

## CONCISE REPORT

# TGF $\beta$ receptor gene variants in systemic sclerosis-related pulmonary arterial hypertension: results from a multicentre EUSTAR study of European Caucasian patients

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## ABSTRACT

**Introduction** Systemic sclerosis (SSc)-related pulmonary arterial hypertension (PAH) has emerged as a major mortality prognostic factor. Mutations of transforming growth factor beta (TGF $\beta$ ) receptor genes strongly contribute to idiopathic and familial PAH.

**Objective** To explore the genetic bases of SSc-PAH, we combined direct sequencing and genotyping of candidate genes encoding TGF $\beta$  receptor family members.

**Materials and methods** TGF $\beta$  receptor genes, *BMPR2*, *ALK1*, *TGFR2* and *ENG*, were sequenced in 10 SSc-PAH patients, nine SSc and seven controls. In addition, 22 single-nucleotide polymorphisms (SNP) of these four candidate genes were tested for association in a first set of 824 French Caucasian SSc patients (including 54 SSc-PAH) and 939 controls. The replication set consisted of 1516 European SSc (including 219 SSc-PAH) and 3129 controls from the European League Against Rheumatism Scleroderma Trials and Research group network.

**Results** No mutation was identified by direct sequencing. However, two repertoried SNP, *ENG* rs35400405 and *ALK1* rs2277382, were found in SSc-PAH patients only. The genotyping of 22 SNP including the latter showed that only rs2277382 was associated with SSc-PAH ( $p=0.0066$ , OR 2.13, 95% CI 1.24 to 3.65). Nevertheless, this was not replicated with the following result in combined analysis:  $p=0.123$ , OR 0.79, 95% CI 0.59 to 1.07.

**Conclusions** This study demonstrates the lack of association between these TGF $\beta$  receptor gene polymorphisms and SSc-PAH using both sequencing and genotyping methods.

## INTRODUCTION

Systemic sclerosis (SSc) is characterised by major vascular involvement. Pulmonary arterial hypertension (PAH) is currently an important challenge in SSc and given the severity of this condition and the poor understanding of its risk factors and pathogenesis, there is an urgent need to identify novel risk factors for the development of SSc-PAH.<sup>1</sup> The identification of mutations in the *BMPR2* gene, and also in other transforming growth factor beta (TGF $\beta$ ) receptor genes in idiopathic PAH and familial PAH has been an important step forward. Indeed, mutations in the *BMPR2* gene, which encodes a type II bone morphogenetic protein receptor of the TGF $\beta$  cell signalling superfamily, underlie the majority of hereditary PAH cases<sup>2</sup> but have also been identified in other disease subtypes including idiopathic PAH and PAH associated with other disorders.<sup>3–4</sup> Mutations in two further receptor members of the TGF $\beta$  signalling superfamily have been identified as uncommon causes of hereditary PAH. Indeed, hereditary haemorrhagic telangiectasia (HHT) is an autosomal dominant vascular disease, caused by heterozygous mutations of either TGF $\beta$  type I receptor activin-like kinase-type 1 (*ALK1*) or of the endoglin gene (*ENG*).<sup>5</sup> A small proportion of HHT patients have PAH that is clinically and histopathologically indistinguishable from other heritable forms of PAH. In rare cases, mutations of *ALK1* appeared to cause idiopathic PAH and hereditary PAH without HHT.<sup>4</sup> Therefore, *BMPR2*, *ALK1* and *ENG* genes, belonging to the TGF $\beta$  superfamily, represent good candidates for the study of genetic susceptibility to SSc-PAH.

Few studies have attempted to identify SSc-PAH genetic risk factors. Despite some relevant prelimin-

ary results, a lack of appropriate cohorts (because of imperfect phenotype and/or insufficient statistical power) has precluded definite conclusions.<sup>6-9</sup>

The aim of this study was to investigate a specific genetic basis favouring the occurrence of PAH in SSc, using a synergistic strategy combining direct sequencing together with genotyping of common variants of candidate genes encoding four TGF $\beta$  receptors: *BMPR2*, *ALK1*, *ENG* and *TGFBR2*.

## PATIENTS AND METHODS

### Study population

All SSc patients and controls were of European Caucasian origin and were provided through the European League Against Rheumatism Scleroderma Trials and Research group (EUSTAR) centres. The discovery set consisted of 824 SSc patients, including 54 SSc-PAH patients and 939 controls from French centres. The replication set consisted of cohorts from other French centres (175 SSc patients including 75 SSc-PAH patients and 438 controls), northern/central Europe (455 SSc, 59 SSc-PAH and 1823 controls), Italy (542 SSc, 33 PAH-SSc and 479 controls) and eastern Europe (344 SSc, 52 SSc-PAH and 389 controls). The characteristics of the study population have been detailed previously.<sup>10</sup> Clinical data collected included age, sex, disease duration and cutaneous SSc subtypes according to the definition of Leroy *et al.*<sup>11</sup> Precapillary PAH diagnosis was established following right heart catheterisation as recommended.<sup>12</sup> All local institutional review boards approved the study, and written informed consent was obtained from all study subjects.

### Direct sequencing

As a first approach, 26 French Caucasian individuals (10 SSc-PAH patients, nine SSc patients without PAH and seven healthy controls) were sequenced for the candidate genes encoding four TGF $\beta$  receptors: *BMPR2*, *ENG*, *ALK1* and *TGFBR2*. Genomic DNA was extracted from blood samples (Qiagen, Courtaboeuf, France). PCR primers were designed using Primer 3 to amplify segments of genomic DNA that included the exons and intron-exon boundaries to detect splice-site variants. PCR products were purified using ExoSAP-IT (USB Corp., Cleveland, Ohio, USA) and sequenced using the BigDye Terminator V3.1 cycle sequencing kit (Applied Biosystems, Foster City, California, USA).

### Genotyping

As a second approach, any single-nucleotide variation detected by direct sequencing in SSc-PAH patients and not present either in controls or PAH-free SSc patients, was tested for association in the genotyping cohort. In addition, Tag single-nucleotide polymorphisms (SNP) with a minor allele frequency (MAF) greater than 5% were genotyped for each of the four TGF $\beta$  receptor genes using the KASpar genotyping system (KBioscience, Hoddesdon, UK) as previously described.<sup>13</sup> Six SNP of the *BMPR2* gene (rs7600694, rs1061157, rs1048829, rs6747756, rs1980153, rs16839127), seven SNP of the *TGFBR2* gene (rs377626, rs1841528, rs2372092, rs3773661, rs9867701, rs11466531, rs11466536), four SNP of the *ALK1* gene (rs706815, rs772003, rs2277382, rs3782479) and five SNP of the *ENG* gene (rs35400405, rs1998923, rs1330684, rs10987746, rs17557600) were chosen according to linkage disequilibrium structure. The average genotype completeness for these variants was above 97% for both the SSc and the control samples.

### Statistical analyses

Statistical analyses were performed as previously described.<sup>13</sup> The Bonferroni correction was applied for all tests performed for SNP marker association with the disease (p value multiplied by n SNP). The analysis of combined data was performed by calculation of the pooled OR under a fixed-effects model (Mantel-Haenszel meta-analysis). No power calculation can be provided for mutation investigations, but regarding common SNP (MAF >5%) and for *ALK1* rs2277382 in particular, the combined sample provides a power of 99.9% to detect an association with SSc and of 52.3% for the SSc-PAH subset, with an OR of 1.5.

## RESULTS

### Sequencing of *TGFBR* genes in cases and controls

No mutation was identified through the sequencing of 38 SSc and 14 control chromosomes (table 1). We identified 17 polymorphisms: 13 SNP listed in public databases and four variants not yet repertoried, none of which were mutations as they were found both in patients and controls. Two variants emerged as interesting candidates for further study. Indeed, the SNP located at codon 14 of exon 1 in the *ENG* gene, known as

**Table 1** Variants identified by direct sequencing of the *ENG*, *ALK1*, *TGFR2* and *BMPR2* genes

Gene	Location	Nucleotide change	Amino acid change	rs number	MAF	SSc-PAH (n=10)	SSc without PAH (n=9)	Controls (n=7)
<i>ENG</i>	Exon 1	c.14 G>A	p.T5M	rs35400405	NA	3	0	0
	Intron 1 (-215)	T>C		rs60683420	NA	0	2	0
	Exon 2	c.227 C>T	p.L69L	rs16930129	0.165	2	4	2
	Intron 2 (+25)	A>C		rs7847860	NA	0	0	2
	Intron 5* (+59)	del G		NA	NA	1	1	1
	Intron 7* (+23)	ins TCCCCC		NA	NA	1	9	4
	Exon 8	c.1029 G>A	p.H343H	rs3739817	0.066	0	5	4
	Intron 13 (-72)	A>G		rs10760503	0.407	3	6	2
<i>ALK1</i>	Promoter (-38)	C>T		rs2277382	0.075	3	0	0
	Intron 3 (+11)	T>C		rs2071218	NA	7	4	5
	Intron 3 (-36)*	T>A		NA	NA	6	5	4
	Intron 5 (+44)*	A>G		NA	NA	0	0	4
<i>TGFBR2</i>	Intron 2 (+7)	A>G	p.N389N	rs1155705	0.336	4	5	3
	Intron 3 (-4)	A>T		rs11466512	NA	8	4	4
	Exon 4	c.1167 C>T		rs2228048	0.027	0	0	1
<i>BMPR2</i>	Intron 6 (-22)	del T	p.R937R	rs11464745	NA	1	2	0
	Exon 12	c.2811 G>A		rs1061157	0.128	3	2	3

\*Novel variants.

*ALK1*, activin A receptor type II-like 1; *BMPR2*, BMP receptor type II; *ENG*, endoglin; MAF, minor allele frequency; NA, not available; PAH, pulmonary arterial hypertension; SSc, systemic sclerosis; *TGFBR2*, TGFbeta receptor type II.

## Basic and translational research

rs35400405, introduces an in-frame ATG sequence downstream of the usual initiating codon that might result in the loss of the first amino acids. This variation was detected in SSc-PAH patients only and none of the controls. The rs2277382 SNP of the *ALK1* gene was found only in three SSc-PAH patients, none of the PAH-free patients and none of the controls. This SNP is located in the promoter of the gene, and has previously been identified as a single-nucleotide variation associated with HHT.<sup>14</sup>

### Association testing of identified variants and common tag SNP of the *TGFβR* genes

#### Discovery set

We investigated the possible association of polymorphisms in the *ALK1*, *TGFBR2*, *BMPR2* and *EGN* genes with SSc and the SSc-PAH subtype by genotyping rs35400405, rs2277382 and 20 tag SNP distributed throughout these genes (table 2). No association was found with SSc in the discovery set. Genetic association was solely observed between the SSc-PAH subset and the *ALK1* rs2277382 SNP (OR 2.13, 95% CI 1.24 to 3.65,  $p_{adj} = 0.0066$ ).

#### Replication set

Following the results obtained in the discovery set, we selected the *ALK1* rs2277382 SNP to be investigated in the EUSTAR replication cohort (table 3). Genotype frequencies of the rs2277382 variant were in Hardy-Weinberg equilibrium in all control populations. However, we did not observe any association between the rs2277382T allele and either SSc-PAH or the SSc subset in these replication sets.

#### Meta-analysis in the European Caucasian population

Meta-analysis of the combined discovery and replication populations (French, northern European, Italian and eastern European) including a total of 2340 SSc patients, 273 SSc-PAH and 4068 controls did not provide evidence for an association between *ALK1* rs2277382 and neither SSc-PAH nor SSc.

## DISCUSSION

Genes encoding TGFβ receptors have been identified as major susceptibility genes in familial and idiopathic forms of PAH. Understanding the genetic differences between idiopathic PAH and SSc-PAH, and also between patients with SSc who do and do not develop PAH, may improve our ability to develop genetic biomarkers of SSc-PAH. This may help to identify these patients earlier in the disease course and to risk stratify patients in order to optimise the management of this devastating condition.

So far, preliminary studies investigating *BMPR2* and *ALK1* have failed to identify variants associated with SSc-PAH by a direct sequencing strategy. However, they were limited by small sample size and heterogeneous definition of PAH.<sup>7-9</sup>

**Table 3** Association study of *ALK1* rs2277382 with SSc and PAH-SSc in the second set of European Caucasian populations and combined analysis including the discovery and replication cohorts

	N	MAF (%)	p Value	OR (95% CI)
French replication cohort				
SSc	175	8.57	0.410	1.21 (0.77 to 1.90)
PAH-SSc	75	8.67	0.524	1.23 (0.66 to 2.29)
Controls	438	7.19	NA	NA
Italians				
SSc	542	8.76	0.870	1.03 (0.75 to 1.40)
PAH-SSc	33	9.09	0.881	1.07 (0.45 to 2.55)
Controls	479	8.56	NA	NA
Northern European				
SSc	455	8.46	0.990	0.10 (0.77 to 1.30)
PAH-SSc	59	11.02	0.331	1.34 (0.74 to 2.41)
Controls	1823	8.48	NA	NA
Eastern European				
SSc	344	5.96	0.570	0.89 (0.58 to 1.35)
PAH-SSc	52	2.88	0.132	0.42 (0.13 to 1.35)
Controls	389	6.68	NA	NA
Meta-analysis in the combined discovery and replication populations in an additive model				
SSc	2340	8.44	0.149	0.91 (0.80 to 1.04)
PAH-SSc	273	9.52	0.123	0.79 (0.59 to 1.07)
Controls	4068	7.72	NA	NA

Northern European: Germany and Poland; Eastern European: Greece, Hungary, Romania, Serbia.

MAF, minor allele frequency; NA, not applicable; PAH, pulmonary arterial hypertension; SSc, systemic sclerosis.

Furthermore, an insertion in intron 7 of the *ENG* gene (6bINS) was reported to be negatively associated with the occurrence of SSc-PAH in a previous work from our group in a small cohort of 280 SSc patients including 29 with PAH and 140 controls.<sup>15</sup> However, until now this result has not been replicated in a larger cohort.

In this study, the *ALK1* rs2277382 and *ENG* rs35400405 SNP were of particular interest because they were detected only in SSc-PAH patients by direct sequencing, the hypothesis being that their minor alleles could be associated with the development of PAH in our cohorts. However, no association was found between these polymorphisms and both the complication that is SSc-PAH and also SSc. This does not rule out the possible implication of other TGFβ signalling pathway genes. Indeed, mutations in the *SMAD* genes have recently been identified in PAH patients and could represent another potential candidate to take into account in the genetics of SSc-PAH in further studies.<sup>16</sup> Furthermore, another limitation may come from the fact that some SSc patients may develop PAH later during the course of the disease.

**Table 2** Association study of *ALK1* rs2277382 and *ENG* rs35400405 SNP in the French discovery cohort

		N	MAF (%)	Fischer's p value	p-adj*	OR (95% CI)
<i>ALK1</i> rs2277382	SSc	824	9.04	0.003	0.066	1.43 (1.13 to 1.81)
	PAH-SSc	54	12.9	0.0003	0.0066	2.13 (1.24 to 3.65)
	Controls	939	6.50	NA	NA	NA
<i>ENG</i> rs35400405	SSc	822	2.49	0.26	NS	1.30 (0.82 to 2.05)
	PAH-SSc	54	3.64	0.22	NS	1.92 (0.67 to 5.49)
	Controls	906	1.93	NA	NA	NA

*ENG*, endoglin gene; MAF, minor allele frequency; NA, not applicable; p-adj\*, adjusted p value after Bonferroni correction for multiple SNP testing (n=22); PAH, pulmonary arterial hypertension; SNP, single-nucleotide polymorphism; SSc, systemic sclerosis.

In conclusion, this study was conducted using a synergistic strategy combining direct sequencing for the identification of potential mutations or rare variants, and genotyping of common variants in a large sample including a replication step. These analyses demonstrate the lack of association between these TGF $\beta$  receptor gene polymorphisms and SSc-PAH.

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