**Association of IL-10 receptor 2 (IL10RB) SNP with systemic sclerosis**

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**Journal or publication title** Biochemical and biophysical research communications

**Volume** 373

**Number** 3

**Page range** 403-407

**Year** 2008-08

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**URL** http://hdl.handle.net/2241/100337

doi: 10.1016/j.bbrc.2008.06.054
Association of IL-10 Receptor 2 (IL10RB) SNP with Systemic Sclerosis

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Abstract
Interleukin-10 (IL-10) signaling has been suggested to play a role in systemic sclerosis (SSc). IL10RB codes for IL-10 receptor 2 (IL-10R2), a component shared in receptor complexes for IL-10, IL-22, IL-26 and interferon (IFN)-λ. In this study, we examined association of IL10RB polymorphism with susceptibility to SSc. Genotype A/A at rs2834167 (47K/K) was significantly increased in diffuse cutaneous SSc (dcSSc) (41.3% in dcSSc, 20.9% in controls, \(P=0.0018\), odds ratio = 2.67). A SNP in the 5’ flanking region of IL10RB, rs999788, also showed association with dcSSc; however, this association was shown to be secondarily caused by linkage disequilibrium with rs2834167. Significant association was not observed in limited cutaneous SSc (lcSSc). Presence of anti-topoisomerase I antibody was also associated with rs2834167A/A genotype \((P=0.0019)\). Serum IL-10 level was significantly associated with the number of rs2834167A allele \((P=0.007)\). These findings suggested that signaling through IL-10R2 may play a causative role in dcSSc.

Key words: systemic sclerosis, IL-10 receptor, polymorphism
Introduction

Systemic sclerosis (SSc) is an autoimmune disease characterized by fibrosis of skin, muscles and other organs as well as vascular damage [1]. SSc is subdivided into limited cutaneous (lcSSc) and diffuse cutaneous SSc (dcSSc) according to the extent of skin sclerosis. Patients with dcSSc have significant and early incidence of internal organ involvement such as lung fibrosis and scleroderma renal crisis. In contrast, lcSSc patients have a low prevalence of internal organ involvement, except for pulmonary hypertension [2]. Specific autoantibodies are associated with distinct types of SSc; anti-topoisomerase I antibody is frequently detected in dcSSc, while anti-centromere antibody is usually observed in lcSSc.

The pathogenesis of SSc is a complex combination of genetic and environmental factors [3]. Several genetic factors such as HLA [4,5], connective-tissue growth factor [6], CD19 [7], interleukin-10 (IL10) [8] and CD22 [9] have been associated with SSc.

IL-10 is secreted by monocytes and lymphocytes, and suppresses production of pro-inflammatory cytokines and activation of T helper 1 (Th1) cells [10]. In addition, IL-10 induces proliferation and differentiation of activated B cells, and stimulates antibody production [11]. IL-10 also promotes proliferation and cytotoxic activity of CD8+ T cells [12]. Several lines of evidence suggested a role of IL-10 in SSc. Serum level of IL-10 was elevated in the patients with dcSSc [13], and was positively correlated with skin and pulmonary fibrosis [8].

Genetic association of IL10 polymorphism with SSc was also reported. Crilly et al. suggested that a haplotype with lower IL-10 production may be associated with dcSSc [14], while other groups reported that a haplotype with high IL-10 production was associated with susceptibility and severity of dcSSc [15,16]. Thus, the role of IL10 polymorphisms in SSc remains controversial.

IL-10 receptor complex is composed of IL-10 receptor 1 (IL-10R1) and IL-10R2. IL-10 first binds to IL-10R1, and induces a conformational change that enables IL-10R2 to interact with IL-10/IL-10R1 complex. This ligand-mediated assembly induces activation of STAT3,
followed by induction of STAT3-responsive genes [17]. IL-10R2 is also a component of IL-22R, IL-26R and interferon (IFN)-λR (IL-28R).

IL-10R2 is encoded by *IL10RB* gene. *IL10RB* is located in a class II cytokine receptor gene cluster together with IFNα receptor 1 (*IFNAR1*), 2 (*IFNAR2*) and IFNγ receptor 2 (*IFNGR2*) on chromosome 21q22. Associations between *IL10RB* polymorphism and several diseases have been reported. Frodsham et al. identified the class II cytokine cluster region as a major susceptibility locus for hepatitis B virus (HBV) persistence, and demonstrated that two nonsynonymous single nucleotide polymorphisms (SNPs), *IFNAR2* F8S and *IL10RB* E47K, were associated with a risk of HBV persistence [18]. *IL10RB* E47K has also been reported to be associated with acute graft-versus-host disease (GvHD) [19]. These observations suggested that *IL10RB* E47K is bears functional significance.

These observations led us to consider *IL10RB* as a potential candidate susceptibility gene for SSc. In the present study, we examined association of *IL10RB* E47K and three other potentially functional SNPs within the same linkage disequilibrium (LD) block with SSc.
Materials and Methods

Patients and controls

Forty-six patients with dcSSc (10 males and 36 females, average age 47.5±16.1 years), 78 patients with lcSSc (4 males and 74 females, average age 54.8±10.8 years), and healthy controls (229 males and 169 females, 28.6±7.3 years) were studied. SSc was classified according to the American College of Rheumatology criteria [20]. Patients and healthy controls were recruited at Kanazawa University, The University of Tokyo and associated laboratories. All patients and healthy controls were unrelated Japanese, living in the central part of Japan. This study was reviewed and approved by the Research Ethics Committees of University of Tsukuba, Kanazawa University and the University of Tokyo.

Genotyping

IL10RB rs2834167 was genotyped using a bead-array system based on xMAP technology (Luminex, Luminex Corp, Austin, TX). The sequences of the primers were as follows: 5'-CACGTGGCCTTTGAAGACATGG-3' and 5'-GCCACGAGAATTCCCAGACAG-3'. These primers were labeled with biotinyl-group at 5'-terminus. The PCR products were subjected to hybridization with Luminex-beads and labeled with streptavidin conjugated phycoerythrin. Each bead was conjugated with the allele specific oligonucleotide probe. The sequences of the probes were as follows: 5'-CTTTTGCCAAAGGGGAACC-3' for A allele and 5'-CTTTTGCCGAAGGGGAACC-3' for G allele. The validity of this method was confirmed by direct sequencing.

IL10RB rs999788, IFNAR2 rs4986956 and rs7279064 were genotyped by direct sequencing. PCR primers were designed by using Primer3 software. The sequences of the primers for rs4986956 and rs7279064 were 5'-TGAGACCAGGCTCAGTCCTTGAA-3' and 5'-GACTTCTGTCCAGTGCTCTC-3', and those for rs999788 were 5'-TGGGTTGATCTTTTGCAGATA-3' and 5'-AAGCGAGGGGAAGTGATGC-3'. PCR products were subjected to sequencing reaction using a BigDye Terminator v3.1 Cycle Sequencing kit, and sequenced by ABI PRISM 3100 Genetic Analyzer (Applied Biosystems,
Foster City, CA).

**ELISA**

Serum levels of IL-10 in the patients with dcSSc were measured by ELISA using Human IL-10 ELISA Set (BD Biosciences Pharmingen, San Diego, CA) following the manufacturer’s instructions.

**Statistical analysis**

Association analyses were conducted by \( \chi^2 \) test for 2×2 and 2×3 contingency tables. When one or more of the variables in the contingency tables was 5 or less, Fisher’s exact test was employed. \( D’ \) values were calculated using Haploview software (http://www.broad.mit.edu/mpg/haploview/download.php). Association of rs2834167 genotype and serum IL-10 levels in 44 patients with dcSSc was analyzed by simple regression analysis. Genotypes G/G, G/A and A/A were encoded as 0, 1 and 2, respectively.
Results

Association of \textit{IL10RB} E47K with dcSSc

We initially focused on \textit{IL10RB} rs2834167 coding for a nonsynonymous substitution E47K, because this SNP was associated with other conditions [18,19] and considered likely to be functional. As shown in Table 1, rs2834167 A/A genotype (47K/K) was significantly increased in SSc under the recessive model ($P=0.038$, odds ratio [OR] =1.61, 95% confidence interval [CI]: 1.02-2.54). The genotype frequency in our controls was compatible with Hardy-Weinberg equilibrium, and was quite similar to that in the 934 Japanese volunteers (G/G 0.30, G/A 0.49, A/A 0.21) in the JSNP database (http://snp.ims.u-tokyo.ac.jp/index.html).

This association was attributable to the dcSSc subgroup, since the difference between dcSSc and controls was highly significant ($P=0.0018$, OR = 2.67, 95% CI : 1.42-5.04), while significant difference was not observed between lcSSc and controls. Similarly, when the patients were classified according to the presence of anti-topoisomerase I and anti-centromere antibodies, significant association of rs2834167 A/A genotype was observed only in the anti-topoisomerase I antibody positive group ($P=0.0019$, OR = 3.00, 95%CI 1.50-5.99).

We next examined whether this SNP is causally associated with dcSSc, or only represents LD with other causal alleles. According to the HapMap database (http://www.hapmap.org/index.html.en), the LD block encompassing rs2834167 extends from \textit{IFNAR2} to exon 2 of \textit{IL10RB}. Within this LD block, three potentially functional SNPs other than rs2834167 are present (Fig. 1). A SNP in the 5' flanking region of \textit{IL10RB}, rs999788, is located at a NF-κB binding site, and a transcription factor binding site prediction program (MATCH, http://www.gene-regulation.com/index.html) predicted that rs999788 T allele, but not C allele, forms the binding site of NF-κB. Two nonsynonymous SNPs of \textit{IFNAR2}, rs4986956 (F8S) and rs7279064 (V10F), are also included in the LD block. Both the \textit{IL10RB} and \textit{IFNAR2} genes have been shown to be associated with HBV persistence [18].
We therefore examined whether these SNPs are associated with SSc. As shown in Table 1, \textit{IL10RB} rs999788 C/C genotype was significantly increased in SSc ($P = 0.028$, OR = 1.65, 95%CI 1.05-2.59). This association was again mainly attributable to dcSSc. On the other hand, significant association was not observed between \textit{IFNAR2} SNPs and SSc. All control genotypes were compatible with Hardy-Weinberg equilibrium.

**Primary contribution of rs2834167**

The two \textit{IL10RB} SNPs associated with SSc, rs2834167 and rs999788, were found to be in strong LD (Fig. 1). To distinguish the contributions, two-locus analysis was performed (Table 2). Subjects were classified into four groups (a-d) with respect to the carriage of the risk genotype at rs2834167 (A/A) and rs999788 (C/C), and each risk of group a-c was compared with that of group d. While the OR was significantly elevated in the group b carrying only the risk genotype at rs2834167, no patient belonged to the group c carrying the risk genotype only at rs999788, suggesting that rs2834167 plays a primary role of the two SNPs. This issue was also addressed by haplotype analysis (Table 3). If rs999788 also contributes to the genetic risk to SSc, then the haplotype containing risk alleles of both SNPs should reveal strong association. Seven haplotypes were inferred, but permutation test revealed no significant difference in the haplotype frequency between dcSSc and controls. These results suggested that rs2834167 encoding E47K, rather than the 5’ flanking region polymorphism rs999788, is primarily associated with dcSSc.

**Association of \textit{IL10RB} rs2834167 polymorphism and serum levels of IL-10**

IL-10 has been shown to induce IL-10 production via STAT3, and also to suppress LPS-induced production of IL-10 by a STAT3-independent pathway [21]. To address the functional significance of \textit{IL10RB} rs2834167 polymorphism, we compared serum IL-10 levels in 44 patients with dcSSc of each genotype (G/G 14, G/A 12, A/A 18). As shown in Fig. 2, serum IL-10 levels were significantly correlated with the number of rs2834167A allele ($P=0.007$).
Discussion

In the present study, we showed that rs2834167 coding for \textit{IL10RB} E47K was associated with susceptibility to dcSSc. \textit{IL10RB} gene has never been studied for the association of SSc. Our findings provided new evidence that supports the causal role of IL-10 or related cytokine signaling in the pathogenesis of SSc.

IL-10 stimulates B cells and T helper 2 (Th2) cells, which may result in production of autoantibodies. In the TSK/+ mouse, a genetic model for human SSc, loss of CD19 significantly decreased skin fibrosis as well as hypergammaglobulinemia and autoantibody production [22]. In addition to the roles on the B cells, IL-10 can promote fibrosis by modulating the Th1/Th2 balance. IL-4, IL-6, and IL-13 stimulate the synthesis of collagen by human fibroblasts [23], while Th1 cytokines such as IFN-\(\gamma\) and TNF-\(\alpha\) suppress collagen production by fibroblasts [23]. Anti-IL-4 treatment [24] and IL-4R\(\alpha\) disruption [25] have been shown to ameliorate fibrosis in TSK/+ mice. In a silica-induced lung fibrosis model, pulmonary overexpression of IL-10 induced profibrotic Th2 cytokines IL-4 and IL-13, and exacerbated lung fibrosis [26]. These observations indicate a crucial role for B cells and Th2 in skin fibrosis in \textit{in vitro} and animal studies.

The role of B cells and Th2 cells have also been shown in human SSc [27]. We previously reported association of SNPs in \textit{CD19} [7] and \textit{CD22} [9] with human SSc. Serum IL-10 level was elevated in dcSSc [13], and was positively correlated with skin and pulmonary fibrosis [8]. These results support that excessive IL-10 signaling may lead to fibrosis, and suggest that \textit{IL10RB} 47K allele product leads to excessive IL-10 signaling.

\textit{IL10} gene is located on 1q31-32; therefore, \textit{IL10} SNPs cannot be in LD with \textit{IL10RB}. Thus, association of serum IL-10 levels with \textit{IL10RB} should be explained by a functional connection between IL-10R signaling and IL-10 induction. IL-10 has been shown to induce IL-10 production via STAT3, and also to suppress LPS-induced production of IL-10 by a STAT3-independent pathway in primary human monocyte - derived macrophages [21]. Two hypotheses can be envisaged on the mechanisms of elevated serum IL-10 in individuals with \textit{IL10RB} 47K/K genotype.
The first possibility is that IL-10R2 E47K substitution induces stronger signals in STAT3-dependent pathway of IL-10 production. IL-10R2 contributes little to IL-10 binding affinity, but acts as an accessory subunit for signaling when IL-10 binds to IL-10R1. The principal function of IL-10R2 appears to be a recruitment of a Jak kinase (Tyk2) into the signaling pathway, which subsequently leads to STAT3 activation [17]. E47K substitution may enhance STAT3 activation and IL-10 production through the influence on the structure of the IL-10 receptor complex, since the SNP E47K introduces a nonconservative substitution in the extracellular domain.

Another possibility is the reduction of suppression of STAT3-independent IL-10 production through IL-10R2. Frodsham et al. previously reported that the mRNA expression level of the \textit{IL10RB} 47K allele was lower than that of 47E allele, and that insufficient inhibition mediated by \textit{IL10RB} 47K resulted in higher LPS-induced TNF-\(\alpha\) release [18]. Similar mechanism is likely to play a role in IL-10 production. The lower mRNA expression of \textit{IL10RB} 47K may be due to the strong LD with the promoter SNP rs999788 C that lacks NF-\(\kappa\)B binding site.

These possibilities are not mutually exclusive. \textit{IL10RB} E47K could also be associated with SSc by mechanisms other than IL-10 signaling. IL-10R2 is a shared component of receptors for IL-10, IL-22, IL-26 and IFN-\(\lambda\) (IL-28, IL-29) [17]. In relation to the pathogenesis of dcSSc, it is remarkable that IL-20R1 that forms IL-26 receptor complexes with IL-10R2 is strongly expressed in skin and lung [28]. IL-22R1 is also expressed in the lung [29]. Furthermore, IFN-\(\lambda\) signaling induces similar repertoire of genes induced by IFN-\(\alpha/\beta\) [30], which have been shown to be upregulated in the peripheral blood expression profile of patients with SSc [31]. Thus, \textit{IL10RB} polymorphism may potentially be involved in the pathogenesis of SSc by multiple pathways.

In conclusion, this study suggested that \textit{IL10RB} E47K contributes to the susceptibility to dcSSc. Because the sample size of SSc patients was small, our present observations need to be replicated in the future. If confirmed, such findings will provide a link between IL-10 or related cytokines and fibrosis.
Acknowledgements

This work was supported by Grant-in-Aid for Scientific Research on Priority Areas ‘Applied Genomics’ from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Grant-in-Aid for Scientific Research (B) from Japan Society for the Promotion of Science (JSPS), grants from the Ministry of Health, Labour and Welfare of Japan, Japan Rheumatism Foundation and The Naito Foundation.
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Figure legends

Figure 1. Organization of *IL10RB* and *IFNAR2* gene in human chromosome 21 and linkage disequilibrium among four SNPs examined in this study, 
(lower) Pairwise $D'$ values among the four SNPs calculated using the genotypes of the control group in this study. Strong LD was observed between *IL10RB* rs2834167 and rs999788 ($D'=0.94$) and also between *IFNAR2* rs4986956 and rs7279064 ($D'=1.0$), but LD between the SNPs of *IL10RB* and *IFNAR2* was moderate.

Figure 2. Association of *IL10RB* rs2834167 G>A (E47K) and serum IL-10 level in patients with dcSSc.
Serum IL-10 level was correlated with the number of rs2834167A (47K) allele ($P = 0.007$, simple regression analysis).
Figure 2

The figure shows the serum IL-10 level (pg/ml) for different genotypes at rs2834167. The equation for the regression line is $y = 4.80x + 8.96$, with a coefficient of determination $r^2 = 0.159$ and a p-value $P = 0.007$.

- rs2834167 G/G: 9.8 ± 4.9 pg/ml
- rs2834167 G/A: 11.8 ± 5.7 pg/ml
- rs2834167 A/A: 19.2 ± 13.5 pg/ml
Table 1. Genotype frequencies of four SNPs in *IL10RB* and *IFNAR2* in patients with SSc and healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>All SSc (n=124)</th>
<th>dcSSc (n=46)</th>
<th>lcSSc (n=78)</th>
<th>controls (n=398)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL10RB</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs2834167G&gt;A (E47K)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G</td>
<td>34 (0.27)</td>
<td>14 (0.31)</td>
<td>20 (0.26)</td>
<td>111 (0.28)</td>
</tr>
<tr>
<td>G/A</td>
<td>53 (0.43)</td>
<td>13 (0.28)</td>
<td>40 (0.51)</td>
<td>204 (0.51)</td>
</tr>
<tr>
<td>A/A</td>
<td>37 (0.30) 1</td>
<td>19 (0.41) 2</td>
<td>18 (0.23)</td>
<td>83 (0.21)</td>
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<tr>
<td><strong>IL10RB 5' flanking region rs999788 T&gt;C</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T/T</td>
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<td>14 (0.30)</td>
<td>19 (0.24)</td>
<td>119 (0.30)</td>
</tr>
<tr>
<td>T/C</td>
<td>53 (0.43)</td>
<td>16 (0.35)</td>
<td>37 (0.48)</td>
<td>195 (0.49)</td>
</tr>
<tr>
<td>C/C</td>
<td>38 (0.31) 3</td>
<td>16 (0.35) 4</td>
<td>22 (0.28)</td>
<td>84 (0.21)</td>
</tr>
<tr>
<td><strong>IFNAR2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs4986956 T&gt;C (F8S)</td>
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<td></td>
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<tr>
<td>T/T</td>
<td>97 (0.78)</td>
<td>38 (0.83)</td>
<td>59 (0.76)</td>
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<tr>
<td>T/C</td>
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<td>7 (0.15)</td>
<td>19 (0.24)</td>
<td>96 (0.24)</td>
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<td>0 (0)</td>
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<td><strong>IFNAR2</strong></td>
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<td></td>
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<tr>
<td>rs7279064 T&gt;G (F10V)</td>
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<tr>
<td>T/T</td>
<td>26 (0.21)</td>
<td>12 (0.26)</td>
<td>14 (0.18)</td>
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<td>T/G</td>
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<td>23 (0.50)</td>
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<td>G/G</td>
<td>31 (0.25)</td>
<td>11 (0.24)</td>
<td>20 (0.26)</td>
<td>129 (0.32)</td>
</tr>
</tbody>
</table>

Genotype frequencies are shown in parentheses.

- rs2834167 A/A against (G/G + G/A): 1 all SSc versus controls, \( P = 0.038 \), OR = 1.61, 95% CI 1.02-2.54.
- dcSSc versus controls: \( P = 0.0018 \), OR = 2.67, 95% CI 1.42-5.04.

- rs999788 C/C against (T/T + T/C): 3 all SSc versus controls, \( P = 0.028 \), OR = 1.65, 95% CI 1.05-2.59.
- dcSSc vs controls: \( P = 0.036 \), OR = 1.99, 95% CI 1.04-3.83.

Other comparisons did not reveal statistical significance.
Table 2. Two-locus analysis support a primary role of *IL10RB* rs2834167A/A (47K/K) genotype

<table>
<thead>
<tr>
<th>group</th>
<th>rs2834167 A/A</th>
<th>rs999788 C/C</th>
<th>dcSSc (n=46)</th>
<th>controls (n=398)</th>
<th>OR</th>
<th>95%CI</th>
<th>P</th>
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<td>a</td>
<td>+</td>
<td>+</td>
<td>16 (0.35)</td>
<td>78 (0.20)</td>
<td>2.35</td>
<td>1.21-4.57</td>
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<tr>
<td>b</td>
<td>+</td>
<td>-</td>
<td>3 (0.07)</td>
<td>5 (0.01)</td>
<td>6.87</td>
<td>1.56-30.30</td>
<td>0.025</td>
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<tr>
<td>c</td>
<td>-</td>
<td>+</td>
<td>0 (0)</td>
<td>6 (0.02)</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>d</td>
<td>-</td>
<td>-</td>
<td>27 (0.58)</td>
<td>309 (0.77)</td>
<td>referent</td>
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</table>

Patients with dcSSc and controls were classified into groups a-d according to the presence of the risk genotypes of rs2834167 and rs999788. Numbers and proportions (in parentheses) of each group among the patients and controls are shown. Odds ratio, 95% confidence interval and *P* value of each group were calculated against group d.
Table 3. Inferred haplotype frequencies in dcSSc and controls.

<table>
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<tr>
<th>haplotype</th>
<th>SNP1</th>
<th>SNP2</th>
<th>SNP3</th>
<th>SNP4</th>
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<td>T</td>
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<td>0.082</td>
<td>0.098</td>
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<td>T</td>
<td>T</td>
<td>G</td>
<td>0.092</td>
<td>0.078</td>
<td>0.999</td>
</tr>
<tr>
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<td>G</td>
<td>C</td>
<td>A</td>
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<td>0.075</td>
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<td>C</td>
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<td>0.028</td>
<td>0.998</td>
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<td>G</td>
<td>T</td>
<td>A</td>
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</tr>
</tbody>
</table>

Haplotype frequencies were inferred using Haploview v4.0. 100,000 permutations were performed for association test.

SNP1: rs4986956 (*IFNAR2* F8S), SNP2: rs7279064 (*IFNAR2* V10F), SNP3: rs999788, SNP4: rs2834167 (*IL10RB* E47K)