A large multicenter analysis of CTGF -945 promoter polymorphism does not confirm association with Systemic Sclerosis susceptibility or phenotype

B Rueda, C Simeon, R Hesselstrand, A Herrick, J Worthington, N Ortego-Centeno, G Riemekasten, V Fonollosa, M C Vonk, F HJ van den Hoogen, J Sanchez-Román, M A Aguirre-Zamorano, R García-Portales, A Pros, M T Camps, M A Gonzalez-Gay, M J Coenen, N Lambert, J L Nelson, T RDJ Radstake and J Martin

Ann Rheum Dis published online 3 Dec 2008;
doi:10.1136/ard.2008.100180

ARD Online First, published on December 3, 2008 as 10.1136/ard.2008.100180

A large multicenter analysis of *CTGF* -945 promoter polymorphism does not confirm association with Systemic Sclerosis susceptibility or phenotype.

Rueda B[1], Simeon C[2], Hesselstrand R[3], Herrick A[4], Worthington J[4], Ortego-Centeno N[5], Riemekasten G[6], Fonollosa V[2], Vonk MC[7], van den Hoogen FHJ[8], Sanchez-Román J[9], Aguirre-Zamorano MA[10], García-Portales R[11], Pros A[12], Camps MT[13], Gonzalez-Gay MA[14], Gonzalez-Escribano MF[15], Coenen MJ[16], Lambert N[17], Nelson JL[18], Radstake TRDJ[19] and Martin J[1].

1 Instituto de Parasitologia y Biomedicina "Lopez-Neyra" (CSIC), Granada, Spain. 2 Servicio de Medinina Interna, Hospital Vall de Ebron, Barcelona, Spain. 3 Department of Rheumatology, Lund University Hospital, S-221 85 Lund, Sweden. 4 Rheumatic Diseases Centre, University of Manchester, Salford Royal NHS Foundation Trust, UK. 5 Servicio de Medicina Interna, Hospital Clinico Universitario, Granada, Spain. 6 Department of Rheumatology and Clinical Immunology, Charité University Hospital, Berlin, Germany. 7 Department of Rheumatology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. 8 Department of Rheumatology, Sint Maartenskliniek, Nijmegen, The Netherlands. 9 Servicio de Medicina Interna. Hospital Virgen del Rocio, Sevilla, Spain. 10 Servicio de Reumatología. Hospital Reina Sofía, Córdoba, Spain. 11 Servicio Medicina Interna. Hospital Virgen de la Victoria, Málaga, Spain. 12 Servicio de Reumatología, Hospital del Mar, Barcelona, Spain. 13 Servicio de Medicina Interna, Hospital Carlos Haya, Malaga, Spain. 14 Servicio de Reumatología, Hospital Xeral-Calde, Lugo, Spain. 15 Servicio de Inmunología, Hospital Virgen del Rocio, Sevilla. 16 Department of Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. 17 INSERM U639, Parc scientifique de Luminy Marseille, France. 18 Department of Medicine, University of Washington, Seattle, Washington, United States of America. 19 Department of Rheumatology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.

Correspondence to:
Javier Martin MD, PhD. Instituto de Parasitologia y Biomedicina "López-Neyra". Consejo Superior de Investigaciones Científicas, Parque Tecnológico Ciencias de la Salud. Avenida del Conocimiento s/n 18100-Armilla (Granada), Spain.
E-mail: martin@ipb.csic.es

ABSTRACT

Objective: In this work we conducted a replication study to investigate whether the -945 *CTGF* genetic variant is associated with SSc susceptibility or specific SSc phenotype.

Methods: The study population comprised of 1180 SSc patients and 1784 healthy controls from seven independent case-control sets of European ancestry (Spanish, French, Dutch, German, British, Swedish and North American). The –945 *CTGF* genetic variant was genotyped using a Taqman 5´ allelic discrimination assay.

Results: First we conducted an independent association study that revealed in all case-control cohorts under study no association of the *CTGF* -945 polymorphism with SSc susceptibility. These findings were confirmed by a meta-analysis that reached a pooled OR of 1.12 (95 % CI 0.99-1.25, *P*=0.06). In addition, the possible contribution of the -945 *CTGF* genetic variant to SSc phenotype was investigated. However, stratification according to SSc subtypes (limited or diffuse), selective autoantibodies (antitopoisomerase I or anti-centromere) or pulmonary involvement reached no statistically significant skewing.

Conclusion: Our results do not confirm previous findings and suggest that the *CTGF* –945 promoter polymorphism does not play a major role in SSc susceptibility or clinical phenotype.

KEY WORDS: Systemic slcerosis, connective tissue growth factor, association study, polymorphism.

INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune disorder characterized by excessive fibrosis, vascular abnormalities and immune system dysfunction that can affect several organs or tissues (mainly skin, lungs and kidneys).[1]
The genetic component of SSc is supported by familial aggregation and ethnic influences, however individual genetic markers or genes have generally not shown reproducible association with SSc susceptibility so far. Most of the candidate gene association studies conducted in SSc are limited by insufficient statistical power due to the small sample sizes analysed and by the lack of replication in independent populations.[1]
Recently, the connective tissue growth factor (CTGF) gene has been suggested as a novel genetic marker for SSc susceptibility. A putative functional single nucleotide polymorphism (SNP) located in the promoter region of *CTGF* gene (rs6918698; -945 C/G) was significantly associated with SSc susceptibility and with certain clinical hallmarks of this condition in a British population.[2] However, these findings were not replicated in a North American case-control set in which the *CTGF* -945 genetic variant showed no association with SSc susceptibility or phenotype.[3]
In view of these controversial results and in order to better understand the role of the *CTGF* gene in SSc pathogenesis, we designed a large replication study including seven independent case-control sets of Caucasian ancestry to investigate whether the -945 *CTGF* genetic variant is implicated in SSc susceptibility or clinical manifestations.

MATERIAL AND METHODS

Patients
The study population consisted of a total of 1180 SSc patients and 1784 healthy controls from seven independent case-control sets of European ancestry (Spanish: 317 SSc and 369 controls; French: 98 SSc and 146 controls, Dutch: 140 SSc and 267 controls; German: 251 SSc and 276 controls; British: 145 SSc and 351 controls and Swedish: 119 SSc and 277 controls; North American: 120 SSc and 98 healthy controls). All the patients fulfilled the 1980 American College of Rheumatology (ACR) classification criteria for SSc.[4]
The control population consisted in unrelated healthy individuals matched by age, sex and ethnicity with the SSc patients groups.
The study was approved by local ethical committees from all the participating centers. Both patients and controls were included in the study after written informed consent.
SSc patients were classified as having limited or diffuse SSc.[5, 6] The seven patients groups were comparable in terms of age, gender and disease duration. Data regarding selective autoantibodies status was not available in all SSc patients: 983 patients were assessed for the presence of anti-topoisomerase I (anti-Scl-70) and 902 for anti-centromere antibodies (ACA). Involvement of the lungs was assessed in 750 SSc patients according to the international guidelines.[7] The presence of pulmonary fibrosis was investigated by a computed tomography scan. Restrictive syndrome and diffusion capacity of the lungs was defined as a forced vital capacity (FVC) < 75% of the predicted value and a diffusion capacity of the lung for carbon monoxide (DLCO) of less than 75% of predicted.
CTGF -945 genotyping.

DNA samples from patients and controls were genotyped for the *CTGF* -945 polymorphism using a Custom-Taqman-SNP-Genotyping-Assay (Applied Biosystems, Foster City, CA, USA) (primers and probes sequences are available under request).
The PCR reaction was performed as follows: 92ºC-10 min, 40 cycles of 92ºC-15 sec and 60 ºC-1:00 min. Post-PCR, the genotype of each sample was automatically attributed in the ABI Prism 7900 Sequence Detection System using the SDS 2.3 software for allele discrimination (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

We tested Hardy-Weinberg equilibrium (HWE) by using the program FINETI (http://ihg.gsf.de/cgi-bin/hw/hwa2.pl). Significance was calculated by 2x2 contingency tables and Fisher's exact test, to obtain p values, odds ratios (OR) and 95% confidence intervals (CI) by using Statcalc software (Epi Info 2002; Centers for Disease Control and Prevention, Atlanta, GA, USA). P values below 0.05 were considered as statistically significant. The analysis of the combined data from all populations was performed using the Stats Direct software. Homogeneity of OR among cohorts was calculated using Breslow-Day and Woolf Q methods and the calculation of the pooled OR was performed under a fixed-effects model (Mantel-Haenszel meta-analysis) or random effects (DerSimonian-Laird) when necessary.
The estimation of the power of the study was performed using the Quanto v 0.5 software (Department of Preventive Medicine University of Southern California, California, USA.)

RESULTS

The *CTGF* -945 genetic variant was analysed in seven populations from Spain, France, The Netherlands, Germany, United Kingdom, Sweden and North America. In all of them, both SSc patients and controls group were found to be in Hardy-Weinberg equilibrium for *CTGF* -945 genotypes.
The individual analysis revealed the same trend in all populations showing no association of the *CTGF* -945 polymorphism with SSc susceptibility considering either allelic or genotypic frequencies (Table 1).
To further investigate the possible role of the *CTGF* -945 polymorphism in SSc susceptibility, we conducted a combined analysis including the seven case-control series. The estimation of the homogeneity between populations revealed that all of them could be combined. Therefore, we performed a meta-analysis under fixed-effects using the Mantel-Haenszel test that reached a p value of 0.06 and pooled OR of 1.12 for the C allele (95 % CI 0.99-1.25). Thus, we confirmed the lack of association of the *CTGF* -945 polymorphism with SSc susceptibility observed in the independent analysis.
The possible contribution of -945 *CTGF* genetic variant to SSc phenotype was also investigated. After stratification of SSc patients according to skin involvement, presence of autoantibodies or lung involvement, no statistically significant skewing was observed (data not shown).

DISCUSSION

Association studies of functional candidate genes represent one of the most powerful and direct approaches to investigate the genetic component of human complex diseases.[8] However, to validate genetic associations the replication of results in independent. populations is mandatory. Only genetic associations consistently reproducible among populations strengthen the confidence in association studies.[9]

In this line, a recent report showed association of the *CTGF* -945 promoter polymorphism with SSc susceptibility in a British population, whereas the same SNP tested in a North American population did not reach any significant association.[2] Due to the controversial results obtained in these studies the role of *CTGF* as a genetic marker for SSc was unclear.

In order to support additional information that could help to clarify the possible contribution of *CTGF* gene in SSc susceptibility, we analysed the -945 *CTGF* promoter polymorphism in a large cohort of SSc patients originating from seven independent replication cohorts. After independent association studies and a meta-analysis, we did not observe a significant association of *CTGF* -945 genetic variant with SSc susceptibility or clinical manifestations.

The lack of association observed in our study is unlikely due to low statistical power since the total sample size analyzed (1180 SSc patients and 1784 controls) represents a power of 99% to detect an association with a genetic marker assuming OR from 1.5 to 2.2 (similar to that observed in the Fonseca et al study), at the 5 % significance level. Furthermore, the replication of the results in seven independent cohorts, supports the notion that our results are unlikely due to the type II error. The discrpancies between our findings and those by Fonseca et al. could be due to differences in the distribution of *CTGF* -945 genotypes. The frequency for the GG genotype observed in our case-control sets range between 24.4% and 33.7%, which is also similar to the frequency of the GG genotype in the USA cohort.[3] However, this gentoype showed a lower frequency in SSc patients from the initial British study (19.8%).[2] Although these differences could be due to different genetic background, this seems not to be the case since we included in our study a British population that showed a frequency of 27.6 % for the GG genotype. Thus, our findings confirm those obtained by Gourh P *et al* that failed to replicate the association of -945 *CTGF* polymorphism with SSc susceptibility or its autoantibody or clinical subgroups.[3]

The excessive scarring process that leads to the most important clinical complications in SSc is still poorly understood. A complex interaction among pro-fibrotic proteins including CTGF, and driven by transforming growth factor betha (TGFβ), is thought to mediate the fibrogenic response. The expression of CTGF is induced by TGFβ and endothelin 1 (ET-1).[10-12] Therefore it could be speculated that the increased expression of CTGF observed in SSc patients [13] might be due to the induction of potent pro-fibrogenic mediators upstream of CTGF, rather than to genetic variants that could alter the activity of the *CTGF* promoter.

Further studies are necessary to understand the exact role of CTGF in the complex cascade that lead to the exacerbated scaring present in SSc and to better characterize which genetic elements regulate CTGF expression.

In summary, through a large replication study we could not confirm a major role of the –945 *CTGF* promoter polymorphism in SSc susceptibility or phenotype expression. Our results, together with previous findings strongly suggest that the *CTGF* -945 polymorphisms does not seem to be a determinant genetic marker for SSc.

ACKNOWLEDGMENTS
We thanks Sofia Vargas for her excellent technical support. This work was supported by grants SAF2006-00398, and in part by Junta de Andalucía, grupo CTS-180. T.R. was supported by the VENI and VIDI laureates from the Dutch National Organization of Research (NWO).

COMPETING INTERESTS
The authors declare that they have no competing interests.

LICENCE FOR PUBLICATION
The Corresponding Author has the right to grant on behalf of all authors and does grant on behalf of all authors, an exclusive licence (or non exclusive for government employees) on a worldwide basis to the BMJ Publishing Group Ltd to permit this article (if accepted) to be published in ARD and any other BMJPGL products and sublicences such use and exploit all subsidiary rights, as set out in our licence (http://ARD.bmjjournals.com/ifora/licence.pdf).

REFERENCES

[1] Agarwal SK, Tan FK, Arnett FC. Genetics and genomic studies in scleroderma (systemic sclerosis). Rheum Dis Clin North Am. 2008;34:17-40.
[2] Fonseca C, Lindahl GE, Ponticos M, Sestini P, Renzoni EA, Holmes AM, et al. A polymorphism in the CTGF promoter region associated with systemic sclerosis. N Engl J Med. 2007;357:1210-20.
[3] Gourh P, Mayes MD, Arnett FC. CTGF polymorphism associated with systemic sclerosis. N Engl J Med. 2008;358:308-9; author reply 9.
[4] Preliminary criteria for the classification of systemic sclerosis (scleroderma). Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Arthritis Rheum. 1980;23:581-90.
[5] LeRoy EC, Medsger TA, Jr. Criteria for the classification of early systemic sclerosis. J Rheumatol. 2001;28:1573-6.
[6] LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA, Jr., et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol. 1988;15:202-5.
[7] Matucci-Cerinic M, D'Angelo S, Denton CP, Vlachoyiannopoulos P, Silver R. Assessment of lung involvement. Clin Exp Rheumatol. 2003;21:S19-23.
[8] Cordell HJ, Clayton DG. Genetic association studies. Lancet. 2005 30;366:1121-31.
[9] Lohmueller KE, Pearce CL, Pike M, Lander ES, Hirschhorn JN. Meta-analysis of genetic association studies supports a contribution of common variants to susceptibility to common disease. Nat Genet. 2003;33:177-82.
[10] Van Beek JP, Kennedy L, Rockel JS, Bernier SM, Leask A. The induction of CCN2 by TGFbeta1 involves Ets-1. Arthritis Res Ther. 2006;8:R36.
[11] Pannu J, Gore-Hyer E, Yamanaka M, Smith EA, Rubinchik S, Dong JY, et al. An increased transforming growth factor beta receptor type I:type II ratio contributes to elevated collagen protein synthesis that is resistant to inhibition via a kinase-deficient transforming growth factor beta receptor type II in scleroderma. Arthritis Rheum. 2004;50:1566-77.
[12] Shi-Wen X, Renzoni EA, Kennedy L, Howat S, Chen Y, Pearson JD, et al. Endogenous endothelin-1 signaling contributes to type I collagen and CCN2 overexpression in fibrotic fibroblasts. Matrix Biol. 2007;26:625-32.
[13] Dziadzio M, Usinger W, Leask A, Abraham D, Black CM, Denton C, et al. N-terminal connective tissue growth factor is a marker of the fibrotic phenotype in scleroderma. Qjm. 2005;98:485-92.

Table 1. Genotype and allele frequencies of *CTGF* -945 promoter polymorphism in the six case-control sets analysed.

Population		CC	CG	GG	Allele C	Allele G	*P* value allele	OR (95%CI)
Spanish	Cases (317)	82 (25.9)	146 (46.1)	89 (28.1)	310 (48.9)	324 (51.1)	0.75	1.03 (0.8-1.3)
	Controls (369)	96 (26.0)	175 (47.4)	98 (26.6)	367 (49.7)	371 (50.3)		
French	Cases (98)	26 (26.5)	46 (46.9)	26 (26.5)	98 (50.0)	98 (50.0)	0.71	1.07 (0.7-1.5)
	Controls (146)	36 (24.7)	69 (47.3)	41 (28.1)	141 (48.3)	151 (51.7)		
Dutch	Cases (140)	32 (22.9)	71 (50.7)	37 (26.4)	135 (48.2)	145 (51.8)	0.63	1.07 (0.8-1.4)
	Controls (267)	56 (21.0)	136 (50.9)	75 (28.1)	248 (46.6)	286 (53.6)		
Swedish	Cases (119)	36 (30.3)	54 (45.4)	29 (24.4)	126 (52.9)	112 (47.1)	0.29	1.17 (0.9-1.6)
	Controls (277)	74 (26.7)	123 (44.4)	80 (28.9)	271 (48.9)	283 (51.1)		
German	Cases (241)	57 (23.7)	112 (46.5)	72 (29.9)	226 (46.9)	256 (53.1)	0.14	1.20 (0.9-1.5)
	Controls (276)	51 (18.5)	132 (47.8)	93 (33.7)	234 (42.4)	318 (57.6)		
British	Cases (145)	39 (26.9)	66 (45.5)	40 (27.6)	144 (49.7)	146 (50.3)	0.11	1.25 (0.9-1.6)
	Controls (351)	62 (17.7)	186 (53.0)	103 (29.3)	310 (44.2)	392 (55.8)		
North American	Cases (120)	31 (25.8)	55 (45.8)	34 (28.3)	117 (48.8)	123 (51.3)	0.36	1.19 (0.8-1.7)
	Controls (98)	21 (21.4)	45 (45.9)	32 (32.7)	87 (44.4)	109 (55.6)		