Analysis of matrix metalloproteinase-9 gene polymorphism -1562 C/T in patients suffering from systemic sclerosis with and without ulcers

EVANGELIA SKARMOUTSOU[1], FABIO D'AMICO[1], MAURIZIO MARCHINI[2], FRANCA STIVALA[1], GRAZIA MALAPONTE[1], RAFFAELLA SCORZA[2] and MARIA CLORINDA MAZZARINO[1]

[1]Department of Biomedical Sciences, University of Catania, via Androne 83, I-95124 Catania; [2]Referral Center for Systemic Autoimmune Diseases, University of Milan and Fondazione IRCCS Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, via Pace 9, I-20122 Milan, Italy

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Abstract. The objective of this study was to determine whether the matrix metalloproteinase-9 (MMP-9) rs3918242 single nucleotide polymorphism may confer susceptibility to systemic sclerosis (SSc) with and without ulcers in an Italian Caucasian population. The MMP-9 rs3918242 functional polymorphism was genotyped in 461 subjects of Italian Caucasian origin: 228 patients with SSc (92 with and 136 without ulcers) and 233 unrelated healthy individuals. The SNP under study was in Hardy-Weinberg equilibrium in the control population. Genotype and allele distributions between SSc patients, with or without ulcers, were not statistically significant (P>0.05). A significant increase of the genotype C/T was observed in male SSc patients without ulcers when compared to patients with ulcers (P=0.04). The MMP-9 rs3918242 functional polymorphism is not associated with susceptibility to SSc. However, the presence of the polymorphism may have a protective effect on the development of ulcers in SSc male patients.

Introduction

Systemic sclerosis (SSc) is a multifactorial disease characterized by endothelial damage, increased proliferation and functional activity of fibroblasts and immune dysregulation (1-3). It is accepted that multiple genetic factors play a key role in conditioning an individual's susceptibility to the disease; they also regulate clinical features and disease evolution as well as the response to therapy (2,4).

Matrix metalloproteinases (MMPs) are endopeptidases that degrade various components of the extracellular matrix (ECM) and basement membranes (5), participating in physiological and pathological processes, such as angiogenesis, wound healing, cancer and arthritis. MMP genes may represent good candidates in diseases whose pathogenesis involves ECM degradation and remodelling (6), such as SSc. In particular, MMP-9, also known as gelatinase B (92 kDa), has been associated with chronic inflammatory autoimmune diseases and various diseases characterized by excessive fibrosis (7).

Recently Wipff *et al* (8) investigated the possible association of six MMP-2, MMP-9 and MMP-14 polymorphisms with the susceptibility to SSc in a French white population. These authors did not find any association between the investigated polymorphisms and SSc susceptibility nor with the major SSc clinical subsets. In their study, they did not include the functional polymorphism rs3918242. This polymorphism of the promoter region of MMP-9 has been characterized functionally: its C/T substitution up-regulates the promoter activity, with a subsequent increase in gene expression (9,10) and serum levels (11).

Patients affected with SSc are classified according to the extent of skin pathological manifestations, ranging from limited to diffuse cutaneous SSc (12), and each year, 30% of patients develop ischemic digital ulcers, necrotic lesions located at the finger distal tips (13,14). Ischemia, thickening of the vessel intima, sclerodactyly and calcinosis are the major factors involved in the pathogenesis of ulcers (15). Digit ulcers are associated with pain and other complications, such as superinfection. Digit ulcers, together with other clinical manifestations of systemic sclerosis, such as the Raynaud's phenomenon, renal crisis and pulmonary arterial hypertension, have a profound impact on the morbidity and mortality of SSc patients. Although the mechanisms involved in vascular diseases of SSc patients are still unknown, several factors, such as endothelial cell injury, immune-mediated cytotoxicity, anti-endothelial antibodies and ischemia-reperfusion, have been implicated (16). Moreover, recently some of us have shown that genetic factors may alter vascular reactivity and structure (17). In particular, MMP-9 has been shown to be involved in vascular damage of idiopathic aneurysms and pulmonary arterial hypertension (18,19). Therefore, we

Correspondence to: Dr Evangelia Skarmoutsou, Department of Biomedical Sciences, University of Catania, via Androne 83, I-95124 Catania, Italy
E-mail: eskarmoutsou@gmail.com

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decided to conduct a case control study to investigate the possible role of the rs3918242 polymorphism in the susceptibility to the SSc with ulcers.

Materials and methods

Subjects. The study was designed as a case-control study. We studied 461 unrelated subjects, 228 patients with SSc (82 females and 10 males with ulcers; 124 females and 12 males without ulcers) and 233 healthy individuals. All patients were of Italian Caucasian origin and were recruited from the Department of Internal Medicine (University of Milan, Italy) and fullfilled the classification criteria of the American College of Rheumatology (20). Patients were classified according to LeRoy *et al* (21). All subjects gave informed consent for the study. The study was approved by the local ethics committee.

DNA extraction. Ten millilitres of venous blood was collected into tubes containing sodium citrate. Genomic DNA was extracted with a commercial DNA isolation kit (Nuclear Laser Medicine, Italy), with a salting out method.

Genotyping of the MMP-9 polymorphism. A total of 125 ng of genomic DNA were used as the template for amplification. The -1562 C/T polymorphism was evaluated on an amplification fragment generated using the primers 5'-GGC ACATAGTAGGCCCTTTAA-3' (forward) and 5'-TCACTC CTTTCTTCCTAGCCA-3' (reverse). PCR amplification was carried out for 1 min at 94˚C, followed by 35 cycles of 30 sec at 94˚C, 30 sec at 57˚C and 30 sec at 72˚C, and a final step at 72˚C for 1 min. PCR products were purified with the Ultrafree-DNA kit (Millipore, Bedford, MA, USA), and 15 ng of purified PCR product was sequenced with the primer. Sequencing was performed with the ABI PRISM BigDye™ Terminator kit (Applied Biosystems, USA) on the automatic sequencer 3100 Genetic Analyzer (Applied Biosystems). Sequences were assembled using the ABI PRISM DNA software 3.7 (Applied Biosystems).

Statistical analysis. The Hardy-Weinberg equilibrium was determined by a χ^2 test with one degree of freedom. Genotype and allele frequencies were examined with the χ^2 test or the Fisher's exact test. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated from 2x2 contingency tables, and the most frequent homozygous genotype or allele was the reference value. A P-value <0.05 was considered to be statistically significant.

Results

The characteristics of the study subjects are reported in Table I. The genotype distributions of MMP-9 rs3918242 SNP did not show deviations from the Hardy-Weinberg equilibrium either in the patients or healthy controls (P>0.05). As shown in Table II, genotype and allele frequencies for the polymorphism under study did not differ significantly between patients affected with SSc and control healthy subjects.

Moreover, comparisons between each disease subset and control subjects did not show significant differences. For the comparisons between the C/T and the C/C genotypes the P-values were 0.12 for subjects with limited cutaneous SSc vs. those with diffuse diseases; 0.32 for subjects with limited disease vs. controls; and 0.19 for subjects with diffuse disease vs. control subjects. For comparisons between the T/T and C/C genotypes, the P-values were 0.75 for subjects with limited vs. diffuse diseases; 0.44 for limited disease vs. control subjects; and 0.88 for diffuse disease vs. control subjects. Furthermore, for comparisons between the T and C alleles, the P-values were 0.19 for limited vs. diffuse diseases; 0.43 for limited disease vs. control subjects; and 0.23 for diffuse disease vs. control subjects.

Table I. Baseline demographic and clinical characteristics.

Variable	Value
Age, years	63.2±13.3
Disease duration, years	15.8±10.9
Observational time, months	48.9±10.9
Males, n(%)	22 (9.6)
dcSSc, n (%)	34 (14.9)
Raynaud's phenomenon, n (%)	228 (100)
Ulcers, n (%)	92 (40.3)
Esophageal involvement, n (%)	102 (44.7)
Lung fibrosis, n (%)	25 (10.9)
Pulmonary hypertension, n (%)	16 (7.0)
Scl70 antibody	104 (45.6)
ACA antibody	78 (34.2)

dcSSc, diffuse cutaneous; Scl70, anti-topoisomerase; ACA, anti-centromere antibody.

In addition, the comparison of the genotype and allele frequencies of the polymorphism between control subjects and SSc patients subgrouped by the presence or absence of ulcers, did not show any significant differences: genotype (C/C, C/T and T/T) distribution was 78.26, 19.56 and 2.18% in SSc patients with ulcers, 75.00, 25.00 and 0.00% in SSc patients without ulcers, 75.97, 23.60 and 0.43% in controls, respectively. The allele distribution was 88.04 (allele C) and 11.96% (allele T) in SSc patients with ulcers, 87.50 (allele C) and 12.50% (allele T) in patients without ulcers, and 87.77 (allele C) and 12.23% (allele T) in controls. Comparisons between the different groups resulted in a P-value >0.05. In particular, for comparisons between the C/T and C/C genotypes, the P-values were 0.24 for patients with vs. patients without ulcers; 0.29 for patients with ulcers vs. control subjects; and 0.44 for patients without ulcers vs. control subjects. For comparisons between the T/T and C/C genotypes, the P-values were 0.17 for patients with vs. patients without ulcers; 0.21 for patients with ulcer vs. the control subjects; and 0.63 for patients without ulcers vs. the controls. For T and C allele comparisons, P=0.49 for patients with vs. patients without ulcers, P=0.52 for patients with ulcers vs. control subjects, and P=0.50 in patients without ulcers vs. control subjects.

Table III reports genotype and allele frequencies in controls and SSc patients subgrouped by gender. The genotype

Table II. Genotype and allele frequencies of MMP-9 -1562 C/T polymorphism (SNP rs3918242) in control subjects and patients with systemic sclerosis subgrouped by the clinical variants, limited and diffuse.

	Patients with systemic sclerosis			Control subjects	Odds ratio (95% CI)
	Limited	Diffuse	Total		P-value[a]
Genotype, n	194	34	228	233	
C/C, n (%)	151 (77.84)	23 (67.65)	174 (76.31)	177 (75.97)	
C/T, n (%)	41 (21.13)	11 (32.35)	52 (22.81)	55 (23.60)	0.96 (0.62-1.48) 0.47
T/T, n (%)	2 (1.03)	0 (0.00)	2 (0.88)	1 (0.43)	2.03 (0.18-22.64) 0.49
Allele, n	388	68	456	466	
C, n (%)	343 (88.40)	57 (83.82)	400 (87.72)	409 (87.77)	
T, n (%)	45 (11.60)	11 (16.18)	56 (12.28)	57 (12.23)	1.00 (0.68-1.49) 0.53

[a]P-value for limited and diffuse vs. control. CI, confidence interval.

frequency comparison between male SSc patients with ulcers and those without ulcers was significantly different: C/C, C/T and T/T distributions were 90.00, 0.00 and 10.00% in male SSc patients with ulcers, 58.33, 41.67 and 0.00% in male SSc patients without ulcers, respectively (P=0.04). The remaining comparisons were not statistically different.

Discussion

In this case-control study we investigated the possible association between the functional rs3918242 SNP and the susceptibility to SSc in patients with or without ulcers.

The functional single nucleotide polymorphism in the MMP gene promoter region may alter the production of proteolytic enzymes, and in turn may be associated with the development and the progression of many diseases (6). In particular, many studies have investigated the association between MMP polymorphisms and the susceptibility to SSc (8,22-24).

The rs3918242 single nucleotide polymorphism in the MMP-9 gene at position -1562 is due to a C/T substitution. Such a substitution results in the loss of binding of a nuclear protein to this region of the MMP-9 gene promoter, which leads to an increased transcriptional activity (9).

Results from our study show that the presence of the polymorphism -1562 C/T is not associated with the susceptibility to systemic sclerosis in a cohort of northern Italian patients. However, the male C/T genotype of the polymorphism under study seems to be over-represented in SSc patients without ulcers in comparison to SSc patients suffering from ulcers. Female groups comparisons did not give similar significant results. Our data may suggest that the C/T genotype may have a role in protecting the SSc male population from the development of ulcers.

It is known that a consistent female excess, about 78% of the affected population, exists for the major connective tissue autoimmune diseases (25). The causes of this gender-dependent bias remain unsolved and some hypotheses have been developed. Gender hormones and reproductive history, X-chromosome inactivation and abnormalities are the most relevant gender-related factors proposed. In particular, hyperprolactinemia, increased frequency of X-chromosome monosomy in peripheral T and B lymphocytes and skewed familial X-chromosome inactivation have been suggested as mechanisms influencing the female predominance in SSc (26-28).

Moreover, it is known that a positive correlation between MMP-9 and gender has been proposed for the susceptibility to some diseases. Male patients affected by type 1 diabetes mellitus have increased urinary MMP-9 (29). Gender difference in MMP-9 concentration was also shown in experimental aortic aneurysm (30) and rat aortic smooth muscle cells (31). Interestingly, other diseases may show gender differences in the MMP-9 -1562/TIMP-1 372 polymorphisms (32). Possible explanations for this gender specific finding may include the hormonal status, which may be able to modify the susceptibility to the disease.

Systemic sclerosis is characterized by a dysregulation of angiogenesis, which leads to failure to replace damaged vessels with a consequent clinical manifestation of ulcers (33,34). Ulcer formation involves an ECM degradation by different MMPs, which appears to be important not only for the maintenance and progression of inflammation, but also for the repair processes of ulcers (35). Interestingly, our results seem to correlate with those of Hulkkonen *et al* (36), who found that the MMP-9 -1562 C/T polymorphism, seemed to protect primary Sjogren's syndrome patients from Raynaud's phenomenon.

MMP-9 plasma levels seem to be increased by the MMP-9 -1562 C/T polymorphism (11), and SSc patients have an altered concentration of serum MMP-9 in comparison to healthy subjects (37). Kikuchi *et al* (38) have found that the activity of serum MMP-9 was decreased in patients with diffuse cutaneous SSc in comparison to patients with the limited form

Table III. Genotype and allele frequencies of MMP-9 -1562 C/T polymorphism in control subjects and patients with systemic sclerosis subgrouped by gender.

				OR (95% CI) P-value		
	SSc-U n (%)	SSc-w/o U n (%)	Control subjects n (%)	SSc-U vs. SSc-w/o U	SSc-U vs. control subjects	SSc-w/o U vs. control subjects
Males						
Genotype, n	10	12	77			
C/C	9 (90.00)	7 (58.33)	56 (72.73)	1	1	1
C/T	0 (0.00)	5 (41.67)	20 (25.97)	NA 0.04[a]	NA 0.07	2.00 (0.57-7.02) 0.22
T/T	1 (10.00)	0 (0.00)	1 (1.30)	NA 0.59	6.22 (0.36-108.63) 0.28	NA 0.89
Allele, n	20	24	154			
C	18 (90.00)	19 (79.17)	132 (85.71)	1	1	1
T	2 (10.00)	5 (20.83)	22 (14.29)	0.42 (0.07-2.46) 0.29	0.67 (0.14-3.07) 0.45	1.58 (0.53-4.67) 0.29
Females						
Genotype, n	82	124	156			
C/C	63 (76.83)	95 (76.61)	121 (77.56)	1	1	1
C/T	18 (21.95)	29 (23.39)	35 (22.44)	0.94 (0.48-1.83) 0.49	0.99 (0.52-1.88) 0.55	1.05 (0.60-1.85) 0.48
T/T	1 (1.22)	0 (0.00)	0 (0.00)	NA 0.40	NA 0.35	NA
Allele, n	164	248	312			
C	144 (87.80)	219 (88.31)	277 (88.78)	1	1	1
T	20 (12.20)	29 (11.69)	35 (11.22)	1.05 (0.57-1.92) 0.50	1.10 (0.61-1.97) 0.43	1.05 (0.62-1.77) 0.48

[a]P-value <0.05; SSc-U, SSc patients with ulcer; SSc-w/o U, SSc patients without ulcer; OR, odds ratio; CI, confidence interval; NA, not applicable. Control subjects, n=233; SSc-U, n=92; SSc-w/o U, n=136.

of SSc and normal subjects. Moreover, MMP-9 serum levels are decreased in SSc patients with pulmonary hypertension in comparison to other subsets of SSc patients (19). As indicated by some authors in other pathological conditions (39,40), our results suggest that MMP-9 may have a protective role in angiogenesis, preventing or delaying the failure of new blood vessel formation in SSc patients.

It is well established that the activity of MMPs is controlled by their specific tissue inhibitors (TIMPs). An increased activity of MMPs could be due to their overexpression or to down-regulation of TIMPs. Interestingly, some of us have previously shown that the TIMP-1 +372 T/C polymorphism may be associated with SSc in male individuals (41). Thus, we could postulate that the MMP/TIMP balance may play an important role in the onset of SSc in male individuals.

However, the major potential limitation of our study is the relatively small male sample size, which could influence the power of the statistical tests. In order to minimize bias by population stratification, we focused our attention only on northern Italian individuals. Furthermore, recruiting male patients is difficult because of the low disease incidence in the male population. Thus, further studies on larger groups of patients are needed to confirm our data.

In summary, the present study shows that the presence of the polymorphism -1562 C/T is not associated with the susceptibility to systemic sclerosis in the studied cohort of northern Italian patients. However, it seems to suggest that the C/T genotype of the MMP-9 -1562 C/T polymorphism may be associated with a smaller risk for susceptibility to developing skin ulcers only in male SSc patients.

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