Association of the TNFa13 microsatellite with systemic sclerosis in Japanese patients

Fujio Takeuchi, Hiromi Nabeta, Monika Füssel, Karsten Conrad, Karl-Heinz Frank

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, (=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 TNFb2, 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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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.17 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 al10 was used with the following minor modifications: sequence first primer (IR 1): CACTCTAGGGCACAGAG, 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,14 were genotyped by the PCR-SSCP (single stranded DNA conformation polymorphism) as described previously.9

**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 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 calculated15 and shown occasionally with r 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% vs 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, 42.60; 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
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 TNFa1-a12 between the patient groups compared with each other and with the controls.

Table 1. Phenotype frequencies of TNFa microsatellite alleles in Japanese SSc (SSc(J)) and German SSc (SSc(G)) and controls

<table>
<thead>
<tr>
<th>Allele</th>
<th>54</th>
<th>102</th>
<th>a-Scl-70*</th>
<th>20</th>
<th>a-Scl-70*+</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>whole</td>
<td>whole</td>
<td>a-Scl-70*</td>
<td>whole</td>
<td>a-Scl-70*</td>
<td>Japanese</td>
</tr>
<tr>
<td></td>
<td>(J)</td>
<td>(G)</td>
<td>(J)</td>
<td>(G)</td>
<td>(J)</td>
<td>69</td>
</tr>
<tr>
<td>a1</td>
<td>2 (3.7)</td>
<td>0</td>
<td>1 (4.5)</td>
<td>0</td>
<td>2 (2.9)</td>
<td>11 (3.5)</td>
</tr>
<tr>
<td>a2</td>
<td>13 (24.1)</td>
<td>22 (44)</td>
<td>5 (22.7)</td>
<td>4 (20)</td>
<td>17 (24.6)</td>
<td>14 (34.52)</td>
</tr>
<tr>
<td>a3</td>
<td>0</td>
<td>1 (2)</td>
<td>0</td>
<td>1 (5)</td>
<td>0</td>
<td>9 (2.9)</td>
</tr>
<tr>
<td>a4</td>
<td>3 (5.6)</td>
<td>4 (8)</td>
<td>1 (4.5)</td>
<td>1 (5)</td>
<td>3 (4.3)</td>
<td>53 (16.9)</td>
</tr>
<tr>
<td>a5</td>
<td>3 (5.6)</td>
<td>4 (8)</td>
<td>1 (4.5)</td>
<td>1 (5)</td>
<td>1 (1.4)</td>
<td>33 (10.5)</td>
</tr>
<tr>
<td>a6</td>
<td>19 (35.2)</td>
<td>14 (28)</td>
<td>6 (27.3)</td>
<td>5 (25)</td>
<td>40 (58.0)</td>
<td>68 (21.7)</td>
</tr>
<tr>
<td>a7</td>
<td>3 (5.6)</td>
<td>7 (14)</td>
<td>2 (9.5)</td>
<td>5 (25)</td>
<td>2 (2.9)</td>
<td>59 (18.8)</td>
</tr>
<tr>
<td>a8</td>
<td>2 (3.7)</td>
<td>1 (2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (0.6)</td>
</tr>
<tr>
<td>a9</td>
<td>3 (5.6)</td>
<td>2 (4)</td>
<td>1 (4.5)</td>
<td>1 (5)</td>
<td>2 (5.7)</td>
<td>15 (4.8)</td>
</tr>
<tr>
<td>a10</td>
<td>14 (25.9)</td>
<td>14 (28)</td>
<td>4 (18.2)</td>
<td>6 (30)</td>
<td>13 (18.8)</td>
<td>84 (26.8)</td>
</tr>
<tr>
<td>a11</td>
<td>14 (25.9)</td>
<td>18 (36)</td>
<td>4 (18.2)</td>
<td>6 (30)</td>
<td>24 (34.8)</td>
<td>81 (25.8)</td>
</tr>
<tr>
<td>a12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4 (1.3)</td>
</tr>
<tr>
<td>a13</td>
<td>23 (42.6)</td>
<td>4 (8)</td>
<td>14 (63.6)</td>
<td>1 (5)</td>
<td>14 (20.3)</td>
<td>21 (6.7)</td>
</tr>
</tbody>
</table>

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

TNFa 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 TNFa was observed before the onset of skin changes. Koch et al reported that TNFa expression was increased on SSc stratum granulosum, especially in early SSc and suggested that TNFa may play a part in the early inflammatory stage of SSc. Systemic sclerosis dermal fibroblasts were reported to be hyperresponsive to TNFa and expression of ICAM-1 was increased to a greater degree in response to TNFa stimulation. Based on these and other reports, we suggest that TNFa 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, we found that TNFa expression was increased in iSSc compared with qSSc and iSSc, respectively. In Japanese patients the expression of TNFá was 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).

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

<table>
<thead>
<tr>
<th>Allele</th>
<th>Number (%)</th>
<th>Diffuse (n=22)</th>
<th>p Value</th>
<th>OR</th>
<th>a-Scl-70* (n=22)</th>
<th>Number (%)</th>
<th>p Value</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>a13</td>
<td>10 (19.2)</td>
<td>16 (72.7)</td>
<td>0.00003</td>
<td>11.2 (3.50, 35.9)</td>
<td>14 (63.6)</td>
<td>0.0035</td>
<td>7.4 (2.42, 22.3)</td>
<td></td>
</tr>
<tr>
<td>*1502</td>
<td>6 (11.5)</td>
<td>14 (63.6)</td>
<td>0.00001</td>
<td>13.4 (3.98, 45.3)</td>
<td>11 (50.0)</td>
<td>0.0073</td>
<td>7.7 (2.32, 25.3)</td>
<td></td>
</tr>
<tr>
<td>a13*1502</td>
<td>6 (11.5)</td>
<td>14 (63.6)</td>
<td>0.00001</td>
<td>13.4 (3.98, 45.3)</td>
<td>11 (50.0)</td>
<td>0.0073</td>
<td>7.7 (2.32, 25.3)</td>
<td></td>
</tr>
</tbody>
</table>

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).
TNFα and TNFα13 in rheumatoid arthritis. In contrast, DRB1*1104 and DRB1*1115 heterozygotes with no TNFα2 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 secretion capacity.

In this study we did not find any significant differences in the distribution of TNFα1-142, especially not in the frequency of TNFα2, between the SSc groups. Unexpectedly, we found associations between the TNFα13 allele and Japanese SS. These associations were most obvious in the a-Scl-70 positive patients. Our results indicate that TNFα13 is a candidate susceptibility factor for SS with a-Scl-70 (and diffuse scleroderma) but not for other SS subgroups.

Our data confirm the increases of DRB1*1502 previously reported for Japanese SSc1 and show LD between this HLA allele and TNFα13. Association analyses indicate that both alleles do not additively contribute to the disease (table 2). This finding leaves the question unanswered as to whether TNFα13 itself contributes to the pathogenesis of SS as a susceptibility allele.

Several reports support the idea that other genes within the MHC including TNF microsatellite might contribute to the pathogenesis of autoimmune disorders independently from HLA genes. This has been suggested for TNFα6 and TNFα1c in rheumatoid arthritis11-15 as well as for TNFα2 in coeliac disease.16

Based on the observed differences of TNFα-SSc associations between Japanese and German it seems more plausible that TNFα13 represents just a genetic marker rather than a susceptibility allele, and TNFα13 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, TNFα13 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 SS.

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