

## Estrogen receptor gene polymorphisms in Japanese patients with systemic sclerosis

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### ABSTRACT

**Objective.** To investigate whether single-nucleotide polymorphisms (SNPs) within the estrogen receptor (ER)  $\alpha$  and  $\beta$  genes are associated with disease susceptibility and clinical presentation in Japanese patients with systemic sclerosis (SSc).

**Methods.** Three SNPs, ER $\alpha$  PvuII T/C, ER $\alpha$  XbaI A/G, and ER $\beta$  RsaI G/A, were genotyped using polymerase-chain reaction combined with restriction fragment length polymorphisms in 103 patients with SSc and 56 race-matched healthy controls. The distribution of the individual ER SNPs in SSc patients with or without SSc-related organ involvement and serum antinuclear antibodies was determined.

**Results.** The frequency of the ER $\alpha$  XbaI GG phenotype was significantly lower in SSc patients than in healthy controls (2% vs. 13%,  $p=0.005$ , odds ratio=0.14, 95%CI 0.03-0.69), and no significant association was detected for the other SNPs. Except in the case of heart involvement accompanying SSc, there was no significant association of the ER SNPs with SSc-related individual organ involvement or with antinuclear antibody profiles. Specifically, the ER $\alpha$  PvuII CC phenotype was significantly more frequent among patients with heart involvement compared with those without it (75% vs. 14%,  $p=0.0001$ , odds ratio=17.4, 95%CI 3.2-94.8).

**Conclusion.** SNPs located within the ER $\alpha$  gene may contribute to disease susceptibility and to certain clinical manifestations of SSc patients.

### Introduction

Systemic sclerosis (SSc) is characterized by thickening of the skin and visceral organs, and by microvascular injury (1). There is increasing evidence that SSc is a complex disorder that results from interactions between genetic factors and environmental stimuli. Polymorphisms within genes encoding various cytokines and extracellular matrixes are associated with susceptibility to SSc (2). In addition, strong associations between the human leukocyte antigen (HLA) class II allele and SSc-related autoantibody production have been demonstrated in various ethnic

groups (3). On the other hand, exposure to environmental factors, such as organic solvents and other chemicals used in industries, has been reported to increase the risk of SSc (4). A familial study has suggested that exposure to occupational inhalation of organic solvents triggers the development of SSc in subjects with certain genetic backgrounds (5). In addition, a recent meta-analysis showed that, among subjects with occupational exposure to organic solvents, men are at higher risk than women for SSc (6). These findings suggest that genetic and environmental factors may interact with each other to promote the development of SSc. Interestingly, some organic solvents are known to bind steroid receptors, with the potential result of alterations in several biological pathways in humans: such reagents are called endocrine-disrupting chemicals (7). The estrogen receptor (ER) is one of the steroid receptors thought to be involved in endocrine disruption. It is a ligand-activated transcription factor that is expressed on a variety of cell types, such as macrophages, lymphocytes, and endothelial cells, and mediates a variety of functions (8). Two homologous subtypes of the ER have been identified in humans: ER $\alpha$  (6q25.1) and ER $\beta$  (14q22-24) (8). Gene polymorphisms within the ER loci are associated with bone mineral density (9), breast cancer (10), and coronary heart disease (11). To evaluate the potential roles of polymorphisms within ER genes in the pathogenesis of SSc, the distribution of three well-characterized ER single-nucleotide polymorphisms (SNPs) was determined in Japanese patients with SSc.

### Materials and methods

#### Patients and controls

We studied 103 unrelated Japanese SSc patients (12 males and 91 females) who were seen at Keio University Hospital or at Den-en Chofu Central Hospital from October 1992 through January 2005. All the patients satisfied the American College of Rheumatology (ACR; formerly the American Rheumatism Association) preliminary classification criteria for SSc (12). None of the patients had occupational exposure to organic solvents. Twenty-five patients had diffuse

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cutaneous SSc and 78 patients were classified as having limited cutaneous SSc. Their mean age at disease onset (first symptom attributable to SSc) was 44.1±13.1 years (range 17-78), and the mean observation period after diagnosis was 12.6±6.1 years (range 3.2-28.6). Fifty-six healthy Japanese volunteers living in the Tokyo area were selected as race-matched healthy controls. Blood samples were obtained from all the subjects after they gave written informed consent, as approved by the Institutional Review Boards.

#### Clinical features

Clinical and laboratory findings were prospectively recorded for all patients. Complete medical histories, physical examinations, and laboratory analyses were performed at the first visit of all the patients, and more limited evaluations were performed during follow-up visits (at least once every 3 months). Information regarding the SSc-related involvement of five major organs: joints, esophagus, heart, kidney, and lung (*i.e.*, pulmonary interstitial fibrosis), was obtained from all patients. The criteria used to define individual organ involvement were as described elsewhere (13).

#### Identification of SSc-related antinuclear antibodies (ANAs)

Serum samples from all the SSc patients were analyzed for three major SSc-related ANAs, including anticentromere, anti-Scl-70/topoisomerase I (topo I),

and anti-U1 ribonucleoprotein (U1RNP) antibodies. These ANAs were detected using indirect immunofluorescence, double immunodiffusion, and RNA immunoprecipitation assays (13).

#### Determination of ER $\alpha$ and ER $\beta$ SNPs

Although the nomenclature for ER gene polymorphisms has been inconsistent and confusing, most results have been derived using the restriction fragment length polymorphism (RFLP) method. We used this method, choosing as specific restriction enzyme sites PvuII and XbaI (one site each) in intron 1 of the ER $\alpha$  gene (10), and the unique Rsa I restriction site in the ligand-binding domain of exon 5 of the ER $\beta$  gene (14). Briefly, genomic DNA samples were isolated from peripheral blood leukocytes using the phenol extraction procedure. The presence or absence of the two SNPs within ER $\alpha$  (PvuII T/C and XbaI A/G) and one SNP within ER $\beta$  (RsaI G/A) were determined using RFLPs combined with polymerase-chain reaction (PCR)-amplified genomic DNA, as described previously (10, 14). The PCR products were digested with the corresponding restriction enzymes, separated on a 1% agarose gel, and stained with ethidium bromide.

#### HLA class II allele genotyping

The HLA-DRB1, DQB1, and DPB1 alleles were identified using RFLPs of PCR-amplified genomic DNA as described previously (15).

#### Statistical analysis

The distribution of the genotype frequencies was assessed using the Hardy-Weinberg equilibrium. Phenotypic frequencies were tested for statistical significance using the 2 x 3 chi-square test. Significant differences (overall  $p < 0.05$ ) were further analyzed by pair-wise comparisons using 2 x 2 chi-square tests. Odds ratios with a 95% confidence interval (CI) were calculated for statistically significant differences. To analyze the interactive effects of ER SNPs and HLA class II alleles, complex interactions for contingency tables were investigated by fitting log-linear models according to the hierarchical principle, as described previously (16).

#### Results

##### ER $\alpha$ and ER $\beta$ SNPs in SSc patients

The phenotypic frequencies of three SNPs within the ER $\alpha$  and ER $\beta$  loci in SSc patients and healthy controls are shown in Table I. The distribution of these phenotypes in SSc patients and healthy controls conformed to the Hardy-Weinberg equilibrium. There was no difference in the distribution of ER $\alpha$  PvuII or ER $\beta$  RsaI SNPs between SSc patients and healthy controls, but the distribution of ER $\alpha$  XbaI was significantly different between them (overall  $p = 0.01$ ). In pair-wise analysis, the frequency of XbaI GG was significantly lower in SSc patients than in healthy controls ( $p = 0.005$ ). ER $\alpha$  PvuII and XbaI SNPs were linked to some extent, but there was no significant difference in nine combined genotypes of these two SNPs between SSc patients and controls. When SSc patients were divided into groups that had diffuse or limited cutaneous SSc, a trend for a decreased frequency of XbaI GG was detected for both disease subsets, but without statistical significance. Among three subgroups, classified by their ER $\alpha$  and ER $\beta$  SNP phenotypes, there were no differences in the frequency of individual DRB1, DQB1, and DPB1 alleles. In addition, we failed to detect any interactive effect of the ER SNPs and HLA class II alleles on the development SSc.

**Table I.** Phenotypic frequencies of three SNPs within the ER $\alpha$  and ER $\beta$  genes in Japanese patients with SSc and race-matched healthy controls.

	SSc (n=103)	Healthy controls (n=56)	Overall $p$ -value
ER $\alpha$ PvuII			
CC	19 (18%)	8 (14%)	0.8
CT	45 (44%)	26 (47%)	
TT	39 (38%)	22 (39%)	
ER $\alpha$ XbaI			
GG	2 (2%)	7 (13%)	0.01*
AG	28 (27%)	9 (16%)	
AA	73 (71%)	40 (71%)	
ER $\beta$ RsaI			
GG	52 (50%)	32 (57%)	0.08
AG	46 (45%)	17 (30%)	
AA	5 (5%)	7 (13%)	

\*In pair-wise analysis, the frequency of ER $\alpha$  XbaI GG in SSc patients was significantly lower than in healthy controls ( $p = 0.005$ , odds ratio=0.14, 95%CI 0.03-0.69).

### Associations of ER $\alpha$ and ER $\beta$ SNPs with SSc-related organ involvement and ANAs

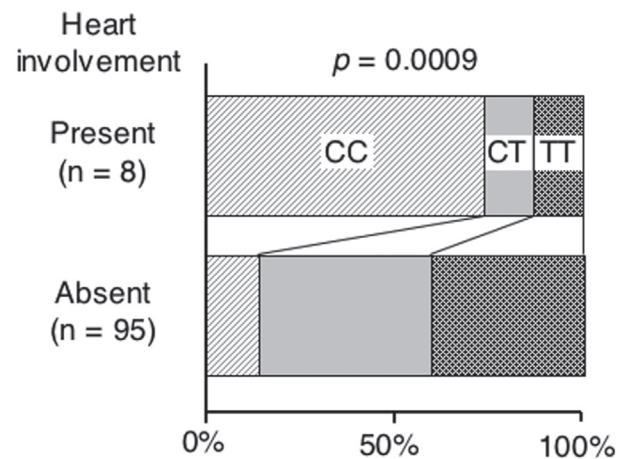
We further evaluated potential associations of the ER $\alpha$  and ER $\beta$  SNPs with organ involvement and SSc-related ANA profiles in the SSc patients. None of the ER SNPs was associated with organ involvement (joint, esophagus, kidney, or lung) or SSc-related ANA (anticentromere, anti-topo I, or anti-U1RNP antibody), but there was a strong association between the ER $\alpha$  PvuII T/C phenotype and heart involvement (overall  $p=0.0009$ ) (Fig. 1).

Heart involvement was observed in eight of the 103 SS patients (8%): five with left ventricular congestive heart failure not attributable to any other condition, such as ischemic heart disease, valvular heart disease, infectious myocarditis, two with conduction defects or arrhythmia requiring treatment, and one with massive pericardial effusion. In pair-wise analysis, the frequency of the PvuII CC phenotype was significantly higher in patients with heart involvement than in those without (75% vs. 14%,  $p=0.0001$ , odds ratio = 17.4, 95%CI 3.2-94.8). In the six patients with heart involvement and the PvuII CC phenotype, four were men, four were positive for anti-topo I antibody, and five had left ventricular cardiomyopathy in the absence of significant stenosis on coronary angiography.

### Discussion

We have examined potential associations between the development and disease manifestation of SSc and three ER gene SNPs, and found that the frequency of XbaI GG is marginally decreased in SSc patients and that PvuII CC was associated with increased risk of heart involvement. These ER $\alpha$  gene polymorphisms were shown to be associated with age at disease onset and with the clinical manifestations of patients with systemic lupus erythematosus (SLE) (17-19). This finding makes sense, because SLE occurs predominantly in women of childbearing age, and sex hormones such as estrogen are known to play a role in SLE onset and perpetuation (20). In contrast, SSc is a disease commonly observed

**Fig. 1.** Distribution of the ER $\alpha$  PvuII SNP phenotype in SSc patients according to the presence or absence of heart involvement. Phenotypic frequencies were tested for statistical significance using 2x3 chi-square test.



in middle-aged women who have declining levels of endogenous estrogens, which suggests that some other disease mechanisms that modulate pathogenic processes through ER gene polymorphisms are likely to be responsible in this patient group. In this regard, estrogens may protect SSc patients against microvascular damage, since estrogen administration prevents the development of isolated pulmonary arterial hypertension (21) and improves peripheral vascular disease in SSc patients (22), although few data on the estrogen treatment have been reported in SSc patients. Alternatively, it is possible that ER gene polymorphisms may modulate the risk of developing SSc in the presence of exposure to organic solvents that are capable of binding to the ER $\alpha$  as endocrine-disrupting chemicals. Although our subjects did not report any occupational exposure to organic solvents, some exposure to such solvents can be experienced by anyone participating in solvent-oriented hobbies, such as painting, gardening with pesticides, and ceramics. This low-grade exposure is also a known risk for developing SSc (23).

The statistical significance of the association between the ER $\alpha$  PvuII CC phenotype and heart involvement in SSc patients was impressive, although the number of patients with heart involvement was relatively small. How this polymorphism alters the pathogenic process of SSc-related heart involvement is unknown. It has been reported that the PvuII C allele produces a binding site for the myb family of

transcription factors, and in the presence of B-myb, there is a four-fold up-regulation of downstream reporter signals in the presence of the PvuII C allele compared with the PvuII T allele (24). Thus, the PvuII C allele could lead to increased sensitivity to ER ligands. Several large cohort studies have shown that the PvuII CC allele is associated with cardiovascular disease (25, 26), and the PvuII TT allele was associated with myocardial infarction, in another study (11). These inconsistent findings may indicate that the effects of ER ligand stimulation in the development of heart disease are not simple. Estrogens are known to exert their cardioprotective effects through a number of indirect and direct ER $\alpha$ -dependent mechanisms. That is, ER ligands can directly modulate the functions of cardiovascular cells, including myocardial cells, endothelial cells, and vascular smooth muscle cells, all of which express ER $\alpha$ , but some of the protective effects of estrogens could also be mediated through systemic effects, such as changes in lipid profile, coagulation, and fibrinolytic systems (27). Interestingly, in the current study, cardiomyopathy without apparent evidence for coronary artery disease in association with the ER $\alpha$  PvuII CC phenotype was detected predominantly in male patients with anti-topo I antibody. These features are consistent with those observed in organic solvent-associated SSc (4), suggesting that the ER $\alpha$  PvuII CC phenotype contributes to increased sensitivity to certain organic solvents.

It is also possible that increased sensitivity to estrogens may have more impact in males whose estrogen levels are much lower than females.

In summary, the associations reported here between certain ER $\alpha$  polymorphisms and SSc may indirectly support the concept that ER ligands, such as estrogens and potential endocrine-disrupting chemicals, are involved in the pathogenesis of SSc. Further large-scale studies of SSc patients, including those with occupational exposure to organic solvents, will be required to confirm the role of ER $\alpha$  gene polymorphisms in SSc pathogenesis.

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