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Association of estrogen and aromatase gene polymorphisms with systemic lupus erythematosus

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Abstract

Experimental and animal studies suggested that estrogens play an important role in the development of systemic lupus erythematosus (SLE) through a variety of mechanisms involved in the regulation of the immune system. The objective of this study was to investigate the association between genetic variations in estrogen metabolic pathway genes, including estrogen receptor α (*ESR1*), estrogen receptor β (*ESR2*), and aromatase (*CYP19A1*), and risk of SLE. We performed a genetic study of SLE among 46 medical record-confirmed female SLE cases and 102 female controls participating in an Internet-based case-control study of SLE. Polymorphisms analysed included: *ESR1* *PvuII*, *XbaI*, and GT repeat; *ESR2* *RsaI*, *AluI*, and CA repeat; and *CYP19A1* *RsaI*, *SfaNI*, and TTTA repeat. We found significant association of the *ESR1* *PvuII* (PP vs. pp, odds ratio (OR): 3.1, 95% confidence interval (CI): 1.1–9.3) and *XbaI* (XX vs. xx, adjusted OR: 3.4, 95% CI: 1.1–10.5) with SLE. Carrying the PPXX genotype conferred the highest risk (PPXX vs. ppxx, OR: 4.6, 95% CI: 1.3–15.9). We also found an association of SLE with the *ESR2* CA repeat (SS vs. LL, OR: 2.8, 95% CI: 1.0–8.0). Our results support a role of estrogen in pathogenesis of SLE and suggested that genetic variants in the estrogen receptor genes might influence susceptibility.

Keywords

aromatase; case-control; estrogen receptor gene; genetic polymorphism; systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown cause that can damage multiple organ systems and has no cure. The two most striking clinical clues to its etiopathogenesis are a propensity to affect women (female gender is the strongest known risk factor for SLE) and familial aggregation. The observation that the incidence of SLE among women is highest during their reproductive years,¹ has implicated estrogens as potential mediators of risk for SLE. Research into this possibility has uncovered a variety of mechanisms through which sex steroid hormones can influence regulation of the immune system.^{2,3} Studies of animal models of SLE have demonstrated striking effects of exogenous estrogens in modulating antibody production and disease severity.^{4–7} In humans with SLE, clinical studies have shown subtle differences in endogenous estrogen metabolism,^{8,9} while epidemiological studies of exogenous estrogen and SLE have tended to support this hypothesis, albeit with less consistent results.^{10–15}

SLE also clusters in families, with a frequency suggesting an important role for genetic susceptibility. Taken together, these observations promote the possibility that variations in estrogen-related genes may be determinants of SLE risk. Furthermore, genetically-based variations in estrogen metabolism are already well described to influence sex hormone levels,^{16,17} biological activity of endogenous and exogenous estrogens,^{18,19} and estrogen-dependent phenotypes from other medical fields.^{20,21}

However, studies of the influence of genetic variations in estrogen pathway genes in relation to SLE are few and inconclusive. Therefore, we conducted a genetic investigation among participants in an established, online-based SLE case-control study²² to test the associations with common polymorphisms in three pivotal genes in the estrogen pathway; estrogen receptor α (*ESR1*) and estrogen receptor β (*ESR2*), which mediate estrogen functions; and aromatase (*CYP19A1*), which converts androgen to estrogen.

Materials and methods

Study design

This study was based on an established online case-control study of SLE. The details of design and validation of this study are published.²² Briefly, we constructed a study website to solicit participants and conduct the study. People searching for one of a set of pre-specified medical key terms using the Google search engine were directed towards our study website, where information about the study was provided along with links to an eligibility screening module, the consent form, and study questionnaires. The genetic study built on existing participants by inviting female participants to further participate in this genetic investigation. Participants of the genetic study were required to sign a new consent form and provide mouthwash samples for genetic analysis. The study received approval from the Institutional Review Board at Tufts Medical Center.

Study population

The source population comprised women living within the United States, aged over 18 years, searching on Google using medical key terms.

Cases

Women searching for lupus-related terms (e.g. lupus, SLE, and systemic lupus erythematosus) using Google were directed to our study website via sponsored links, where they answered a set of SLE screening questions soliciting a recent (<15 years prior) physician diagnosis of SLE, use of appropriate SLE medications (corticosteroids, anti-malarials, azathioprine, cyclophosphamide, and methotrexate), and non-use of other rheumatic disease medications (gold, sulphasalazine, and penicillamine). SLE candidates that passed the screening were requested to sign a release form for obtaining medical records from their physicians/rheumatologists. An individual whose combined chart review and physician's checklist documented the presence of four or more criteria for American College of Rheumatology (ACR) criterion classification of SLE^{23,24} was classified as a SLE case.

Controls

These were women recruited through the study website over the same period as the case recruitment, using terms derived from a list of medical disorders most frequently searched through Google. Nine most commonly entered terms for diseases not known to share risk factors with SLE and not exclusive to men (e.g. migraine, hypertension, sinusitis, and fibroids) were used initially and were later extended a list to 80 common medical key terms from which 10 key terms were randomly selected every 4 weeks. A questionnaire screening for connective tissue diseases was used to determine the eligibility of control.

Data collection

All participants completed an online questionnaire, which included standardized questions derived from previous lupus studies^{25,26} and covered detailed information on demographics, socioeconomic status, medications, co-morbidities, disease epiphenomena, and common environmental exposures. Questions were constructed to ascertain life-time exposure, current exposure, and exposure before diagnosis (cases) or before the reference year (controls). For each new control participant, a computational subroutine was applied to assign a reference year in lieu of date of diagnosis from the real time frequency distribution of diagnosis year among currently enrolled cases. For genetic analysis, a supplemental questionnaire was also completed to collect additional information regarding menstrual history and hormonal exposure.

Biospecimen collection

Mouthwash samples were collected through the mail using a mouthwash collection kit, which included detailed, self-explainable instructions for sample collection procedure. This method has been approved to be efficient and effective for collecting biospecimen for DNA analysis.²⁷ Participants were requested to collect the mouthwash sample following the instructions and to send the sample back to us through prepaid, first class mail within 24 hours of the sample collection. Within 12 hours of reception, samples were transferred to -70°C for storage until DNA extraction.

DNA extraction and genotyping analysis

DNA isolation and quantification was conducted at BioServe Biotechnologies, Ltd. (Laurel, Maryland, USA). Extracted DNA samples were quantitated using a SpectraMax Plus spectrophotometer manufactured by Molecular Devices (now part of MDS Analytical Technologies).

We genotyped six single nucleotide polymorphisms (SNPs), which are also restriction fragment length polymorphisms (RFLPs, DNA sequences that can be detected by the presence of fragments of different lengths after digestion with specific restriction endonucleases. The specific restriction endonucleases, such as *Xba*I, *Pvu*II, etc., listed below, have been commonly used to name the polymorphisms), including *ESR1 Pvu*II (rs2234693) and *Xba*I (rs9340799), *ESR2 Rsa*I (rs1256049) and *Alu*I (rs4986938), and *CYP19A1 Rsa*I (rs700518) and *Sfa*NI (rs700519), and three microsatellite tandem repeats (multiple copies of the same short DNA of very variable length), including *ESR1* GT repeat (variable copies of dinucleotide GT tandem repeats), *ESR2* CA repeat (variable copies of dinucleotide CA tandem repeats), and *CYP19A1* TTTA repeat (variable copies of tetranucleotide TTTA tandem repeats). Genotyping of SNPs was performed by BioServe Biotechnologies, Ltd. using the MassARRAY iPLEXTM platform. Three positive controls, three negative controls, and 10% duplicated sample controls were included for each assay. The mass spectrometer data were acquired and automatic genotype calls were made using the Sequenom Typer software. All automatic calls were manually reviewed for genotype accuracy. Genotyping of the microsatellite tandem repeats was performed using the GeneScan method.

Statistical analysis

Hardy–Weinberg equilibrium was determined among control samples for each polymorphism. Univariate logistic regression model and multivariate logistic regression model with age and race adjusted were used to estimate the association between polymorphisms and SLE. For each SNP analysis, the most common homozygote genotype was used as the baseline and the ORs were calculated for each alternative genotype. The

trend test p -value was obtained to evaluate the allelic dose-response effects. For microsatellite tandem repeats analysis, we used the commonly reported cut point for each polymorphism to dichotomize the allele into long (L) and short (S) allele and then analysed the genotype using the same method as SNPs. Interactions between gene and oral contraception use was tested by including the interaction term in the logistic regression model. All analyses were performed using SAS v9.1 (SAS Institute Inc., Cary, North Carolina). A two-sided test with $p < 0.05$ was considered statistically significant.

Results

Participant characteristics

Of the 124 chart-confirmed SLE cases and 693 controls participating in the online case-control study of SLE, 55 cases and 119 controls consented to participate in the genetic study of SLE and completed the supplemental questionnaire. Forty-six consented cases and 102 controls provided mouthwash samples. DNA was successfully extracted from all samples. The successful rates of genotyping each polymorphism ranged from 90% to 99%. All genotypes were in Hardy-Weinberg equilibrium among controls. The basic characteristics of the cases and controls are shown in Table 1. Ninety percent of the participants were white, 3% were African-American, and 2% were Hispanics. Cases and controls were of similar demographic features (such as age, race/ethnicity, and region of residence) and socioeconomic status (such as education level and household income). The participants of the genetic analysis also showed overall similarity to the whole population of the parent online case-control study.²²

Estrogen gene polymorphisms and the risk of SLE

ESR1 *PvuII* and *XbaI* polymorphisms were both significantly associated with SLE, with the *PvuII* P and *XbaI* X alleles conferring approximately a two to threefold increase in risk (see Table 2). *PvuII* and *XbaI* are in strong linkage, so we analysed combined *PvuII* and *XbaI* genotypes. Compared to women with the most common homozygous genotype, ppxx, those carrying PPXX had an OR for SLE of 4.6 (95% CI: 1.3–15.9). For women carrying heterozygous genotypes for either one or both SNPs, such as PpXx, Ppxx, PPXx, the risks for SLE were also increased, with ORs in the range of 2.1–2.6. The *ESR1* GT repeat was not associated with SLE.

For the *ESR2* CA repeat, compared with longer alleles (L, ≥23 repeats), the shorter allele (S, <23 repeats) was marginally associated with increased risk of SLE (LS vs. LL, adjusted OR: 1.9, 95% CI: 0.7–5.0; SS vs. LL, adjusted OR: 2.8, 95% CI: 1.0–8.0; p -value trend for numbers of L allele, 0.048) (Table 2). The two SNPs, *RsaI* and *AluI*, were not found to be associated with SLE risk.

We genotyped three polymorphisms, *RsaI*, *SfaNI*, and TTTA repeat, in *CYP19A1*, none of which were associated with SLE (Table 2).

Discussion

Our study confirms that polymorphisms in estrogen receptor genes are associated with SLE and provides further evidence that estrogens and their metabolites mediate in part the increased risk for this disorder in women. We found the *PvuII* P and *XbaI* X alleles of *ESR1* to be most strongly associated with SLE, with the PPXX genotype conferring the greatest magnitude of risk. SLE was also associated, albeit more weakly, with the estrogen receptor gene β (*ESR2*) CA shorter repeat (<23 repeats), but not with three polymorphisms in the aromatase gene (*CYP19A1*) that we tested.

The intronic *PvuII* and *XbaI* polymorphisms have been studied extensively in estrogen-related disorders. Although the results from individual studies are heterogeneous, a meta-analysis of these two polymorphisms in relationship with osteoporosis evidenced a protective association between the *XbaI* XX genotype in bone mineral density (BMD) and fracture risk,²⁰ suggesting that the X allele is associated with higher biological activities of estrogen, probably through more efficient expression of *ESR1* or a more active type of estrogen receptor α . Endogenous estrogen level and exogenous estrogen exposure have been found to enhance autoimmunity^{3,28} and increase the risk of SLE.²⁹ Animal studies have also showed that deficiency of estrogen receptor α attenuated autoimmune disease in lupus-prone mice.^{30,31} Therefore, genetic variations associated with enhanced estrogen/estrogen receptor activities are expected to increase the risk of SLE. Our finding of the *XbaI* X allele being associated with increased risk of SLE is consistent with this expectation. Our findings are also consistent with Lee *et al.*'s report, among 137 Korean female SLE cases and 268 female controls, which found that the *PvuII* P allele was over-represented by 24% in SLE cases compared to controls, and the *XbaI* X allele was 14% more frequent among childhood onset SLE than controls and adult onset SLE.³² We also tested two 5'-dinucleotide repeat polymorphisms that have been implicated in other estrogen-related disorders, TA repeat and GT repeat, but found no association with SLE.

Estrogen receptor β has also been implicated in autoimmune diseases. Disruption of *ESR2* in mice resulted in an increased propensity toward autoimmune diseases.³³ The polymorphic CA repeat has been broadly investigated in relation to various disorders,^{16,34} however, no study of this polymorphism has been conducted in SLE. However, two studies among US populations found a consistent association between the CA repeat and BMD. Scariano *et al.* in a study of postmenopausal women from New Mexico, observed a higher BMD in subjects with shorter alleles (CA < 25) as compared with those having longer alleles.³⁵ The Framingham Study offspring cohort also observed that subjects homozygous for a high number of repeats (CA $\geq$ 23) had the lowest femoral BMD.²¹ These results suggest that the shorter alleles are associated with higher estrogen activity. Since in our study we found that these shorter alleles (CA < 23) are also associated with increased risk of SLE, it is logical to infer that increased estrogenic activity may mediate the increase in risk for SLE. Two *ESR2* SNPs, *RsaI* and *AluI*, have also been related to ovulatory dysfunction and endometriosis,¹⁷ both disorders known to be linked to SLE. However, we did not find any significant associations of these two SNPs with SLE in our sample.

Aromatase is the key enzyme in converting androgen to estrogen. In postmenopausal women, peripheral aromatization of testosterone is an important source of estrogen.^{36,37} The intronic TTTA repeat in the aromatase gene (*CYP19A1*) has been reported to relate to aromatase activity and the consequent estrogen levels and BMD, as well as associated disorders, such as fracture and osteoporosis. Carrying an allele with a high number of repeats showed a greater aromatase activity, higher circulating estradiol level, higher lumbar BMD, and lower fracture risk.^{38–40} The *RsaI* polymorphism (silent mutation of Valine (80) that does not result in the change in amino acid sequence of aromatase) was also linked to serum estradiol level and BMD in postmenopausal women, with the G allele associated with lower serum estradiol concentration, lower BMD, and higher prevalence of osteoporosis and fractures.⁴¹ The TTTA repeat^{42,43} and an *SfaNI* (Arg264Cys, change amino acid arginine at 264 to cysteine)⁴⁴ are also associated with risk of sex steroid-related cancers, such as breast, prostate, and endometrial cancer. However, in this study we did not find any association between these three *CYP19A1* polymorphisms with SLE risk.

This study contains limitations. The size of the SLE case group was small, restricting statistical power to detect weaker associations, and associations with less common polymorphisms (such as *ESR2 RsaI* and *CYP19A1 SfaNI*). Also, due to the small sample

size, we lacked power to detect modest levels of interactions between genes and estrogen exposures. While previous studies have suggested a link between genetic variations in estrogen receptor α and particular clinical features of SLE,^{45,46} the number of the SLE cases in our study was not large enough to verify that association. In addition, because of the number of statistical tests carried out, the results should be interpreted with caution.

We used an Internet-based methodology to perform this study, which is a novel approach in our field. The Internet-based approach of the parent study conformed to all critical aspects of a case-control study design, including recruitment of cases and controls from the same source population. We have published the methodological aspects of that study in detail, including its high level of feasibility and construct validity.²² Nested within the existing online case-control study of SLE, this genetic investigation has inherited the strength as well as the limitations of the parent study.²²

Another important consideration is that the results of genetic analyses among population studies can be confounded by racial factors. However, the two groups were broadly similar in most respects, including self-reported race and ethnicity, and we additionally adjusted for race in our analyses. While self-reported race/ethnicity does not provide the highest level of information on participants' genetic background, it is efficient and sufficiently informative for classification of genetic clusters.⁴⁷ Based on these considerations, we do not view race as having a confounding effect in our results. Also, probably because of our Internet-based recruitment approach, the sample largely comprised white participants. While this would not affect the validity of our results, it could limit the generalizability of the findings to other racial groups.

This study illustrates the feasibility of conducting genetic studies using the Internet. Since the nested recruitment may have limited the sample size, we recommend that future Internet-based genetic studies of SLE leverage the power of the World Wide Web through direct recruitment, as in the parent study.

In conclusion, we have found associations between *ESR1 XbaI* and *PvuII* and SLE, and between *ESR2* CA repeat and SLE. Our study supports a role of estrogen in pathogenesis of SLE and indicates that genetic variants in the estrogen receptor genes influence SLE susceptibility.

Acknowledgments

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Table 1

Basic characteristics of study subjects

	Cases (46)	Controls (102)	p-value
Race			
White	39 (85%)	94 (92%)	0.1
African-American	4 (9%)	1 (1%)	
More than one race	2 (4%)	3 (3%)	
Others ^a	1 (2%)	4 (4%)	
Ethnicity			
Non-Hispanic	45 (98%)	97 (95%)	0.5
Hispanic	1 (2%)	2 (2%)	
Did not say	0 (0%)	3 (3%)	
Age			
Mean (SD)	42.9 (11.8)	45.7 (11.1)	0.2
Region of Residence			
North-east	10 (22%)	28 (27%)	0.7
Midwest	9 (20%)	20 (20%)	
South	12 (26%)	30 (29%)	
West	15 (33%)	24 (24%)	
Education Level ^b			
High school graduate or less	5 (11%)	10 (10%)	0.5
Some college education	20 (44%)	32 (33%)	
College graduate	11 (24%)	31 (32%)	
Professional or graduate school	9 (20%)	25 (26%)	
Household Income ^c			
Less than US\$25 K	8 (21%)	18 (22%)	0.6
US\$25 K–US\$50 K	13 (33%)	21 (25%)	
US\$50 K–US\$100 K	11 (28%)	33 (40%)	
More than US\$100 K	7 (18%)	11 (13%)	

^aOthers included Asians, American Indians and Alaska Natives, Native Hawaiians and Other Pacific Islanders.

^bOne of the cases and four of the controls did not report an education level.

^cSeven of the cases and 19 of the controls did not report a household income.

Table 2Associations between *ESR1*, *ESR2*, and *CYP19A1* polymorphisms and SLE risk

Gene	Polymorphism	Genotype	Case (%)	Control (%)	OR (95% CI) ^a	p-trend
<i>ESR1</i>	<i>Pvu</i> II (rs2234693)	pp	9 (20)	38 (38)	1.0 (ref)	
		Pp	26 (57)	48 (48)	2.2 (0.9–5.4)	
		PP	11 (24)	15 (15)	3.1 (1.1–9.3)	0.03
	<i>Xba</i> I (rs9340799)	xx	14 (30)	48 (48)	1.0 (ref)	
		Xx	24 (52)	44 (44)	1.8 (0.8–4.0)	
		XX	8 (17)	9 (9)	3.4 (1.1–10.5)	0.03
	<i>Pvu</i> II & <i>Xba</i> I	ppxx	9 (20)	38 (38)	1.0 (ref)	
		Ppxx	5 (11)	8 (8)	2.6 (0.7–10.1)	
		PpXx	21 (46)	39 (39)	2.2 (0.9–5.6)	
		PPXx	3 (7)	5 (5)	2.1 (0.4–11.7)	
<i>ESR2</i>	(GT) ⁿ L ₁ ≥ 16 S ₁ < 16	PPxx	0	2 (2)	N/A	
		PpXX	0	1 (1)	N/A	
		PPXX	8 (17)	8 (8)	4.6 (1.3–15.9)	
		LL	19 (41)	48 (47)	1.0 (ref)	
		LS	20 (43)	36 (35)	1.5 (0.7–3.3)	
		SS	7 (15)	18 (18)	1.2 (0.4–3.4)	0.6
		CC	45 (98)	91 (90)	1.0 (ref)	
		CT	1 (2)	10 (10)	0.2 (0.03–1.9)	
		TT	0	0	N/A	
		CC	17 (37)	41 (41)	1.0 (ref)	
<i>CYP19A1</i>	(CA) ⁿ L ₁ ≥ 23 S ₁ < 23	CT	20 (43)	43 (43)	1.2 (0.5–2.7)	
		TT	9 (20)	17 (17)	1.3 (0.5–3.6)	0.6
		LL	7 (15)	31 (30)	1.0 (ref)	
		LS	22 (48)	44 (43)	1.9 (0.7–5.0)	
		SS	17 (37)	27 (26)	2.8 (1.0–8.0)	0.048
		TT	16 (35)	25 (25)	1.0 (ref)	
		CT	17 (37)	51 (50)	0.7 (0.3–1.6)	
		CC	13 (28)	26 (25)	1.0 (0.4–2.6)	1

Gene	Polymorphism	Genotype	Case (%)	Control (%)	OR (95% CI) ^a	p-trend
	S/zN1 (rs700519)	GG	41 (89)	94 (94)	1.0 (ref)	
		AG	3 (7)	6 (6)	1.1 (0.3–4.8)	
		AA	2 (4)	0 (0)	N/A	
	(TTTA) _n L: _n ≥ 9 S: _n < 9	SS	26 (57)	43 (42)	1.0 (ref)	
		LS	11 (24)	47 (46)	0.4 (0.2–1.0)	
		LL	9 (20)	12 (12)	1.4 (0.5–4.0)	0.9

^aThis has been adjusted for age and race.

CI: confidence interval, L: long allele, N/A: not applicable, OR: odds ratio, S: short allele.