Matrix metalloproteinase gene polymorphisms and susceptibility to systemic sclerosis

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ABSTRACT. The major pathological hallmark of the systemic sclerosis (SSc) is skin and internal organ fibrosis, which results from normal tissue architecture alterations and extracellular matrix (ECM) protein deposition. ECM components are degraded by matrix metalloproteinases (MMP). Promoter region polymorphisms in MMP genes may influence gene expression, resulting in an imbalance between ECM protein production and degradation. Here, we analyzed *MMP1 -1607 1G/2G* (rs1799750), *MMP3 -1171 5A/6A* (rs3025058), and *MMP9 -1562 C/T*

(rs3918242) polymorphisms in relation to susceptibility to SSc and its clinical features. The patient group included 98 individuals with longstanding or recently diagnosed disease, meeting the American College of Rheumatology or LeRoy and Medsger criteria for SSc; the control group included 100 healthy blood donors. All participants were of European descent. Genotyping was performed by polymerase chain reaction followed by restriction digestion. Genotype and allele frequencies of MMP polymorphisms were similar between the two groups. In secondary analyses, significantly higher frequency of 1G/2G genotype from *MMP1* polymorphism was observed for patients testing positive for antinuclear autoantibodies (P = 0.007), while 1G/1G genotype was associated with interstitial lung disease development (P = 0.018). The 6A/6A genotype from *MMP3* polymorphism was absent in patients with calcinosis (P = 0.011), while the *MMP3* 5A/5A genotype correlated with the presence of anti-topoisomerase I antibodies (P = 0.009) and reduced diffusing capacity for carbon monoxide (P = 0.024). These results suggest that MMP polymorphisms are not associated with SSc susceptibility, although *MMP1* and *MMP3* variants are associated with specific SSc clinical and laboratory features.

Key words: Systemic sclerosis; Matrix metalloproteinase; MMP1; MMP3; MMP9; Genetic polymorphisms

INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune connective tissue disease, characterized by microvascular dysfunction, immunological alterations with autoantibody production, and skin and internal organ fibrosis (Varga and Abraham, 2007). SSc pathogenesis is multifactorial and not completely understood, but the disease can be triggered in a genetically susceptible individual by exposure to some environmental factors (Katsumoto et al., 2011; Luo et al., 2013). A marked feature of SSc is the autoantibody production and, although its actual role in the disease pathogenesis is not clear, it is known to be linked to the clinical features of SSc (Hamaguchi, 2010; Graf et al., 2012).

The major pathological hallmark of SSc is fibrosis (Katsumoto et al., 2011). The fibrotic process is characterized by the deposition of extracellular matrix (ECM) proteins on normal tissue, which alters the tissue architecture and results in a loss of tissue functionality and organ failure (Bhattacharyya et al., 2011; Katsumoto et al., 2011). Matrix metalloproteinases (MMP) are enzymes that are able to degrade ECM components (Peng et al., 2012). These are involved in normal physiological processes, as well as in pathological processes such as cell proliferation, apoptosis, inflammation, aging, cancer, and arthritis (Johnson et al., 2001; Peng et al., 2012). Polymorphisms in the promoter region of MMP genes may influence gene expression and, consequently, result in an imbalance between production and degradation of ECM proteins (Peng et al., 2012). Of the polymorphisms described for MMP genes, an insertion/deletion of a guanine at *MMP1* in the position -1607 results in one allele with a single guanine (1G) and another with two guanines (2G) (Johnson et al., 2001). The 2G allele demonstrates a two- to ten-fold increase in the expression of MMP-1 (Johnson et al., 2001).

Moreover, in the -1171 position of the *MMP3* promoter region, an insertion of one adenosine gives rise to alleles having either six (6A) or five adenosines (5A); it has been previously reported that individuals with the 6A/6A genotype may have decreased MMP-3 expression (Chaudhary et al., 2010). Finally, *MMP9* harbors a C-to-T transition at the -1562 position; the T allele has been associated with higher transcriptional activity compared to the C allele (Skarmoutsou et al., 2011).

MMP genetic polymorphisms and levels of gene expression have been associated with several diseases, including chronic obstructive pulmonary disease, coronary heart disease, rheumatoid arthritis, and systemic lupus erythematosus (Schirmer et al., 2009; Scherer et al., 2010; Bahrehmand et al., 2015; Jiang et al., 2016; Ma et al., 2015, 2016). Different studies have addressed the MMP serum levels and MMP gene expression in SSc (Young-Min et al., 2001; Kikuchi et al., 2002; Toubi et al., 2002; Meng et al., 2008; Brown et al., 2012; Frost et al., 2012). However, studies on the influence of genetic polymorphisms in *MMP1*, *MMP3,* and *MMP9* on SSc susceptibility are few (Johnson et al., 2001; Marasini et al., 2001; Skarmoutsou et al., 2011).

In the present study, we analyzed the frequency of *MMP1 -1607 1G/2G* (rs1799750), *MMP3 -1171 5A/6A* (rs3025058), and *MMP9 -1562 C/T* (rs3918242) polymorphisms, as well as a potential association between these polymorphic variants and clinical features of the disease, in SSc patients from Southern Brazil.

MATERIAL AND METHODS

Patients and controls

The SSc patient group was recruited from Hospital de Clínicas de Porto Alegre (HCPA, Porto Alegre, RS, Brazil) and was composed of individuals with longstanding or recently diagnosed disease, meeting the American College of Rheumatology criteria for SSc or the LeRoy and Medsger criteria for early forms of disease (LeRoy and Medsger, 2001). Patients with overlapping syndromes were excluded, except those with definite SSc diagnosis and secondary inflammatory myopathy or Sjögren's syndrome. The control group consisted of healthy blood donors from the urban population of Porto Alegre. Individuals with acute or chronic diseases, as well as a family history of genetic diseases, were excluded from this study. All patients and controls were ethnically classified as of European descent (i.e., Caucasians). This study was approved by the Ethics Committee of HCPA and Universidade Luterana do Brasil. All participants provided written informed consent.

Clinical evaluation

The procedures used for diagnosis, identification and classification of the clinical features were performed as described previously by Bredemeier et al. (2004). Pulmonary high-resolution computed tomography (HRCT) was performed during breath holding after deep inspiration in the supine position, without using an intravenous contrast agent. All HRCT scans were assessed for the presence of interstitial lung disease (ILD) (ground-glass opacities, reticular pattern, and honeycombing) by two radiologists. Spirometry and diffusing capacity for carbon monoxide (DLCO) test were also performed for most patients. Forced vital capacity (FVC) and DLCO were considered to have reduced when presenting <80 and <75% of the predicted values, respectively. Doppler echocardiography was used to estimate the pulmonary systolic arterial pressure; those with values of ≥40 mmHg were considered to have pulmonary hypertension (PH).

Molecular analysis

Peripheral blood samples were collected, and genomic DNA was extracted from them using a salting-out method (Lahiri and Nurnberger, 1991). MMP polymorphisms were evaluated by polymerase chain reaction (PCR), followed by restriction fragment length polymorphism (RFLP) analysis. Primers and conditions for the analysis of *MMP1 -1607 1G/2G* (rs1799750), *MMP3 -1171 5A/6A* (rs3025058), and *MMP9 -1562 C/T* (rs3918242) polymorphisms have previously been described by Dunleavey et al. (2000a,b) and Morgan et al. (2003), respectively. The resulting amplified 119 and 111-bp PCR products of *MMP1 -1607 1G/2G* (rs1799750) and *MMP3 -1171 5A/6A* (rs3025058) analyses, respectively, were digested using *Xmn*I restriction enzyme (New England Biolabs, Ipswich, MA, USA). The amplified 436-bp fragment products of *MMP9 -1562 C/T* (rs3918242) analysis were digested using *Sph*I enzyme (New England Biolabs). Genotypes from all polymorphisms were determined by visualization on 10% polyacrylamide gel stained with silver nitrate. Positive and negative control samples were included in all analyses.

Statistical analysis

Allele and genotype frequencies were estimated by direct counting. The chi-square test was performed to compare the observed genotype frequencies with the assumptions for the Hardy-Weinberg equilibrium. The chi-square or Fisher exact test were performed to compare allele and genotype frequencies between patients and controls, and the clinical variables between genotypes. Residual analysis was used to identify the categories responsible for a significant chi-square test. All tests were two-tailed and the results were considered statistically significant when the P value was less than 0.05. Data was analyzed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA) and WinPepi version 11.43 (Abramson, 2011).

RESULTS

We evaluated 98 SSc patients and 100 healthy blood donors with a mean age of 49.7 ± 14.9 and 44.1 ± 7.3 years, respectively. Limited cutaneous SSc was the most prevalent disease subtype (59.2%). Calcinosis and telangiectasia affected 23.5 and 68.4% of the patients, respectively. Anti-nuclear antibodies (ANA) were present in 85.7% of the patients, with 39.8%, positive for anti-centromere antibodies, and 22.4%, positive for anti-topoisomerase I antibodies. Reduced FVC and DLCO were observed in 36.7 and 85.4% of the patients, respectively. ILD affected 66.7% of the patients, and 14.6% of the patients presented PH (Table 1).

Data are represented as numbers (percentages in parentheses), except as otherwise indicated. *Data not available for all patients. dcSSc: diffuse cutaneous SSc; lcSSc: limited cutaneous SSc; ssSSc: sine-scleroderma SSc; ANA: anti-nuclear antibodies; FVC: forced vital capacity; DLCO: carbon monoxide diffusing capacity; HRCT: high resolution computed tomography.

The genotype frequencies of polymorphisms were in accordance with the expected frequencies for Hardy-Weinberg equilibrium in all groups. Allele and genotype frequencies of MMP polymorphisms were similar between patients and controls (Table 2).

Table 1. Demographic, clinical, and laboratory data of SSc patients.

Characteristics	SSc patients
Male/female	11 (11.2)/87 (88.8)
Age in years (mean ± SD)	49.7 ± 14.9
SSc subtypes	
dcSSc	25 (25.5)
lcSSc	58 (59.2)
ssSSc	15 (15.3)
Duration of disease in years, median (25-75% quartiles)	9 (3.5-20.0)
Raynaud's phenomenon	98 (100.0)
Total skin score, median (25-75% quartiles)	5.5 (2-14.2)
Calcinosis	23 (23.5)
Telangiectasia	67 (68.4)
ANA ≥1:80	84 (85.7)
Anti-centromere antibodies	39 (39.8)
Anti-topoisomerase I antibodies	22 (22.4)
Reduced FVC (N = 90)*	33 (36.7)
Reduced DLCO (N = 89)*	76 (85.4)
Interstitial lung disease on HRCT (N = 93)*	62 (66.7)
Pulmonary hypertension (N = 89)*	13 (14.6)

Table 2. Comparison of allele and genotype frequencies of *MMP1 -1607 1G/2G*, *MMP3 -1171 5A/6A*, and *MMP9 -1562 C/T* polymorphisms between SSc patients and healthy controls.

Polymorphism	SSc patients N (%)	Controls N (%)	P
MMP1 -1607 1G/2G	98	100	
Alleles:			
1G	77 (39.3)	86 (43.0)	0.48
2G	119 (60.7)	114 (57.0)	
Genotypes:			
1G/1G	14 (14.3)	19 (19.0)	0.68
1G/2G	49 (50.0)	48 (48.0)	
2G/2G	35 (35.7)	33 (33.0)	
MMP3 -1171 5A/6A	90	100	
Alleles:			
5A	92 (51.1)	90 (45.0)	0.26
6A	88 (48.9)	110 (55.0)	
Genotypes:			
5A/5A	24 (26.7)	24 (24.0)	0.36
5A/6A	44 (48.9)	42 (42.0)	
6A/6A	22 (24.4)	34 (34.0)	
MMP9 -1562 C/T	92	100	
Alleles:			
C	169 (91.8)	184 (92.0)	0.99
T	15 (8.2)	16 (8.0)	
Genotypes:			
CC	78 (84.8)	84 (84.0)	0.69
CT	13 (14.1)	16 (16.0)	
TT	1 (1.1)	-	

Analyses of the clinical characteristics of patients, according to genotypes of *MMP1*, *MMP3*, and *MMP9* polymorphisms are presented in Table 3. Significant differences were observed concerning the genotype frequencies of *MMP1*, and the presence of ANA (P = 0.007) and ILD (P = 0.018). Among patients positive for the ANA test, an increased frequency of 1G/2G genotype was observed, while the frequency of 2G/2G genotype was significantly decreased (95.9 and 71.4%, respectively). Furthermore, all patients with 1G/1G genotype presented ILD.

Table 3. Comparison of clinical and laboratory data of SSc patients stratified by genotypes of *MMP1 -1607 1G/2G*, *MMP3 -1171 5A/6A*, and *MMP9 -1562 C/T* polymorphisms.

Characteristic	*MMP1*			P	*MMP3*			P	*MMP9*		P
	1G/1G (N = 14)	1G/2G (N = 49)	2G/2G (N = 35)		5A/5A (N = 24)	5A/6A (N = 44)	6A/6A (N = 22)		CC (N = 78)	CT+TT (N = 14)	
Diffuse form	4 (28.6)	13 (26.5)	8 (22.9)	NS	7 (29.2)	11 (25.0)	4 (18.2)	NS	20 (25.6)	2 (14.2)	NS
Skin score ≥10	5 (35.7)	20 (40.8)	10 (28.6)	NS	9 (37.5)	14 (31.8)	7 (31.8)	NS	27 (34.6)	4 (28.6)	NS
Calcinosis	4 (28.6)	15 (30.6)	4 (11.4)	NS	8 (33.3)	13 (29.5)	0 (0.0)[a]	**0.011**	21 (26.9)	1 (7.1)	NS
Telangiectasia	10 (71.4)	34 (69.4)	23 (65.7)	NS	19 (79.2)	29 (65.9)	14 (63.6)	NS	54 (69.2)	10 (71.4)	NS
ANA ≥1:80	12 (85.7)	47 (95.9)[a]	25 (71.4)[a]	**0.007**	20 (83.3)	40 (90.9)	18 (81.8)	NS	68 (87.2)	12 (85.7)	NS
ACA	5 (35.7)	22 (44.9)	12 (34.3)	NS	8 (33.3)	19 (43.2)	9 (40.9)	NS	33 (42.3)	4 (28.6)	NS
Anti-topoisomerase I	3 (21.4)	10 (20.4)	9 (25.7)	NS	11 (45.8)[a]	6 (13.6)[b]	4 (18.2)	**0.009**	18 (23.1)	3 (21.4)	NS
Reduced FVC*	7/14 (50.0)	14/45 (31.1)	12/31 (38.7)	NS	10/21 (47.6)	13/43 (30.2)	8/19 (42.1)	NS	27/72 (37.5)	4/13 (30.8)	NS
Reduced DLCO*	12/14 (85.7)	37/44 (84.1)	27/31 (87.1)	NS	21/21 (100)[b]	35/42 (83.3)	13/19 (68.4)[b]	**0.024**	60/71 (84.5)	11/13 (84.6)	NS
ILD on HRCT*	13/13 (100)[b]	28/48 (58.3)	21/32 (65.6)	**0.018**	18/22 (81.8)	29/44 (65.9)	12/20 (60.0)	NS	51/75 (68.0)	9/13 (69.2)	NS
Pulmonary hypertension	1/13 (7.7)	6/43 (14.0)	6/33 (18.2)	NS	3/20 (15.0)	8/41 (19.5)	2/22 (9.1)	NS	10/73 (13.7)	3/12 (25.0)	NS

Data are reported as numbers (percentages in parentheses), except as otherwise indicated. *Data not available for all patients; values represent number of patients with the indicated abnormalities over the number of patients that had the respective complementary examination, with percentages given in parentheses. ANA: anti-nuclear antibodies; ACA: anti-centromere antibodies; FVC: forced vital capacity; DLCO: carbon monoxide diffusing capacity; ILD: interstitial lung disease; HRCT: high resolution computed tomography. [a]Adjusted residual $P < 0.01$. [b]Adjusted residual $P < 0.05$.

Genotypes from the *MMP3 -1171 5A/6A* polymorphism were also significantly associated with specific clinical features. None of the SSc patients who developed calcinosis presented the 6A/6A genotype (P = 0.011). Anti-topoisomerase-I antibodies were present in 45.8 and 13.6% of the patients with 5A/5A and 5A/6A genotypes, respectively (P = 0.009). Moreover, significant differences in genotype frequencies were observed in patients with reduced DLCO (P = 0.024). All patients with the 5A/5A genotype had reduced DLCO, while the frequency of this condition was significantly decreased (68.4%) in patients with 6A/6A genotype. Finally, the variants of *MMP9 -1562 C/T* polymorphism were not associated with the clinical features of the patients.

DISCUSSION

ECM components include elastic fibers, collagen fibers, glycoproteins (fibronectin and laminin), and mucopolysaccharides. A physiological balance between the synthesis, deposit in the extracellular environment, and degradation of these molecules is required (Robert et al., 2016). MMP enzymes degrade ECM components, promoting this required balance (Johnson et al., 2001; Peng et al., 2012). Disruption of this balance results in the fibrotic process, a pathological hallmark of SSc, which causes loss of tissue functionality and the failure of affected organs (Bhattacharyya et al., 2011; Katsumoto et al., 2011; Robert et al., 2016). In this study, we investigated the role of *MMP1 -1607 1G/2G*, *MMP3 -1171 5A/6A*, and *MMP9 -1562 C/T* polymorphisms in SSc susceptibility and the influence of polymorphic variants on the clinical features of the disease. The results showed no association between MMP polymorphisms and SSc susceptibility. However, genetic variants of *MMP1 -1607 1G/2G* and *MMP3 -1171 5A/6A* polymorphisms significantly influenced the clinical and laboratory features of the disease.

In accordance with data from a previous study performed by Johnson et al. (2001), similar frequencies for *MMP1 -1607 1G/2G* polymorphism were observed between patients and controls. Nevertheless, in the present study, ANA were more prevalent among patients with the 1G/2G genotype than among patients with the 2G/2G genotype. Furthermore, the 5A/5A genotype from *MMP3 -1171 5A/6A* polymorphism was linked to an increased risk for the presence of anti-topoisomerase I. It has been reported that genetic factors are associated with specific ANA, suggesting that the genetic background may contribute to the autoantibody profile (Hamaguchi, 2010).

A significant association was observed between the 1G/1G genotype of *MMP1* and ILD development. ILD is characterized by alveolar epithelial cell damage, activation of fibroblasts, and excessive accumulation of ECM proteins in the lung parenchyma, which ultimately results in pulmonary fibrosis (Rosas et al., 2008; Murray et al., 2012). It has been reported that the 1G allele is associated with low transcriptional activity of *MMP1* (Johnson et al., 2001), and in this context, it may result in the accumulation of ECM proteins, contributing to ILD development. Pulmonary fibrosis in SSc is associated with substantial morbidity and mortality in SSc (Rosas et al., 2008).

Our data from the analysis of *MMP3 -1171 5A/6A* polymorphism disagree with that from a previous study by Marasini et al. (2001) which suggested that the 6A allele is involved in SSc susceptibility. However, in our study, none of the SSc patients that developed calcinosis presented the 6A/6A genotype. Calcinosis is characterized by formation of calcium deposits in skin and subcutaneous tissues, and it is frequently observed in connective tissues diseases. Factors that trigger the development of calcinosis are not completely understood. They may

include damage to the ECM proteins, alterations in the mechanisms responsible for inhibition of the calcification process, and abnormalities in the mitochondrial calcium and phosphate levels (Gutierrez and Wetter, 2012). It has been previously reported that the 6A allele has two-folds lower promoter activity than the 5A allele (Chaudhary et al., 2010). This lower transcriptional activity may be responsible for protecting against calcinosis development in SSc patients with the 6A/6A genotype. Of note and contrary to the individuals who were at a higher risk of reduced DLCO due to their 5A/5A genotype, those bearing the 6A/6A genotype were at a lower risk of reduced DLCO. In SSc patients, reduced DLCO generally indicates the presence of ILD and PH (Murray et al., 2012). Therefore, the 6A/6A genotype can be suggested as a genetic protection factor in the development of clinical complications from SSc, such as calcinosis and reduced DLCO.

Some studies also detailed serum or gene expression levels of *MMP1*, *MMP3*, and *MMP9* in SSc. For example, an increased serum level of MMP1, but not of MMP3, was observed among SSc patients; although, MMP1 serum levels were not attributed to SSc (Toubi et al., 2002). In another study, serum levels of tissue inhibitors of metalloproteinases (TIMP) 1 were significantly increased in SSc patients, especially those with the diffuse form in early disease (Young-Min et al., 2001). TIMP are important regulatory enzymes that are involved in MMP degradation (Murphy, 2011). A study performed with South African SSc patients reported a significant decrease in *MMP1* expression and an increase in *TIMP-1* expression (Frost et al., 2012). Concerning MMP9, it has been previously demonstrated that expression levels are lower for MMP9 and higher for TIMP-1 in skin lesions of patients with diffuse SSc (dcSSc), as compared to those with normal skin (Meng et al., 2008). Furthermore, a study reported significantly decreased MMP9 activity in serum of dcSSc patients compared with that of healthy controls (Kikuchi et al., 2002). Despite such data, and in accordance with previous data from molecular analysis, our results suggest that there is no association between *MMP9 -1562 C/T* polymorphism and SSc susceptibility or clinical features of the disease.

Genetic polymorphism, gene transcription, posttranslational modification, and inhibition of TIMP are all possible regulatory mechanisms of MMP (Peng et al., 2012). Thereby, the accumulation of ECM components, which results in tissue fibrosis, is not only related to polymorphisms or altered MMP gene expression, but may also be due to increase in TIMP enzyme activity, which blocks the action of MMP and inhibits matrix degradation (Murphy, 2011; Peng et al., 2012).

Our study has some limitations. Our sample was composed of prevalent cases of SSc and, therefore, more severe and aggressive cases may not have been adequately represented. Another limitation of the present study is the relatively small sample size, and this could be explained, in part, by the exclusion of individuals of African descent or of a mixed ethnic background. The major strengths of our study are the prospective data collection and the thorough clinical and laboratory evaluation of almost all patients.

To our knowledge, this is the first study that evaluated the association of polymorphisms in MMP genes in Brazilian SSc patients. In conclusion, this study suggests that there is no association between MMP polymorphisms and SSc susceptibility; although, genotypes from *MMP1 -1607 1G/2G* and *MMP3 -1171 5A/6A* polymorphisms were associated with specific clinical features of the disease, such as the presence of ANA, calcinosis, and ILD.

Conflicts of interest

The authors declare no conflict of interest.

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