Association Study of Serotonin Transporter Gene (SLC6A4) in Systemic Sclerosis in European Caucasian Populations

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ABSTRACT. ***Objective.*** Serotonin is a key contributing factor in pulmonary arterial hypertension (PAH) by inducing pulmonary arterial smooth muscle cell (PA-SMC) proliferation. This relates specifically to the internalization process in PA-SMC of the serotonin transporter (SLC6A4 or 5-HTT). A long (L)/short (S) (44 base pair insertion) functional polymorphism within the promoter of the transporter *SLC6A4* gene has been reported to be associated with familial and idiopathic PAH. Our objective was to determine whether polymorphisms of *SLC6A4* confer susceptibility to SSc and its vascular phenotype.

Methods. Three Tag single-nucleotide polymorphisms (SNP) (rs2066713, rs1042173, rs6354) chosen using Hapmap and linkage disequilibrium data were genotyped in a total cohort of 667 SSc patients (56 with PAH, 207 with digital ulcerations) and 447 controls. All individuals were of French Caucasian origin. L/S polymorphism genotyping was determined by polymerase chain reaction in a random subgroup of 364 SSc patients (34 with PAH, 138 with digital ulcerations) and 218 controls.

Results. Three polymorphisms (L/S, rs2066713, rs1042173) were in Hardy-Weinberg equilibrium in the control population, but rs6354 deviated. Allelic and genotypic frequencies for these 3 polymorphisms were similar in SSc patients and controls. Subphenotype analyses of subsets with PAH and digital ulceration did not detect any difference between SSc patients compared to controls.

Conclusion. These results from a large cohort of European Caucasian SSc patients do not support the implication of *SLC6A4* in the pathogenesis of SSc and its vascular subphenotypes. However, serotonin pathways remain good candidates to contribute to the vasculopathy of SSc. (First Release April 15 2010; J Rheumatol 2010;37:1164–7; doi:10.3899/jrheum.091156)

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Systemic sclerosis (SSc) is a connective tissue disease characterized by early generalized microangiopathy. Its most severe vascular complication is pulmonary arterial hypertension (PAH) that has emerged as a leading cause of death[1]. Bone morphogenetic protein receptor type 2 gene (*BMPR2*) has been identified as a major gene of the famil-

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ial form of PAH. However, some other candidate genes have been advocated including the serotonin transporter *SLC6A4* (or *5-HTT*)[2]. Indeed, pulmonary arterial smooth muscle cells (PA-SMC) from patients with idiopathic PAH have a greater proliferative response to serotonin transduced by the 5-HTT pathway than patients free of PAH[3]. Moreover, a long (L)/short (S) (44 base pair insertion) functional polymorphism within the promoter region of *SLC6A4* gene (17q11.1-q12), increasing the rate of transcription, has been identified[4]. This polymorphism has been found to be associated with different subtypes of PAH[5,6]. PA-SMC from homozygous LL PAH patients proliferate more in response to 5-HTT than those without this homozygous genotype[3].

Clinical and experimental data suggest that the pathogenesis of SSc is multifactorial, involving both genetic and environmental factors. Regarding SSc-related PAH, only scarce genetic data are available, related to *Endoglin*[7], *NOS2*[8], *CX3CR1*[9], and *SDF-1* genes[10], but to date there has been no independent replication. We investigated whether *SLC6A4* gene may be implicated in the pathogenesis of SSc and in particularly its microvascular complications, PAH and digital ulcerations.

MATERIALS AND METHODS

A total of 1170 unrelated individuals were included: 667 SSc patients (56 having precapillary PAH diagnosed by catheterization, 207 having a history of digital ulcerations, and 17 with concomitant PAH and digital ulcerations) and 511 healthy matched controls, all of European Caucasian origin. The whole population has been genotyped for 3 *SLC6A4* Tag single-nucleotide polymorphisms (SNP: rs1042173, rs2066713, rs6354) using the KASpar Genotyping system (Kbioscience, Hoddesdon, UK)[11,12]. These 3 TagSNP account for the common genetic diversity according to HapMap data. In a second study, using classical direct genotyping by polymerase chain reaction[4], a randomly selected subsample of 364 SSc patients (34 with PAH and 138 with a history of digital ulcerations) and 218 healthy controls were genotyped for the *SLC6A4* L/S polymorphism.

The ethics committee of Cochin Hospital approved the study and all subjects gave written informed consent.

The screening and definition of patients with PAH were performed as reported[13]. Patients identified by (1) an echocardiographically estimated systolic pulmonary artery pressure (PAP) > 40 mm Hg; (2) DLCO < 50% of predicted in the absence of pulmonary fibrosis; or (3) unexplained dyspnea, were offered right-heart catheterization. Confirmed PAH was defined as a resting mean PAP ≥ 25 mm Hg with pulmonary capillary wedge pressure ≤ 15 mm Hg.

Statistical analysis. Hardy–Weinberg equilibrium was investigated with a chi-square test with 1 degree of freedom. Power calculations were driven through an asymptotic non-central chi-square approach and provided a power of 81% to detect association for a genotype of 38% frequency with an OR of 1.5 at the 5% significance level. Fisher's exact test was used to compare allele and genotype frequencies.

RESULTS

All demographic data and disease characteristics of SSc patients are given in Table 1. Two of the 3 SNP studied (rs1042173 and rs2066713) were in Hardy-Weinberg equilibrium for the control group, but the rs6354 variant deviated and was excluded from subsequent analyses. Regarding the 2 TagSNP, no difference was found between allelic and genotypic frequencies of patients and controls (Table 2). The sub-phenotype analyses, especially those related to vascular involvement, did not detect any *SLC6A4* rs1042173 and rs2066713 allelic or genotypic association (Table 2). Further, the frequency of the S allele was not different in SSc patients compared to controls (Table 3). In the SSc-PAH and digital ulcerations subgroups, the allelic and genotypic frequencies of the L/S polymorphism were similar in patients and in controls (Table 3). Intra-cohort analyses did not reveal any difference between SSc patients with and those without the vascular trait for any of the 3 variants.

DISCUSSION

SSc-related PAH has emerged as a leading and lethal complication of the disease. Identification of PAH risk factors is a key issue to improve outcomes in SSc. Evidence of a key role of the serotonin transporter in PAH has been provided recently: (1) 5-HTT expression was shown to be increased in lungs of patients with idiopathic PAH[14]; (2) serotonin induces greater activation of PA-SMC proliferation in patients with PAH[4]; (3) mice disrupted for the *SLC6A4* gene developed less severe hypoxic PAH than wild-type controls[15]; and (4) an association between the functional L/S polymorphism and PAH was reported[4-6].

Table 1. Characteristics of the European Caucasian cohort of patients with systemic sclerosis (SSc) for the different *SLC6A4* polymorphisms.

Patients, n (%)	SSc Cohort for *SLC6A4* Tag SNP Markers, n = 667	SSc Cohort for *SLC6A4* I/D, n = 364
Age, yrs ± SD	57.4 ± 12.1	56 ± 8
Female, n (%)	573 (86)	303 (83)
Disease duration, yrs ± SD	10.8 ± 8	11.5 ± 9
Diffuse cutaneous subtype, n (%)	208 (31)	163 (33)
Pulmonary arterial hypertension, n (%)	56 (8)	34 (9)
Digital ulcerations, n (%)	207 (31)	138 (38)
Renal crisis, n (%)	6 (1)	4 (1)
Positive antitopoisomerase I antibody, n (%)	153 (23)	75 (21)
Positive anticentromere antibody, n (%)	258 (39)	116 (32)

Table 2. Genotype and allelic frequencies of the *SLC6A4* rs1042173 and rs2066713 Tag SNP. Data are no. (%).

		SSc, n = 659	PAH, n = 52	Digital Ulcerations, n = 207	Controls, n = 447	p and OR (95% CI) Values for Allelic Comparisons
rs1042173						
Allele	T	342 (53)	31 (61)	98 (50)	243 (55)	$p_1 = 0.21$, OR_1 0.90 (0.75–1.06)
						$p_2 = 0.29$, OR_2 1.25 (0.82–1.90)
	G	307 (47)	20 (39)	98 (50)	196 (45)	$p_3 = 0.09$, OR_3 1.43 (0.94–2.17)
						$p_4 = 0.09$, OR_4 0.81 (0.64–1.03)
Genotypes	T/T	179 (28)	17 (33)	51 (26)	141 (32)	
	G/T	325 (50)	28 (55)	95 (48)	204 (47)	
	G/G	145 (22)	6 (12)	50 (26)	94 (21)	
rs2066713						
Allele	C	415 (63)	29 (57)	125 (62)	279 (62)	$p_1 = 0.89$, OR_1 1.01 (0.85–0.21)
						$p_2 = 0.26$, OR_2 0.79 (0.52–1.19)
	T	247 (37)	22 (43)	76 (38)	168 (38)	$p_3 = 0.19$, OR_3 0.76 (0.09–1.14)
						$p_4 = 0.99$, OR_4 1.01 (0.78–1.27)
Genotypes	C/C	263 (40)	18 (35)	81 (40)	181 (40)	
	C/T	304 (46)	23 (44)	89 (44)	196 (44)	
	T/T	95 (14)	11 (21)	31 (16)	70 (16)	

SSc: systemic sclerosis; PAH: pulmonary arterial hypertension. OR_1: SSc vs controls; OR_2: PAH-SSc vs controls; OR_3: SSc with vs SSc without PAH; OR_4: SSc with digital ulcerations vs controls.

Table 3. Genotype and allelic frequencies of the *SLC6A4* L/S polymorphism. Data are no. (%).

		SSc, n = 364	PAH, n = 34	Digital Ulcerations, n = 138	Controls, n = 218	p and OR (95% CI) Values for Allelic Comparisons
Allele	S	161 (44)	15 (43)	68 (49)	103 (47)	$p_1 = 0.28$, OR_1 0.88 (0.69–1.11)
						$p_2 = 0.45$, OR_2 0.82 (0.49–1.38)
	L	203 (56)	19 (57)	70 (51)	115 (53)	$p_3 = 0.78$, OR_3 0.93 (0.56–1.54)
						$p_4 = 0.71$, OR_4 1.06 (0.78–1.43)
Genotypes	S/S	71 (20)	6 (18)	33 (24)	55 (25)	
	L/S	180 (49)	17 (50)	69 (50)	97 (45)	
	L/L	113 (31)	11 (32)	36 (26)	66 (30)	

SSc: systemic sclerosis; PAH: pulmonary arterial hypertension. OR_1: SSc vs controls; OR_2: PAH-SSc vs controls; OR_3: SSc with vs SSc without PAH; OR_4: SSc with digital ulcerations vs controls.

Our results show that the *SLC6A4* polymorphisms we studied do not contribute to susceptibility to SSc and its main microvascular consequences. The large sample size of our cohort provides strong power (> 80%) for global analysis of SSc, and the focus on European Caucasian individuals avoids a heterogeneity bias. In idiopathic PAH, the LL genotype of the *SLC6A4* promoter polymorphism was present in homozygous form in 65% of patients, but in only 27% of controls[4]. Results from our control group are close to these and support the validity of our findings. Conversely, the discrepancies we observed between the SSc-PAH findings and previous results in PAH studies may be explained by specificities of SSc-PAH compared to familial or idiopathic PAH, including differences in genetic background. Despite inclusion of a large number of patients, and due to the scarcity of PAH, the statistical power was lower for this subset and this may lead to a type 2 error. In addition, as the PAH phenotype can change during the disease course, a prospective followup of the SSc cohort is needed to clarify the phenotype and improve sub-phenotype analyses. However, our cohort was the largest used to date for an association study related to vascular sub-phenotype; in addition PAH was diagnosed for our study using the "gold standard," that is, catheterization.

To conclude, common variants of the *SLC6A4* gene, including the L/S polymorphism, were found not to be associated with overall SSc or with SSc-related PAH and/or digital ulcerations. Rare variants and other serotonin pathways should now be investigated.

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