CONCISE REPORTS

Association of the TNFa13 microsatellite with systemic sclerosis in Japanese patients

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Abstract

***Objectives*—To elucidate the contribution of microsatellite polymorphisms of TNFa and TNFb alleles to the pathogenesis of systemic sclerosis (SSc) by comparing the allele distribution among populations with different HLA susceptibility genes in SSc.**

***Methods*—TNFa and TNFb microsatellite polymorphisms were determined by PCR in 54 Japanese and 50 German SSc patients and in normal controls. HLA-DR genotyping was carried out by PCR-SSCP.**

***Results*—The frequency of TNFa13 was significantly increased in Japanese SSc (p=0.011, OR=8.53, 95% confidence intervals (95%CI)=2.46, 32.51, and p<1.0 × 10E-5, OR=10.35, 95%CI=4.88, 22.09) and SSc with antitopoisomerase I antibody (a-Scl-70) (p=0.021, OR=33.25, 95%CI=3.39, 800.76, and p<1.0 × 10E-5, OR=24.42, 95%CI=8.40, 72.83), compared with the German patient group and German controls, respectively. This increase was not only attributable to a higher prevalence of TNFa13 in Japanese compared with Germans (p=0.005, OR=3.55, 95%CI=1.60, 7.85) but was also caused by an increase in SSc, especially in the a-Scl-70 positive patients (p=0.028, OR=6.88, 95%CI=1.16, 22.60) compared with Japanese controls. TNFa13 was positively in linkage disequilibrium with HLA-DRB1*1502 (LD=0.053, *t*=2.69). Association analysis indicated that both TNFa13 and DRB1*1502 might have comparable probabilities of being susceptibility factors for SSc with a-Scl-70 in Japanese. Prevalences of TNFa6 and 13 were significantly increased and prevalences of TNFa2, and 7 were significantly decreased in Japanese controls as compared with German controls.**

***Conclusion*—TNFa13 is a genetic marker for SSc with a-Scl-70 in Japanese patients. Various differences in the prevalences of TNFa alleles between Japanese and German controls were established.**

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Systemic sclerosis (SSc) is a well known autoimmune disease of unknown aetiology. Two major types, SSc with diffuse scleroderma and SSc with limited scleroderma,[1 2] are clinically defined. Both forms show associations with antitopoisomerase I (a-Scl-70) and anticentromere (ACA) antibodies, respectively. However, these autoantibodies do not exactly match the clinical types of the disease.

The contribution of genetic factors to SSc has been demonstrated by several family studies and associations with specific HLA antigens have been reported. In Japanese, HLA-DRB1*1502-DRB5*0102 haplotype and DRB1*0802 were found to be associated with diffuse scleroderma and a-Scl-70 positive SSc.[3 4] In white populations, associations with DR3 and DR5, especially in a-Scl-70 positive patients with DRB1*1104 and DRB1*11/15 have been shown.[5–7] Briggs *et al* reported associations of HLA A1-B8-C4AQ0-DR3 haplotype and DQA2, an allele that was in linkage disequilibrium (LD) with both DR3 and DR11.[8] So far, the susceptibility gene (or genes) within the extended HLA-haplotypes has not been identified unequivocally.

On the other hand, several studies investigating tumour necrosis factor α (TNFα) in the course of SSc[9 10] have suggested a pathogenic role for this cytokine in disease progression, and, reproducible differences in TNFα secretory capacities in people with different HLA types have been shown.[11] Consequently, genes within the MHC-encoded TNF region have been investigated for their role as potential susceptibility genes. Besides other polymorphisms within the MHC class III region the TNF microsatellites TNFa and TNFb (which map 3.5kB from the TNFα gene and the related lymphotoxin α and β genes) have gained special attention.

In a recent German study[6] some of us investigated the genetics of scleroderma with and without quartz dust exposure (qSSc and iSSc, respectively). We reported that the typing of HLA class II, the TNF-308 promoter region, and the TNFa and b microsatellite alleles revealed immunogenetic differences, especially in the a-Scl-70 positive patients, among the patient groups in the use of HLA and TNF alleles markedly differentiated by their TNF secretory capacity. The TNF microsatellite allele a2, reported to be associated with an increased TNFα production capacity,[12] was differently found in the two a-Scl-70 positive groups.

A helpful approach in clarifying the contribution of TNF alleles in SSc (with or without quartz/metal dust exposure) might be to compare the TNF alleles in populations with different HLA susceptibility genes prevalent in a-Scl-70 positive SSc.

In a first step we compared the distribution of TNFa and TNFb microsatellites in 54 Japanese and 50 German idiopathic SSc (iSSc) patients and in the corresponding controls. Unexpectedly, we found TNFa13, a rare allele in white populations, to be associated with Japanese SSc. As expected, the recruitment of Japanese SSc patients with a history of quartz/metal dust exposure has proved to be difficult.

Methods

PATIENTS

Unrelated iSSc patients from Japan (n=54, 52 women and two men) and Germany (n=50, 46 women and four men) were analysed. All patients with SSc fulfilled the criteria of the American Rheumatism Association (ARA).[1] Randomly selected, unrelated, healthy Japanese and German people were studied as controls (n=69 and n=314, respectively). All patients and controls were ethnic Japanese or Germans, respectively. Familial analysis was not available in this study for either patients or controls. The Japanese patients were aged (mean (SD)) 52.5 (11.4) years. Twenty two patients (40.7%, all women) were positive for a-Scl-70 and 10 patients (18.5%) were positive for ACA. There was no occupational history of quartz/metal dust exposure. Twenty two (40.7%) patients had SSc with diffuse scleroderma (age 51.3 (9.3); all women, 16 of them were a-Scl-70 responders). The German patients were diagnosed at the Departments of Dermatology in Dresden (Medical Faculty at the Technical University), Bochum (St Joseph Hospital at the Ruhr University), and Berlin (Charité). When analysing the occupational history of patients believed to have iSSc, we found that seven of them were employed at jobs associated with increased exposure to quartz dust: two were stone masons, two were pit coal miners, two were building workers with quartz dust exposure, and one woman was exposed to scouring powder. These seven patients were not included in this study. The German patients were aged 54.2 (9.6). Twenty patients (40%; 18 women, two men) were positive for a-Scl-70 and 10 patients (20%; nine women, one man) were positive for ACA.

AUTOANTIBODY DETERMINATIONS

In Japanese patients antinuclear antibodies were detected by an indirect immunofluorescent method using Hep-2 cell specimens as nuclear antigen (Quantafluor Test Kit (HEp-2), Kallestad, Chaska, USA). A-Scl-70 was detected by a double immunodiffusion method (immunoprecipitation method in agarose gel) using rat thymus extract as nuclear antigen (ENA-3 Test, MBL, Nagoya, Japan). These methods are routinely used in the Central Laboratory Service of Tokyo University Hospital.[13] For the detection of a-Scl-70 in German patients immunodiffusion against extractable nuclear antigens (Biolab Deutschland, Hürth, Germany), immunoblotting with antigen preparations from Hep-2 cells (AID, Strasberg, Germany) and two enzyme linked immunosorbent assays with purified antigen (Orgasan, Mainz, Germany) and recombinant antigen (ELIAS, Freiburg, Germany) were used. ACA was recognised as a characteristic, discrete, speckled, nuclear staining pattern on Hep-2 cells in both institutions.

TNF MICROSATELLITE TYPING

For TNFa and TNFb microsatellite typing the method described by Jongeneel *et al*[14] was used with the following minor modifications: sequence first primer (IR 1): CACTCCAGCCTAGGCACAGAG, use of 200 μM of primer 1 and 2; 30 amplification cycles were performed in the last run.

GENOTYPING OF HLA ANTIGENS

Genotyping of HLA-DRB1* alleles was performed by conventional PCR-SSO. HLA-DR15, DR16, and DR8, respectively, all of which have been shown to be associated with Japanese SSc,[3 4] were genotyped by the PCR-SSCP (single stranded DNA conformation polymorphism) as described previously.[3]

STATISTICAL ANALYSIS

Using the EPI-INFO statistical program Fisher's exact test (two tailed) was used for all comparisons, as well as odds ratios (OR) and exact 95% confidence intervals (95%CI) for the same 2 × 2 tables. Significantly different probability values (p) between the groups ($p<0.05$) were Bonferroni adjusted for the number of tests in case multiple comparisons were made. The LD was calculated[15] and shown occasionally with *t* value.

Results

Table 1 shows the phenotype frequencies of TNFa alleles of Japanese and German SSc and of the controls. No significant differences were found in the distribution of TNFa1-a12 between the SSc groups compared with each other and with the controls. The only significant difference was present in the TNFa13 distribution. The frequency of TNFa13 was significantly increased in the Japanese whole SSc group compared with the German whole SSc group and German controls (p=0.011, OR=8.53, 95%CI=2.46, 32.51; p<1.0 × 10E-5, OR=10.35, 95%CI=4.88, 22.09, respectively) but only moderately increased compared with Japanese controls (42.6% *v* 20.3%, the difference being not statistically significant). The prevalence of TNFa13 was also significantly increased in Japanese SSc with a-Scl-70 compared with the German a-Scl-70 positive SSc, Japanese controls, and German controls (p=0.021, OR=33.25, 95%CI=3.39, 800.76; p=0.028, OR=6.88, 95%CI=1.16, 22.60; p<1.0 × 10E-5, OR=24.42, 95%CI=8.40, 72.83, respectively). In the German SSc groups no significant associations of any of the TNFa alleles were observed when compared with German controls, but a significant decrease of TNFa2 was

Table 1 Phenotype frequencies of TNFa microsatellite alleles in Japanese SSc (SScJ) and German SSc (SScG) and controls

	SSc (%)				*Controls*		
TNFa	*whole (J) 54*	*whole (G) 50*	*a-Scl-70* (J) 22*	*a-Scl-70* (G) 20*	*Japanese 69*	*German 314*	*Comparison of controls p Value*
a1	2 (3.7)	0	1 (4.5)	0	2 (2.9)	11 (3.5)	NS
a2	13 (24.1)	22 (44)	5 (22.7)	4 (20)	17 (24.6)	142 (45.2)	0.0218, OR=0.4 (95% CI=0.21, 0.74)
a3	0	1 (2)	0	1 (5)	0	9 (2.9)	NS
a4	3 (5.6)	4 (8)	0	1 (5)	3 (4.3)	53 (16.9)	NS
a5	3 (5.6)	4 (8)	1 (4.5)	1 (5)	1 (1.4)	33 (10.5)	NS
a6	19 (35.2)	14 (28)	6 (27.3)	5 (25)	40 (58.0)	68 (21.7)	<1.0 × 10E-5, OR=4.99 (95% CI=2.79, 8.96)
a7	3 (5.6)	7 (14)	2 (9.5)	5 (25)	2 (2.9)	59 (18.8)	0.0142, OR=0.13 (95% CI=0.02, 056)
a8	2 (3.7)	1 (2)	0	0	0	2 (0.6)	NS
a9	3 (5.6)	2 (4)	1 (4.5)	1 (5)	5 (7.2)	15 (4.8)	NS
a10	14 (25.9)	14 (28)	4 (18.2)	6 (30)	13 (18.8)	84 (26.8)	NS
a11	14 (25.9)	18 (36)	4 (18.2)	10 (50)	24 (34.8)	81 (25.8)	NS
a12	0	0	0	0	0	4 (1.3)	NS
a13	23 (42.6)	4 (8)	14 (63.6)	1 (5)	14 (20.3)	21 (6.7)	0.005, OR=3.55 (95% CI=1.60, 7.85)

TNFa frequencies in SSc (whole), a-Scl-70*, and controls were compared between the Japanese and German population. No significant differences were found in the distribution of TNF a1-a12 between the patient groups compared with each other and with the controls.
p Values of the comparisons of the controls were shown after correction by total number of comparison (13 for TNFa). p Values of the comparisons of TNFa13 frequencies between the patient groups and with the controls were additionally adjusted by the factor 6 (number of the comparisons below).
whole SSc(J) *v* whole SSc(G): p=0.011, OR=8.53 (95% CI=2.46, 32.51)
whole SSc(J) *v* control(J): p=NS, OR=2.91 (95% CI=1.22, 7.00), (without correction p=0.0074)
whole SSc(J) *v* control(G): p<1.0 × 10E-5, OR=10.35 (95% CI=4.88, 22.09)
a-Scl-70*(J) *v* a-Scl-70*(G): p=0.021, OR=33.25 (95% CI=3.39, 800.76)
a-Scl-70*(J) *v* control(J): p=0.028, OR=6.88 (95% CI=1.16, 22.60)
a-Scl-70*(J) *v* control(G): p<1.0 × 10E-5, OR=24.42 (95% CI=8.40, 72.83)

found when we compared the a-Scl-70 positive with the a-Scl-70 negative patients (p=0.04, OR=0.17, 95%CI=0.04, 072, data not shown).

Associations between TNFa alleles and HLA-DRB1* (*15, *16 and *08) were calculated for the Japanese control group. A significant association was observed between TNFa13 and DRB1*1502 (p=0.0001; data not shown) and the LD was 0.053 (t =2.69). No associations were found between any of the TNFa alleles and DRB1*08. In each SSc group no significant association of any of the TNFb alleles was observed.

TNFa microsatellite allele distribution in Japanese and German normal controls are also given in table 1. In Japanese, TNFa6 and 11 are the major TNFa alleles, while in Germans TNFa2 and 10 prevail. The TNFa2 and 7 are significantly decreased in Japanese controls as compared with German controls. In contrast, TNFa6 and 13 are significantly increased in Japanese. TNFa13, which is the fourth most frequent allele in Japanese controls (20.3%), is a relatively minor allele in German controls (6.7%). Compared with the TNFa alleles, the distribution patterns of TNFb microsatellite alleles do not differ that much between Japanese and German controls (data not shown).

In this study, the significant HLA-DRB1* associations in the SSc of both populations were reconfirmed.[3 4 6] In Japanese patients the prevalences of DRB1*1502 were 38.9% (p=0.0016; OR= 4.87, 95%CI =1.77, 13.4) and 50.0% (p= 0.0007; OR = 7.67, 95%CI= 2.33, 22.3) in whole SSc and SSc with a-Scl-70, respectively, as compared with 11.5% in normal controls (data not shown). In German patients DRB1*1104 was significantly increased in iSSc with a-Scl-70 compared with the controls (p=0.04; OR=11.0, 95%CI=2.68, 45.93). All five DRB1*11/15 heterozygotes were positive for a-Scl-70, which was significant at p=0.02 (OR=12.43, 95%CI=3.65, 40.04).

Discussion

TNFα is a potent modulator of the immune response and an important mediator of inflammation. In salivary glands of very early stages of SSc the expression of TNFα was observed before the onset of skin changes. Koch *et al* reported that TNFα expression was increased on SSc stratum granulosum, especially in early SSc and suggested that TNFα may play a part in the early inflammatory stage of SSc.[16] Systemic sclerosis dermal fibroblasts were reported to be hyperresponsive to TNFα and expression of ICAM-1 was increased to a greater degree in response to TNFα stimulation.[17] Based on these and other reports[9 10] we suggest that TNFα might play a part in the pathogenesis of scleroderma.

In a recent study of German scleroderma patients with and without exposure to quartz/metal dust, qSSc and iSSc, respectively,[6] we found differences in the distribution of HLA class II and TNFa microsatellite genes, especially in the a-Scl-70 positive patients. HLA-DRB1*0301 (DR17) and TNFa2, components of the extended haplotype HLA-DQA1*0501; B1*0201; DRB1*0301; TNF-308.2; TNFa2/b2, had a decreased frequency in iSSc a-Scl-70 positive compared with

*Table 2 Effect of coexistence of TNFa13 and HLA-DRB1*1502 on SSc*

	Normal (n=52) Number (%)	*Diffuse (n=22)*			*a-Scl-70 (n=22)*		
		Number (%)	*p Value*	*OR*	*Number (%)*	*p Value*	*OR*
a13	10 (19.2)	16 (72.7)	0.00003	11.2 (3.50, 35.9)	14 (63.6)	0.00035	7.4 (2.42, 22.3)
*1502	6 (11.5)	14 (63.6)	0.00001	13.4 (3.98, 45.3)	11 (50.0)	0.00073	7.7 (2.32, 25.3)
a13*1502	6 (11.5)	14 (63.6)	0.00001	13.4 (3.98, 45.3)	11 (50.0)	0.00073	7.7 (2.32, 25.3)

a-Scl-70 negative patients, but a significantly increased frequency in qSSc a-Scl-70 positive compared with controls and with iSSc a-Scl-70 positive patients. In contrast, DRB1*1104 and DRB1*11/15 heterozygotes with no TNFa2 were prevalent in only the iSSc a-Scl-70 positives compared with controls. The HLA and TNF alleles in these patient groups were reported to be associated with markedly differentiated TNF secretory capacity.[12]

In this study we did not find any significant differences in the distribution of TNFa1-a12, especially not in the frequency of TNFa2, between the SSc groups. Unexpectedly, we found associations between the TNFa13 allele and Japanese SSc. These associations were most obvious in the a-Scl-70 positive patients. Our results indicate that TNFa13 is a candidate susceptibility factor for SSc with a-Scl-70 (and diffuse scleroderma) but not for other SSc subgroups.

Our data confirm the increases of DRB1*1502 previously reported for Japanese SSc[3 4] and show LD between this HLA allele and TNFa13. Association analyses indicate that both alleles do not additively contribute to the disease (table 2). This finding leaves the question unanswered as to whether TNFa13 itself contributes to the pathogenesis of SSc as a susceptibility allele.

Several reports support the idea that other genes within the MHC including TNF microsatellites might contribute to the pathogenesis of autoimmune disorders independently from HLA genes. This has been suggested for TNFa6 and TNFc1 in rheumatoid arthritis[18 19] as well as for TNFa2 in coeliac disease.[20]

Based on the observed differences of TNFa-SSc associations between Japanese and Germans it seems more plausible that TNFa13 represents just a genetic marker rather than a susceptibility allele, and TNFa13 might link to the HLA-susceptibility haplotype DRB1*1502, DRB5*0102 and/or another MHC susceptibility gene. With regard to the starting point of this study (and provided that a sufficient number of patients with a history of quartz/metal dust exposure and/or male patients can be recruited in Japan) the more restricted use of the DRB1*1502, DRB5*0102, TNFa13 haplotype for Scl-70 responses in Japanese seems to be a good prerequisite for studying environmental and/or sex related effects in the pathogenesis of SSc.

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1 Masi AT, Rodman GP, Medsger TA Jr, Altman RD, D'Angelo WA, Fries JF, *et al.* Preliminary criteria for the classification of systemic (scleroderma). Arthritis Rheum 1980;23:581–90.
2 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.
3 Takeuchi F, Nakano K, Yamada H, Hong GH, Nabeta H, Yoshida A, *et al.* Association of HLA-DR with progressive systemic sclerosis in Japanese. J Rheumatol 1994;21:857–63.
4 Kuwano M, Kaburaki J, Okano Y, Inoko H, Tsuji K. The HLA-DR and DQ genes control the autoimmuno response to DNA topoisomerase I in systemic sclerosis (scleroderma). J Clin Invest 1993;92:1296–301.
5 Morel PA, Chang HJ, Wilson JW, Conte C, Saidman SL, Bray JD, *et al.* Severe systemic sclerosis with anti-topoisomerase I antibodies is associated with an HLA-DRw11 allele. Hum Immunol 1994;40:101–10.
6 Frank KH, Füssel M, Conrad K, Rihs HP, Koch R, Gebhardt B, *et al.* Different distribution of HLA class II and tumor necrosis factor alleles (TNF-308.2, TNFa2 microsatellite) in anti-topoisomerase I responders among scleroderma patients with and without exposure to quartz/metal dust. Arthritis Rheum 1998;41:1306–11.
7 Reveille JD. Molecular genetics of systemic sclerosis. Curr Opin Rheumatol 1995;7:522–8.
8 Briggs D, Stephens C, Vaughan R, Welsh K, Black C. A molecular and serologic analysis of the major histocompatibility complex and complement component C4 in systemic sclerosis. Arthritis Rheum 1993;36:943–54.
9 Kantor TV, Friberg D, Medsger TA JR, Buckingham RB, Whiteside TL. Cytokine production and serum levels in systemic sclerosis. Clin Immunol Immunopathol 1992;65: 278–85.
10 Hasegawa M, Fujimoto M, Kikuchi K, Takehara K. Elevated serum tumor necrosis factor-α levels in patients with systemic sclerosis: association with pulmonary fibrosis. J Rheumatol 1996;24:663–5.
11 Jacob CO, Fronek Z, Lewis GD, Koo M, Hansen JA, Mc Devitt HO. Heritable major histocompatibility complex II-associated differences in production of tumour necrosis factor-α: relevance to genetic predisposition to systemic lupus erythematosus. Proc Natl Acad Sci USA 1990;87: 1233–7.
12 Pociot F, Briant L, Jongeneel CV, Molvig J, Worsaae H, Abbal M, *et al.* Association of tumour necrosis factor (TNF) and class II major histocompatibility complex alleles with the secretion of TNF-alpha and TNF-beta by human mononuclear cells: a possible link to insulin-dependent diabetes mellitus. Eur J Immunol 1993;23:224–31.
13 Takeuchi F, Nabeta H, Hong GH, Matsuta K, Tokunaga K, Tanimoto K, *et al.* C4A and C4B null alleles are genetic markers of different types of systemic sclerosis in Japanese patients. Clin Exp Rheumatol 1998;16:55–60.
14 Jongeneel CV, Briant L, Udalova IA, Sevin A, Nedospasov SA, Cambon-Thomsen A. Extensive genetic polymorphism in the human tumor necrosis factor region and relation to extended HLA haplotypes. Proc Natl Acad Sci USA 1991;88:9717–21.
15 Mittal KK. The HLA polymorphism and susceptibility to disease. Vox Sang 1976;31:161–73.
16 Koch AE, Kronfeld-Harrington LB, Szekanecz Z, Cho MM, Haines GK, Harlow LA, *et al.* In situ expression of cytokines and cellular adhesion molecules in the skin of patients with systemic sclerosis. Pathobiology 1993;61: 239–46.
17 Cho MM, Jimenez SA, Johnson BA, Harlow LA, Burrows JC, Koch AE. In vitro cytokine modulation of intercellular adhesion molecule-1 expression on systemic sclerosis dermal fibroblasts. Pathobiology 1994;62:73–81.
18 Hajeer AH, Worthington J, Silman AJ, Olliver WER. Association of tumor necrosis factor microsatellite polymorphisms with HLA-DRB1*04-bearing haplotypes in rheumatoid arthritis patients. Arthritis Rheum 1996;39: 1109–14.
19 Mulcahy B, Waldron-Lynch F, McDermott MF, Adams C, Amos CI, Zhu DK, *et al.* Genetic variability in the tumor necrosis factor-lymphotoxin region influences susceptibility to rheumatoid arthritis. Am J Hum Genet 1996;59:676–83.
20 McManus R, Moloney M, Borton M, Finch A, Chuan YT, Lawlor E, *et al.* Association of celiac disease with microsatellite polymorphisms close to the tumor necrosis factor genes. Hum Immunol 1996;45:24–31.