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Analysis of TIMP-1 Gene Polymorphisms in Italian Sclerodermic Patients

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Systemic sclerosis (SSc) is an autoimmune disease characterized by skin and internal organs fibrosis due to an extracellular matrix (ECM) accumulation of type I collagen. The turnover of the ECM is dependent on the balance between matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs). The disruption of this balance is involved in SSc because higher serum TIMP-1 levels have been demonstrated in SSc patients than in controls. On this basis, we analyzed three polymorphisms: $-19A>G$, $+261C>T$, and $+372T>C$ of the TIMP-1 gene in SSc patients (67 females, eight males) and controls (29 females, nine males). The C allele of the $+372T>C$ single nucleotide polymorphism (SNP) was observed at a higher frequency in male patients than in healthy individuals ($P = 0.02$), while no differences were observed in the female subjects. Our findings suggest that the $+372T>C$ polymorphism of the TIMP-1 gene is associated with SSc in male individuals. No association with the clinical characteristics of SSc Italian patients and TIMP-1 gene polymorphisms was observed. Thus, the role of TIMP-1 gene in predisposition to SSc remains controversial. J. Clin. Lab. Anal. 20:173–176, 2006. © 2006 Wiley-Liss, Inc.

Key words: systemic sclerosis; tissue inhibitor of matrix metalloproteinases; single nucleotide polymorphism; clinical characteristics

INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune disease characterized by fibrosis of the skin and of the internal organs. SSc has been observed more frequently in women than in men (4:1) of 30–50 years (1). SSc patients have autoantibodies to topoisomerase I (ScL-70), antinuclear (ANA), anticentromere and anti RNA polymerase antibodies (2). SSc is divided into different subtypes: diffuse SSc (dSSc) and limited SSc (lSSc).

In diffuse SSc, skin induration is present in hands, feet, face, forearms, upper arms, and trunk. In limited SSc, the earliest diagnostic sign is Raynaud's phenomenon; skin thickening is limited to hands, feet, face, and/or forearms. lSSc also includes the subtype "calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia" (CREST). There is also a localized scleroderma (LS) or MORPHEA, in which fibrotic lesions are confined to the skin, without systemic involvement. No Raynaud's phenomenon is present in MORPHEA. Moreover, there is a form of SSc indicated as ss (sine scleroderma), characterized by a systemic involvement without a cutaneous disease. The

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early scleroderma (early SSc) is another form of SSc that begins with Raynaud's phenomenon and, within three years, develops into a systemic form.

The pathogenesis of systemic sclerosis still remains unknown, although there is evidence that it is probably multifactorial. The mechanisms implicated in SSc include exposure to viral infection (3), genetic predisposition and an association between SSc autoantibodies and HLA alleles (4).

The fibrosis is caused by an excessive accumulation of extracellular matrix (ECM), especially of type I collagen. It has been demonstrated that fibroblasts from scleroderma patients produce increased amount of type I collagen (5).

Matrix metalloproteinases (MMPs) are endopeptidases that are able to degrade ECM. Physiologically, turnover of the ECM is dependent on the balance between MMPs and the specific tissue inhibitors of matrix metalloproteinases (TIMPs). Disruption of this balance, due to the reduction of MMPs and/or the increase of TIMPs serum levels, may be responsible for the net accumulation of ECM in SSc.

Several studies have demonstrated higher serum TIMP-1 levels in SSc patients than in controls (6). TIMP-1 has a mitogenic activity for both normal and SSc fibroblasts. However, the mitogenic response to TIMP-1 in SSc fibroblasts is significantly greater than in those from controls (7).

To our knowledge, no previous studies have been performed to examine TIMP-1 polymorphisms in SSc. As a consequence, the aim of our work is to investigate the possible impact of the TIMP-1 gene polymorphism on the development of SSc and its clinical manifestations.

MATERIALS AND METHODS

Subjects

Seventy-five scleroderma patients (eight males, 67 females) were recruited at the Department of Internal Medicine, University of Milan, Italy, between 1999 and 2004. All patients had systemic sclerosis according to American College of Rheumatology (ARC) criteria for the classification of SSc and the median age at onset was 45 ± 13.7 (range 20–70 years). Subjects with overlap syndromes were excluded. As controls, 38 ethnically matched healthy subjects (nine males, 29 females) were enrolled. None of the patients or healthy donors had any evidence of other autoimmune diseases or malignancies which could influence the TIMP-1 results. Written informed consent was obtained from patients and normal controls. The study was performed according to the guidelines of the local ethical committee. Clinical features of patients are summarized in Table 1.

TABLE 1. Clinical Characteristics of Sclerodermic Patients

	Females n (%)	Males n (%)
Diffuse systemic sclerosis	15 (22.3)	4 (50.0)
Localized systemic sclerosis	37 (55.2)	3 (37.5)
Early scleroderma	3 (4.5)	1 (12.5)
Systemic sclerosis without scleroderma	8 (12.0)	–
Localized systemic sclerosis+crest	3 (4.5)	–
Morphea	1 (1.5)	–
Total	67	8

Sequence Analysis

DNA from peripheral blood samples of patients and healthy donors was collected at the Department of Biomedical Sciences, University of Catania, Italy. A total of 200 ng of genomic DNA were used as the template for amplification. Three single nucleotide polymorphisms (SNPs) have been described in the TIMP-1 gene in the Caucasian population (8): $-19A>G$ in the 5′ UTR of the gene, $+261C>T$ in exon 4, and $+372T>C$ in exon 5.

Polymorphism at position –19 was evaluated on an amplification fragment generated using the primers T1-For (5′-GAA TAG TGA CTG ACG TGG AGG-3′) and T1-Rev (5′-CAG GCC AAG CTG AGT AGA CAG-3′). The polymerase chain reaction (PCR) conditions were 35 cycles consisting of 94°C for 30 sec, 62°C for 30 sec, and 68°C for 30 sec, followed by one cycle at 72°C for 7 min.

For polymorphism at position +261, we used the primers T4-For (5′-GGA TTA TGT CAG TAA AAG AGA C-3′) and T4-Rev (5′-GTG GTT GCT AGG CCG CG-3′). The PCR conditions were 35 cycles consisting of 94°C for 30 sec, 58°C for 30 sec, and 68°C for 30 sec, followed by one cycle at 72°C for 7 min.

Polymorphism at position +372 was evaluated using the primers T5-For (5′-GCC AAT CAC AAG CTG CTT GTC G-3′) and T5-Rev (5′-GGA ATG GCC CCG GGA AGG AT-3′). The PCR conditions were 35 cycles consisting of 94°C for 30 sec, 70°C for 30 sec, and 68°C for 30 sec, followed by one cycle at 72°C for 7 min.

PCR products were purified with the Ultrafree-DNA kit Millipore (Bedford, MA) and 30 ng of purified PCR product was sequenced with the primers T1-For, T4-For and T5-For for the first, second and third fragment respectively. Sequencing was performed with the ABI PRISM® BigDyeTM Terminator kit (Applied Biosystems, Foster City, CA) on an automatic sequencer (Applied Biosystems 3100 Genetic Analyzer) according to the manufacturer's instructions. Sequences were assembled using the ABI Prism® DNA software 3.7 (Applied Biosystems).

Statistical Analysis

As TIMP-1 is located on the X chromosome, allele frequencies for males and females were separately analyzed. We detected no deviation from Hardy-Weinberg equilibrium in any of the groups. The chi-squared (χ^2) test was used to determine differences in genotype and allele frequency between patients and controls, and between controls and patients with different clinical forms. Potential relationships between TIMP-1 mutations and other patient characteristics were examined by the χ^2 test or Fisher's exact test. A P value less than 0.05 by a two-tailed test was defined to be statistically significant.

RESULTS

The genomic DNA was analyzed corresponding to the three SNPs: −19A > G, +261C > T and +372T > C. We identified only SNP +261C > T and +372T > C in our control group. To investigate the involvement of the TIMP-1 gene, we analyzed these two polymorphisms in SSc patients.

No statistical significance was observed in allele and genotype distribution of the SNP +261C > T between patients and controls, both in men and in women. The same results were obtained when the group of patients were subdivided into subgroups with different clinical forms. No differences among patients from different subgroups were observed (data not shown). For +372T > C SNP, a statistically significant difference in the frequency of this polymorphism between the male control group and male patients was observed ($P = 0.02$; Fisher's exact test) (Table 2). The same results were not obtained for the female groups (Table 3). The sequence of each PCR product was compared with that of GenBank accession number NT_011584.

DISCUSSION

SSc is characterized by the accumulation of ECM, due to an overproduction of type I collagen from fibroblasts.

TABLE 2. Genotype Frequencies of the TIMP-1 +372T > C SNP in Male*

Genotype	SSc patients n (%)	Controls n (%)	P value
C	7 (87.5)	3 (30.0)	
T	1 (12.5)	7 (70.0)	0.02
Total	8	10	

*The χ^2 test or Fisher's exact test was used to calculate P values.

TABLE 3. Genotype Frequencies of the TIMP-1 +372T > C SNP in Female*

Genotype	Controls n (%)	SSc patients n (%)	P value
CC	4 (13.8)	10 (14.9)	
CT	17 (58.6)	31 (46.3)	
TT	8 (27.6)	26 (38.8)	0.50
Total	29	67	

*The χ^2 test or Fisher's exact test was used to calculate P values.

Turnover of ECM is regulated by the balance between MMPs and TIMPs.

MMP-1 is one of the most important MMPs involved in the catalytic process of type I collagen. Johnson et al. (9) studied the −16071G/2G polymorphism present in the promoter region of the MMP-1 gene in SSc patients. They did not find any association between SSc and the MMP-1 genotype (9). Fibroblasts from SSc patients produce less MMP-3 than normal dermal fibroblasts (10). Consistent with these findings, there are preliminary results from a study on the MMP-3 promoter polymorphism that revealed that individuals heterozygous and homozygous for the 6A allele (5A/6A and 6A/6A), which result in a weaker promoter activity, are more susceptible to SSc than those with a 5A/5A genotype. However, no association was observed between the MMP-3 genotype and clinical manifestations of SSc (11).

Kuroda et al. (12) have demonstrated increased mRNA levels of type I and III procollagen, MMP-1, MMP-3, and TIMP-1 in skin fibroblasts from SSc patients with an early stage (less than 1 year duration of disease), compared to controls. However, in midstage (2 to 4 years duration) differences between patients and controls remained only for the expression of messenger RNA (mRNA) encoding procollagen I and TIMP-1 (12).

It has been demonstrated that serum levels of TIMP-1 are higher in SSc patients than in controls. TIMP-1 may play a double role in the fibrotic process in SSc: on one hand, it may act as a growth factor for fibroblasts (which are the major producers of type I collagen); on the other hand, it may reduce the turnover of ECM, inhibiting the activity of MMPs. In order to clarify whether SNPs in the TIMP-1 gene have a role in SSc, we investigated polymorphisms at −19A > G, +261C > T, and +372T > C. We did not find any statistical difference between patients and controls in the female group, even when we divided the group of patients into subgroups with different clinical presentations. Even if we had a small number of male patients, due to the rarity of this disease among males, we obtained statistically significant result comparing the frequency of

distribution of +372T>C in male SSc patients group and in male controls. Our results are in agreement with previous data reported by Susol et al. (13), in which an association between SSc and the microsatellite DXS426, marker for TIMP-1, was revealed. In particular, the allele frequency distribution for DXS426 was significantly different when male SSc patients were compared with male controls, but not female SSc with female controls, indicating that predisposing genetic factors differ between male and female disease (13). Transcriptional regulation of the TIMP-1 gene is influenced by several response elements (14–15).

To our knowledge, this is the first report analyzing the TIMP-1 polymorphism in SSc patients. Our results indicate that the analysis of allele and genotype frequencies of TIMP-1 is not associated with clinical characteristics of SSc in Italian patients. Since high TIMP-1 serum levels are associated with SSc (6), the role of TIMP-1 polymorphisms in SSc remains controversial.

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REFERENCES

1. Chen K, See A, Shumack S. Epidemiology and pathogenesis of scleroderma. Australas J Dermatol 2003;44:1–7.
2. Sato S, Fujimoto M, Hasegawa M, Takehara K. Altered blood B lymphocyte homeostasis in systemic sclerosis: expanded naive B cells and diminished but activated memory B cells. Arthritis Rheum 2004;50:1918–1927.
3. Hamamdzic D, Kasman LM, LeRoy EC. The role of infectious agents in the pathogenesis of systemic sclerosis. Curr Opin Rheumatol 2002;14:694–698.
4. Johnson RW, Tew MB, Arnett FC. The genetics of systemic sclerosis. Curr Rheumatol Rep 2002;4:99–107.
5. Uitto J, Bauer EA, Eisen AZ. Scleroderma. Increased biosynthesis of triple helical type I and type III procollagens associated with unaltered expression of collagenase by skin fibroblasts in culture. J Clin Invest 1979;64:921–930.
6. Young-Min SA, Beeton C, Laughton R, et al. Serum TIMP-1, TIMP-2, and MMP-1 in patients with systemic sclerosis, primary Raynaud's phenomenon, and in normal controls. Ann Rheum Dis 2001;60:846–851.
7. Kikuchi K, Kadono T, Furue M, Tamaki K. Tissue inhibitor of metalloproteinase 1 (TIMP-1) may be an autocrine growth factor in scleroderma fibroblasts. J Invest Dermatol 1997;108:281–284.
8. Krex D, Rohl H, Konig IR, Ziegler A, Schackert HK, Schackert G. Tissue inhibitor of metalloproteinases-1, -2, and -3 polymorphisms in a white population with intracranial aneurysms. Stroke 2003;34:2817–2821.
9. Johnson RW, Reveille JD, McNearney T, et al. Lack of association of a functionally relevant single nucleotide polymorphism of matrix metalloproteinases-1 promoter with systemic sclerosis (scleroderma). Genes Immunol 2001;2:273–275.
10. Bou-Gharios G, Osman J, Black C, Olsen I. Excessive matrix accumulation in scleroderma is caused partly by differential regulation of stromelysin and TIMP-1 synthesis. Clin Chim Acta 1994;231:69–78.
11. Marisini B, Casari S, Zeni S, Turri O, Biondi ML. Stromelysin promoter polymorphism is associated with systemic sclerosis. Rheumatology 2001;40:475–476.
12. Kuroda K, Shinkai H. Gene expression of Types I and II collagen, decorin, matrix metalloproteinases and tissue inhibitors of metalloproteinases in skin fibroblasts from patients with systemic sclerosis. Arch Dermatol Res 1997;289:567–572.
13. Susol E, Rands AL, Herrick A, et al. Association of markers for TGFbeta3, TGFbeta2 and TIMP1 with systemic sclerosis. Rheumatology 2000;39:1332–1336.
14. Aicher WK, Alexander D, Haas C, et al. Transcription factor early growth response 1 activity up-regulates expression of tissue inhibitor of metalloproteinases 1 in human synovial fibroblasts. Arthritis Rheum 2003;48:348–359.
15. Dean G, Young DA, Edwards DR, Clark IM. The human tissue inhibitor of metalloproteinases (TIMP)-1 gene contains repressive elements within the promoter and intron 1. J Biol Chem 2000; 275:32664–32671.