doi: 10.3325/cmj.2009.50.117

Association of Estrogen Receptor α Gene Polymorphisms with Cytokine Genes Expression in Systemic Lupus Erythematosus

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Aim To analyze the association of estrogen receptor α (ORα) gene polymorphisms with cytokine genes expression in patients with systemic lupus erythematosus (SLE) and controls.

Methods Genomic DNA was extracted and polymorphisms of XbaI (XX, Xx, or xx genotype) and PvuII (PP, Pp, or pp) in intron 1 of ORα gene were detected by polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) method. The messenger RNA (mRNA) levels of interleukin (IL)-10, IL-4, interferon (IFN)-γ, and IL-2 were assessed by semiquantitative reverse transcription polymerase chain reaction (RT-PCR).

Results In patients with SLE with PpXx genotype, IL-10 and IL-4 mRNA expression was higher ($P<0.001$ and $P=0.013$, respectively), while in patients with SLE with Ppxx genotype IFN-γ and IL-2 mRNA expression was lower than in controls ($P<0.001$). There was no significant difference in mRNA expression of 4 cytokines among controls with various genotypes.

Conclusion ORα gene polymorphism may be associated with the expression of IL-10, IL-4, IL-2, and IFN-γ in patients with SLE.

Received: October 27, 2008

Accepted: February 27, 2009

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Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by polyclonal B cell activation and overproduction of pathogenic autoantibodies (1,2). Although etiopathology of SLE is not clearly understood, the mechanism seems to be affected by sex hormones. Epidemiologically, there is a female predominance in the number of diseased of about 90%. The peak incidence of SLE occurs in the reproductive period, whereas the onset of SLE in postmenopause, characterized by loss of estrogen production, is relatively uncommon (3).

Estrogen must combine with target tissue cytolymph or intranuclear estrogen receptor in order to produce a marked effect. However, estrogen receptor α (ORα) produces effects by binding to a specific estrogen response element, which is cis-acting enhancer located within the regulatory regions of target genes. Human ORα gene, also named *ESR1*, is located on chromosome 6q25, which comprises 8 exons separated by 7 intronic regions and spans more than 140 kilobases (4,5). In intron 1, there are 2 polymorphism sites: the PvuII polymorphism, caused by a T/C transition and located approximately 0.4 kb upstream of exon 2, and the XbaI polymorphism (XX, Xx, or xx genotype), located approximately 50 bp away from the PvuII polymorphism (PP, Pp, or pp genotype) site. Distribution of ORα gene polymorphism has been related to sex, disease onset age, and clinical symptoms (6-8). For example, unusual PvuII C and XbaI G alleles have been associated with a milder form of SLE, characterized by skin manifestations, later onset, and less organ damage (7). The frequency of the combined ppXx genotype was greater in patients with childhood onset SLE than in controls or patients with adult onset SLE (8). Correlation of ORα gene polymorphism with some definite clinical manifestation suggests that ORα gene polymorphism probably serve as a genetic background of SLE. In addition, it has been demonstrated that imbalance between Th1 and Th2 cytokine production plays a key role in the induction and development of several autoimmune diseases. In patients with SLE, serum levels of Th2 cytokines, such as interleukin (IL)-4, IL-6, and IL-10, are elevated, while a decrease in production of Th1 cytokines, including IL-2 and interferon (IFN)-γ, is observed (9-12). However, there have been no reports on the relationship between the ORα gene polymorphism and the cytokines expression in patients with SLE. So, we analyzed the relationship between PvuII and XbaI restriction fragment length polymorphism (RFLP) of ORα and the expression of a few cytokine genes in patients with SLE.

PARTICIPANTS AND METHODS

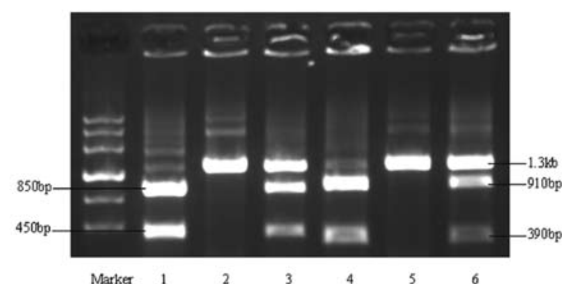
Study participants

The present study included 378 women – 157 healthy controls and 221 patients with SLE. patients with SLE were recruited from the Department of Rheumatism, Shandong Provincial Hospital, whereas the controls were selected from the staff members of Shandong University from March to December 2007. The inclusion criterion for patients with SLE was the fulfillment of at least 4 of American College of Rheumatology classification criteria (13,14). The age of patients was 27.6 ± 7.4 and that of controls was 28.3 ± 5.4. All participants gave their informed consent before enrollment. The study was approved by the ethics committee of Shandong Provincial Hospital and the Shandong University.

Estrogen receptor gene α polymorphism analysis

Genomic DNA was extracted from whole blood (EDTA treated) using the TIANamp blood DNA kit supplied by Tiangen (Beijing, China). To genotype the PvuII and XbaI restriction polymorphic sites in intron 1 of ORα gene, polymerase chain reaction (PCR) and specific oligonucleotide primers were used. The upstream primer sequence was 5'-CTG CCA CCC TAT CTG TAT CTT TTCCTA TTC TCC-3', and downstream primer sequence was 5'-TCT TTC TCT GCC ACC CTG GCG TCG ATT ATC TGA-3'. This pair of primers predicts a production of 1.3 kb (15). The reaction was carried out under the following conditions: at 95°C for 7 minutes, 35 cycles at 94°C for 30 seconds, at 62°C for 40 seconds, at 72°C for 90 seconds, and finally at 72°C for 10 minutes. After amplification, the PCR products were digested with 10 units PvuII or XbaI at 37°C for 7 hours and electrophoresed

Figure 1.



The genotype of estrogen receptor α gene after PvuII or XbaI digesting. 1 – pp genotype; 2 – PP genotype; 3 – Pp genotype; 4 – xx genotype; 5 – XX genotype; 6 – Xx genotype.

TABLE 1. The primer sequence of interleukin-10, interferon-γ, interleukin-4, interleukin-2, and β-actin

Cytokine	The primer sequence	
Interleukin -10	5′-TTA CCT GGA GGA GGT GAT GC-3′	5′-TGG GGG TTG AGG TAT CAG AG-3′
Interferon-γ	5′-GCA GAG CCA AAT TGT CTC CT-3′	5′-ATG CTC TTC GAC CTC GAA AC-3′
Interleukin -4	5′-CTG CAA ATC GAC ACC TAT TA-3′	5′-GAT CGT CTT TAG CCT TTC CA-3′
Interleukin -2	5′-TGC CAC AAT GTA CAG GAT GC-3′	5′-GCC TTC TTG GGC ATG TAA AA-3′
β-actin	5′-CTC CAT CCT GGC CTC GCT GT-3′	5′-GCT GTC ACC TTC ACC GTT CC-3′

in 1% ethidium bromide-agarose gel. PvuII digestion resulted in genotypes PP (1.3kb), Pp (1.3kb, 850bp, 450bp), and pp (850bp, 450bp), while XbaI digestion resulted in genotypes XX (1.3kb), Xx (1.3kb, 910bp, 390bp), and xx (910bp, 390bp) (Figure 1).

Total RNA extraction for molecular studies

Mononuclear cells were isolated from 5 mL fresh human peripheral blood with Lymphocytes Separation Medium (LSM, Krackeler Scientific, Albany, NY, USA). Total RNA from 5×10^6 peripheral blood mononuclear cells (PBMC) was obtained from all patients by a procedure described elsewhere (16).

Synthesis of complementary DNA (cDNA)

Synthesis of cDNA was performed by heating total RNA from 5×10^6 PBMCs at 72°C for 10 minutes, cooling it on ice, and adjusting it to a total volume of 20 µL containing 0.5 µmol dNTPs, 3 µg of random primers, 10 U RNase inhibitor, and 200 U M-MLV reverse transcriptase. Samples were incubated at 37.5°C for 60 minutes, heated to 95°C for 10 minutes in order to inactivate traces of reverse transcriptase activity, and stored at -20°C.

Analysis of cytokine gene expression by semiquantitative PCR method

With cDNA as the template, PCR was carried out with primers for IL-10, IFN-γ, IL-4, IL-2, and β-actin (Table 1). The procedure conditions were pre-denaturation at 95°C for 7 minutes; 35 cycles of denaturation at 94°C for 60 seconds, annealing at 64°C (IL-10), 52°C (IFN-γ), 60°C (IL-4), 60°C (IL-2) for 30 seconds, extension at 72°C for 60 seconds, and the final cycle of further 10 minutes at 72°C. Each cytokine was amplified with β-actin at the same time in order to detect the cytokine's quantity by ratios of the cytokine and β-actin.

Statistical methods

Genotype frequencies were calculated. Results from the control and test groups were compared using the χ^2 test or Fisher exact test for small samples. In each group, the observed distribution of homozygotes and heterozygotes was in agreement with the Hardy-Weiberg equilibrium. $P > 0.05$ indicated that the groups had the same genetic equilibrium and that the data were drawn from the same Mendelian population. Differences in cytokines expression data (mean ± standard deviation) among the various ORα genotypes were tested by Kruskal-Wallis test. Differences between patients with SLE and controls were determined by t test or Mann-Whitney test for the group of skewed distribution or heterogeneity of variance. $P < 0.05$ was considered statistically significant. SPSS statistical package, version 13.0 for Windows (SPSS Inc., Chicago, IL, USA) was used.

RESULTS

Distribution of ORα genotypes in patients with SLE

The distribution of genotypes in both patients and controls was in agreement with the Hardy-Weinberg equilibrium ($P > 0.05$). There was no significant difference in the allele frequency of PvuII or XbaI RFLPs between control and SLE group (Table 2). Genotypes ppXX and ppXx were not found in any participant and PpXX was not found in any

TABLE 2. Allele and genotype frequencies (No., %) of estrogen receptor α (ORα) polymorphism in controls and patients with systemic lupus erythematosus (SLE)

	SLE (n = 221)	Control (n = 157)	*P**
For PvuII			
Allele P	160 (36.2)	92 (29.3)	0.051
p	282 (63.8)	222 (70.7)	
Genotype PP	34 (15.4)	18 (11.5)	0.153
Pp	92 (41.6)	56 (35.7)	
pp	95 (43.0)	83 (52.9)	
For XbaI			
Allele X	93 (21.0)	52 (16.6)	0.134
x	349 (79.0)	262 (83.4)	
Genotype XX	10 (4.5)	7 (4.5)	0.171
Xx	73 (33)	38 (24.2)	
xx	138 (62.4)	112 (71.3)	

*Hardy-Weiberg equilibrium test.

TABLE 3. Distribution of estrogen receptor α (ORα) genotypes in patients with systemic lupus erythematosus (SLE) and controls (No., %)

Genotype	SLE	controls	P
PPXX	14 (6.3)	6 (3.8)	0.354
PPXx	22 (10.0)	9 (5.7)	0.183
PPxx	5 (2.3)	0	0.079†
PpXX	0	3 (1.9)	0.071†
PpXx	55 (24.9)	30 (19.1)	0.212
Ppxx	36 (16.3)	36 (22.9)	0.112
ppXX	0	0	NA
ppXx	0	0	NA
ppxx	89 (40.3)	73 (46.5)	0.247

*NA –not applicable.
†Fisher exact test for small samples.

SLE patient. Ppxx, PpXx, and ppxx were found to be 3 major genotypes in patients with SLE and controls (Table 3).

PBMC cytokine mRNA expression

The levels of IL-10 and IL-4 mRNA in patients with SLE was significantly higher than in controls ($P<0.001$). There were no significant differences between the two groups in the level of IFN-γ and IL-2 mRNA (Figure 2 and Table 4).

TABLE 4. Cytokine expression (relative expression to β-actin, mean ± standard deviation) in the peripheral blood mononuclear cells of patients with systemic lupus erythematosus (SLE) and controls

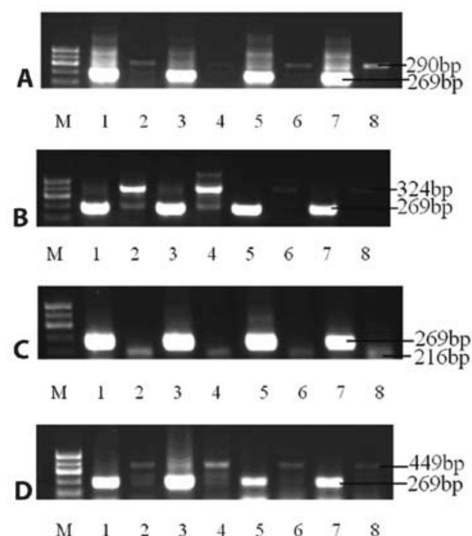
Cytokine	Controls (n = 157)	Patients with SLE (n = 221)
Interleukin-10	0.24 ± 0.06	0.37 ± 0.21*
Interleukin-4	0.38 ± 0.12	0.43 ± 0.16*
Interferon-γ	0.39 ± 0.17	0.40 ± 0.18
Interleukin-2	0.31 ± 0.09	0.31 ± 0.10

*$P<0.01$ by *t* test for independent samples comparing controls with patients with SLE.

The relationship between the ORα gene polymorphism and cytokines

The level of IL-10 and IL-4 mRNA in patients with SLE with PpXx genotype was much higher than in controls. The level of IFN-γ and IL-2 mRNA in patients with SLE with Ppxx genotype was lower than in controls (Table 5). Furthermore, cytokine gene expression was significantly different in patients with SLE with different ORα genotypes (Kruskal-Wallis test) (Table 6). The level of IL-10 mRNA in patients with PpXx was higher than in patients with PPXX, PPXx, Ppxx, and ppxx genotype ($P=0.018$, $P<0.001$, $P<0.001$, $P<0.001$, respectively), while the level of IL-4 mRNA in patients with PpXx genotype was higher than in patients with PPXX, Ppxx, and ppxx ($P=0.027$, $P=0.030$, $P=0.021$, respectively). The level of IFN-γ mRNA in patients with Ppxx genotype was lower than in patients with PPXX, PPXx, PPxx, PpXx, and ppxx ($P=0.009$, $P=0.010$, $P=0.046$, $P=0.003$, $P<0.001$, respectively), while the level of IL-2 mRNA in patients with Ppxx genotype was lower than in patients with PPXX, PPXx, PpXx, and ppxx ($P=0.035$, $P=0.042$, $P=0.001$, $P=0.001$, respectively, Mann-Whitney test).

Figure 2.



The mRNA expression of Th1 (interferon-γ and interleukin-2) and Th2 (interleukin-10 and interleukin-4) cytokines. (**A**) interferon-γ; (**B**) interleukin-10; (**C**) interleukin-2; (**D**) interleukin-4. Lane 1, 3, 5, 7 is the β-actin's expression. Lane 2 and lane 4 shows systemic lupus erythematosus patients' cytokine expression, whereas lane 6 and lane 8 show controls' cytokine expression.

DISCUSSION

Our study showed that ORα genotypes Ppxx, PpXx, and ppxx were 3 major genotypes both in patients with SLE and control participants. However, there was no significant difference in the distribution of these 3 ORα genotypes between patients and controls. We also found that the level of IL-10 and IL-4 mRNA in patients with SLE was significantly higher than in controls, while there was no significant difference in the level of IFN-γ and IL-2 mRNA between the groups. We further analyzed the association of the ORα gene polymorphisms with the Th1 and Th2 cytokine expression. The level of IL-10 mRNA in patients with SLE with PpXx genotype was higher than in healthy con-

TABLE 5. Cytokine expression (relative expression to β-actin, mean ± standard deviation) and estrogen receptor α (ORα) gene polymorphisms in patients with systemic lupus erythematosus (SLE) and control

	IL-10			IL-4			IFN-γ			IL-2		
Genotypes	SLE	controls	*P*	SLE	controls	*P*	SLE	controls	*P*	SLE	controls	*P*
PPXX	0.36 ± 0.18	0.23 ± 0.05	0.101	0.37 ± 0.14	0.35 ± 0.10	0.729	0.37 ± 0.13	0.38 ± 0.13	0.861	0.32 ± 0.09	0.31 ± 0.11	0.988
PPXx	0.26 ± 0.07	0.24 ± 0.05	0.393	0.45 ± 0.17	0.36 ± 0.08	0.165	0.36 ± 0.13	0.36 ± 0.07	0.958	0.29 ± 0.07	0.27 ± 0.08	0.654
PPxx	0.38 ± 0.22	NA	NA	0.39 ± 0.04	NA	NA	0.32 ± 0.01	NA	NA	0.28 ± 0.04	NA	NA
PpXX	NA	0.25 ± 0.10	NA	NA	0.27 ± 0.03	NA	NA	0.35 ± 0.07	NA	NA	0.37 ± 0.06	NA
PpXx	0.49 ± 0.23	0.27 ± 0.06	<0.001†	0.48 ± 0.16	0.39 ± 0.13	0.013‡	0.38 ± 0.18	0.37 ± 0.08	0.012†	0.31 ± 0.09	0.30 ± 0.08	0.485
Ppxx	0.28 ± 0.06	0.26 ± 0.05	0.147	0.41 ± 0.12	0.37 ± 0.12	0.214	0.27 ± 0.11	0.41 ± 0.05	<0.001†	0.25 ± 0.05	0.33 ± 0.12	<0.001†
ppxx	0.27 ± 0.10	0.25 ± 0.06	0.512	0.43 ± 0.17	0.38 ± 0.13	0.097	0.47 ± 0.26	0.41 ± 0.10	0.410	0.34 ± 0.13	0.31 ± 0.08	0.702

*NA – not applicable.
†Mann-Whitney test.
‡*t* test.

TABLE 6. Statistical significance of cytokine expression between patients with different genotypes in systemic lupus erythematosus

	P (Mann-Whitney test)			
Genotypes	interleukin-10	interleukin-4	interleukin-2	interferon-γ
PPXX & PPXx	0.061	0.150	0.597	0.987
PPXX & PPxx	0.823	0.823	0.754	1.000
PPXX & PpXx	0.018	0.027	1.000	0.858
PPXX & Ppxx	0.072	0.331	0.035	0.009
PPXX & ppxx	0.350	0.296	0.736	0.256
PPXx & PPxx	0.146	0.650	0.880	0.786
PPXx & PpXx	<0.001	0.321	0.554	0.627
PPXx & Ppxx	0.335	0.409	0.042	0.010
PPXx & ppxx	0.110	0.482	0.325	0.206
PPxx & PpXx	0.259	0.237	0.567	0.482
PPxx & Ppxx	0.301	0.954	0.301	0.046
PPxx & ppxx	0.277	0.811	0.522	0.358
PpXx & Ppxx	<0.001	0.030	0.001	0.003
PpXx & ppxx	<0.001	0.021	0.639	0.065
Ppxx & ppxx	0.091	0.861	0.001	<0.001

trols. There was also difference in IL-10 mRNA expression among patients with SLE with different ORα genotypes. In SLE group, the level of IL-10 mRNA in patients with PpXx genotype was significantly higher than in patients with PPXX, PPXx, Ppxx, and ppxx. IL-10 is a multifunctional cytokine that plays a central role in the pathogenesis of SLE, including regulation of growth and differentiation of B cells and auto-antibody production (17). Furthermore, the serum level of IL-10 showed positive correlation with SLEDAI and anti-double stranded (ds)DNA and negative correlation with C3, C4, and lymphopenia (18). The level of IL-4 mRNA in patients with PpXx genotype was higher than in patients with PPXX, Ppxx, and ppxx genotypes. IL-4 and IL-10 are predominantly produced by Th2 cells. An overproduction of Th2 cytokines, which resulted in B-cell hyperactivity, has been demonstrated in patients with SLE (19). Our results suggested that Th2 cytokines were predominant in patients with SLE with the PpXx genotypes.

The level of IFN-γ mRNA in patients with SLE with Ppxx genotype was much lower than in controls. In SLE group, the level of IFN-γ mRNA in patients with Ppxx genotype was lower than in patients with PPXX, PPXx, PPxx, PpXx, and ppxx genotypes, while the level of IL-2 mRNA in patients with Ppxx genotype was lower than in patients with PPXX, PPXx, PpXx, and ppxx genotypes. A previous study reported that patients with SLE disease activity index (SLEDAI)>10 had significantly fewer Th1 cells than controls or patients with SLEDAI<10, which suggested that the imbalance of Th1/Th2-type cytokines in SLE mainly occurred due to the decrease in Th1-type cells and may relate to lupus disease activity (20). Our results indi-

cate that Th2 cytokines were predominant in patients with SLE with the Ppxx genotype, which resulted from the decrease in Th1 cytokines. These conflicting findings might be attributed to differences between the studies in the stage of the disease and timing of treatment. Tokano et al (21) reported that the concentration of IFN-γ (determined using the sandwich ELISA kit) was increased in the serum in the active stage of SLE. However, Chen et al (20) reported that patients with SLE disease activity index (SLEDAI>10 had significantly fewer CD4+ or CD8+ T cells producing IFN-γ (determined by flow-cytometry following whole-blood culture) than patients with SLEDAI=0, SLEDAI between 1 and 10, or healthy controls. In our study, the patients were all in the active stage of SLE with SLEDAI>10.

Since the validity of these findings may be compromised by the relatively limited size of our patient group, molecular mechanism by which the ORα gene polymorphisms influence the Th1/Th2 cytokine expression need to be explored in future studies, which would include more participants.

SLE is a multifactorial autoimmune disease with pathogenesis influenced by genetic factors. Polymorphisms occur frequently throughout the human genome and in some cases are known to alter either the expression or the function of a gene product (22). The association of ORα gene polymorphisms with breast cancer, hypertension, osteoporosis, and osteoarthritis has been reported in recent studies (23-27). The association of genetic polymorphism, which may alter the Th1/Th2 balance, with susceptibility to SLE has been previously reported (28). Our study confirmed this by showing that the ORα gene polymorphisms could influence the expression of IL-10, IL-4, IL-2, and IFN-γ in SLE. Our result indicates that the Th2 cell was predominant in patients with SLE with PpXx and Ppxx genotypes. Since SLE is a heterogeneous disease and its pathologic mechanisms are difficult to understand, it is important to bear in mind that it may be closely related to ORα gene polymorphisms.

Acknowledgments

This work was supported by the Shandong Province Healthy Department (2001CA20BA1) and the Natural Science Foundation of Shandong Province (Y2006C116) to Z.M. Lu.

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