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The -2518A>G promoter polymorphism in the CCL2 gene is not associated with systemic sclerosis susceptibility or phenotype: Results from a multicenter study of European Caucasian patients

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ABSTRACT

A single nucleotide polymorphism (SNP) of the gene encoding monocyte chemoattractant protein–1 (MCP-1, CCL2) has previously been suggested to be involved in the susceptibility of systemic sclerosis (SSc). Here we have tested whether the -2518A>G CCL2 variant is associated with SSc susceptibility and/or phenotype using a cohort of SSc patients (n = 345). Clinical data from SSc patients attending rheumatology clinics in the Netherlands and Germany was collected DNA was obtained after informed consent. The control group used (n = 272) was randomly recruited from comparable geographic regions. The -2518A>G SNP in CCL2 (rs1024611) was determined using a Taqman SNP Genotyping assay. The genotype distribution was found to be similarly distributed among SSc patients and healthy controls. In addition, no association could be detected between the genotype and the presence of antinuclear antibodies, anticentromere antibodies, and antitopoisomerase antibodies or pulmonary involvement. Our results demonstrate that the functional variant -2518A>G of CCL2 is not implicated in the susceptibility or phenotype of SSc.

1. Introduction

Systemic sclerosis (SSc) is a connective tissue disease of unknown etiology, which is characterized by excessive deposition of collagen in the skin and various other organs and is clinically known as fibrosis. One of the first characteristic histologic features observed in the skin from SSc patients is the presence of infiltrating macrophages, T cells, B cells and fibroblasts implying the implication the local secretion of chemokines. Chemokines comprise a family of small secreted proteins that act as chemotactic ligands through interaction with seven membrane spanning chemokine receptors.

Monocyte chemoattractant protein–1 (MCP-1, CCL2) is a multifunctional inflammatory chemokine belonging to the C-C chemokines. CCL2 is predominantly produced by monocytes but also macrophages, fibroblasts, endothelial cells and keratinocytes are also able to secrete CCL2. The roles of CCL2 have been thoroughly investigated and it has found that its biologic activities extend beyond that of chemo-attraction as it can also directly induce cell activation and fibrosis. In this light, CCL2 has been demonstrated to be upregulated in a variety of fibrotic conditions including idiopathic pulmonary fibrosis, systemic sclerosis, and bleomycine-induced experimental scleroderma [1–4]. In addition, the direct role of CCL2 in the fibrotic process has been further substantiated by the observations that CCL2 is able to directly increase type I collagen gene expression by the modulation of TGFβ expression [5], and that mice lacking CCL2 receptor (CCR2) are protected from fluorescein isothiocynate- and bleomycine-induced lung fibrosis [6]. More recently, SKL-2841, a small-molecule antagonist of CCL2 and MIP-1 was shown to significantly suppress the inflammation of inflammatory cells in bleomycin-induced scleroderma, further attesting to the pivotal role of CCL2 in fibrosis [7].

In SSc patients, increased serum CCL2 levels were found to correlate with clinical symptoms such as pulmonary fibrosis [2,8,9]. More recently, CCL2 protein levels were suggested as a useful tool for risk stratification in early-stage disease [10]. Simi-

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larly, supernatants collected from peripheral blood mononuclear cells (PBMCs) from SSc patients had significantly higher spontaneous production of CCL2 and the protein was highly expressed in SSc skin and isolated skin fibroblasts [2,11,12]. Interestingly, and in contrast to mice, a recent report from Distler *et al.* suggested a role for IL-4 in the pro-fibrotic effect of CCL2; however no direct effect on fibroblast behaviour could be observed [13]. Various single nucleotide polymorphisms (SNPs) have been described in the CCL2 gene located on chromosome 17q11.2–q12. One of these SNPs in the promoter of the gene changing an A to G at position -2518 (-2518A>G, rs1024611) has been functionally related to CCL2 levels. So far, this SNP has been associated with CCL2 expression in chronic hepatitis C infection [14], pulmonary tuberculosis [15], reduced insulin sensitivity in nondiabetic individuals, and systemic lupus erythematosus [16]. So far, three studies have studied the potential role of the CCL2 gene in SSc leading to much controversy. Karerr *et al.* were the first to describe a positive association between CCl2 functional variant and SSc susceptibility using an exceptional small number ($n = 18$) of SSc patients [17]. After this study, two recent reports showed an absence of association between SSc susceptibility using small cohorts of UK and Slovak patients ($n = 94$ and 48, respectively) [18,19].

Because numerous reports suggest for a role for CCL2 in SSc, we investigated whether the CCL2 SNP is associated with susceptibility to and/or the phenotype of SSc. In contrast to previous findings on the protein level, we could not prove an association between the -2518A>G CCL2 variant and SSc or disease phenotype using a large cohort of patients with well-documented SSc.

2. Subjects and methods

2.1. Study subjects

Patients at European League Against Rheumatism Scleroderma Trials and Research Group centers (Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands; St. Maartens Kliniek Nijmegen, Nijmegen, the Netherlands; and Charité—University Medicine Berlin, Berlin, Germany) were included in this study ($n = 345$). The control group ($n = 272$) was randomly recruited from a comparable population. SSc was classified as either a limited subtype or a diffuse subtype according to the extent of the skin involved, as proposed by Leroy *et al.* [20]. The following clinical data were collected for ascertainment of clinical phenotype: age, gender, disease duration, and presence of autoantibodies (antitopoisomerase and anticentromere). Involvement of the lungs was assessed according to international guidelines [21]. Pulmonary fibrosis was investigated by a computed tomographic scan. Restrictive syndrome and diffusion capacity of the lungs was defined as a forced vital capacity <75% of the predicted value and a diffusion capacity for carbon monoxide of <75% of predicted (based on age, gender, height, and ethnic origin). The control population consisted of unrelated healthy individuals recruited in the same geographic region as SSc patients and matched by sex and ethnicity with the SSc patients groups. From these individuals, DNA was extracted from blood using salt extraction [22]. All patients gave written informed consent, and the ethics committee of each hospital approved the study.

2.2. Polymerase chain reaction

The -2518A>G SNP in CCL2 (rs1024611) was determined using a Taqman SNP Genotyping assay (assay ID, C_2590362_10, Applied Biosystems, Nieuwekerk aan de Ijssel, the Netherlands). Reactions were performed according to the manufacturer's protocol using 10 ng of DNA. Genotypes were generated automatically using the 7500 Fast Real-Time PCR Instrument (Applied Biosystems, Nieuwekerk aan de Ijssel, the Netherlands).

2.3. Statistical analysis

Test for deviation from Hardy Weinberg equilibrium was performed by means of Chi-square tests in controls only. A χ^2 test was performed using SPSS, version 14.0 statistical package (SPSS Inc., Chicago, IL) to test for association of the genotype with SSc by comparing the genotype frequencies of cases and controls. To test for association with a specific disease phenotype, χ^2 tests were performed to compare the frequencies of the genotypes between patients with a limited and diffuse phenotypes, and tests were conducted as to whether lung fibroses was associated with a specific genotype.

3. Results

In the current study we genotyped 345 SSc patients and 272 healthy controls for the -2518 A>G CCL2 variant. Table 1 summarizes characteristics of the SSc patients and healthy controls. The mean age (±SD) of the SSc patients was 56 ± 13 years, the percentage of female individuals was 81.7%, the percentage of patients having limited phenotype of SSc was 62.6%, and the presence of anti nuclear antibodies was 94.5%, which is in line with previous genetic studies in European populations [23,24]. The genotype and allele distribution among the SSc samples from the Netherlands and Germany was similar, and no deviation from Hardy-Weinberg equilibrium was observed in the healthy control group (data not shown).

The genotype and allele distribution was found to be similarly distributed among SSc patients and healthy controls and also within clinical phenotypes between German and Dutch patients (Table 2). Moreover, the -2518 GG genotype that was previously suggested to be associated with SSc susceptibility and increased CCL2 levels in fibroblasts was represented equally among patients having diffuse or limited disease, between patients with or without computed tomography–proven lung fibrosis, or patients with or without decreased lung capacity. Also no association could be detected between the genotype and the presence of antinuclear antibodies, anticentromere antibodies, and antitopoisomerase antibodies.

4. Discussion

Systemic sclerosis is characterized by the involvement of inflammatory cells implying the role of chemokines. Currently, accumulating evidence suggests a role for CCL2 proteins in SSc. A recent report by Carulli *et al.* demonstrated that CCL2 protein levels were consistently and significantly elevated in the circulation of SSc patients [10]. Moreover, high CCL2 levels tended to be associated with internal organ involvement in early SSc, particularly pulmonary hypertension and cardiac involvement. In addition, Antonelli *et al.* also observed increased levels of CCL2 protein in the serum of SSc patients early after its diagnosis [9]. Interestingly, here it was found that CCL2 levels were quite stable throughout disease

Table 1
Demographic variables of patients and healthy controls

Characteristic	Healthy controls	SSc patients
N	272	345
Age, years, mean (SD)	42 (12)	56 (13)
Female (%)	43.8%	81.7%
Limited phenotype (%)		62.6%
Disease duration, months, mean (SD)		112 (92)
Positivity ANA antibodies	—	94.5%
Positivity anticentromere antibodies	—	34.8%
Positivity antitopoisomerase antibodies	—	22.3%
Pulmonary fibrosis on CT scan	—	34.8%
Low FVC (<75% predicted)	—	20.9%
Low DLCO (<75% predicted)	—	61.7%

ANA, anti-nuclear antibodies; DLCO, diffuse capacity of the lung for carbon monoxide; FVC, forced vital capacity; SSc, systemic sclerosis.