td>1 (8.3%)</td>
<td>67 (23.9%)</td>
</tr>
<tr>
<td>6bINS/6bINS</td>
<td>11 (3.9%)</td>
<td>11 (4.4%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>7 (5%)</td>
</tr>
<tr>
<td>6bINS/WT</td>
<td>86 (30.7%)</td>
<td>80 (31.9%)</td>
<td>6 (20.7%)</td>
<td>5 (21.7%)</td>
<td>1 (16.7%)</td>
<td>53 (37.8%)</td>
</tr>
<tr>
<td>WT/WT</td>
<td>183 (65.4%)</td>
<td>160 (63.7%)</td>
<td>23 (79.3%)</td>
<td>18 (78.3%)</td>
<td>5 (83.3%)</td>
<td>80 (57.1%)</td>
</tr>
<tr>
<td>6bINS/WT and 6bINS</td>
<td>97 (34.6%)</td>
<td>91 (36.3%)</td>
<td>6 (20.7%)</td>
<td>5 (21.7%)</td>
<td>1 (16.6%)</td>
<td>60 (42.9%)</td>
</tr>
</tbody>
</table>

\*SSc, patients with PAH compared with controls; PAH, pulmonary arterial hypertension; PF, pulmonary fibrosis.
Conclusion

Our results show an association between a polymorphism of the gene encoding endoglin and SSc-related PAH. However, the sample size of our study and the heterogeneity of the phenotypes will require confirmation from other cohorts. Nevertheless, in line with the recent evidence of the role of mutations in receptor members of the TGF-β superfamily in idiopathic PAH, our results could further support a putative role of this family, and particularly endoglin in this vascular devastating condition of SSc. The sequencing of endoglin is ongoing to further evaluate other gene variations.

Nomenclature

We referred to GenBank accession No. U17156 regarding the homo sapiens endoglin gene, exon 7 and GenBank accession No. AH006911 regarding the 6-bp DNA insert. Results were interpreted while the sense strand was read in the 5′ to 3′ direction.

Key messages

- An endoglin gene variant in intron 7 is negatively associated with SSc-related PAH.
- Genetic variations in the receptor members of the TGF-β superfamily are highly suspected to play a key role in this vascular devastating condition.

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The authors have declared no conflicts of interest.

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