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Association of a cytosine-adenine repeat polymorphism in the estrogen receptor *β* gene with occurrence and severity of rheumatoid arthritis

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ABSTRACT

We investigated the influence of the cytosine-adenine (CA) dinucleotide repeat polymorphism in intron 6 of estrogen receptor *β* (ER*β*) gene on rheumatoid arthritis (RA) risk. One hundred and ninety-three RA patients and 77 control subjects with osteoarthritis (OA) were recruited. The CA repeat polymorphism was assayed by a dye-terminator cycle sequencing analysis. No statistically significant difference in the mean number of CA repeats between the RA and OA patients was observed (RA: 21.47, OA: 21.23, *P* = 0.324). The alleles were categorized according to the number of repeats: short (S, ≦21) and long (L, ≧22), in which the genotypes SS, SL, and LL were observed. No significant differences were observed for the allele and genotype distributions of this polymorphism in both patient groups. The RA patients were classified according to RA severity: mild (least erosive disease) and severe (more erosive and mutilating disease). Again, no significant difference in genotype frequency between these groups was observed, even after stratifying by sex. The present study indicates that additional studies are needed to clarify the roles of this polymorphism, estrogen, and ER in the development of autoimmune diseases.

Keywords: Polymorphism; Rheumatoid Arthritis; Sex; Estrogen Receptor *β*; CA Repeat

1. INTRODUCTION

Rheumatoid arthritis (RA) is the most common chronic autoimmune disorder, characterized by chronic inflammation and destruction of the synovial joints, leading to progressive joint deterioration and disability [1]. RA is characterized as a complex genetic disease, meaning that several genes and environmental and stochastic (chance) factors act in concert to cause the pathological events [2]. Principal among the list of known risk factors is sex, as the rate of RA in females is 2 to 3 times higher than that in males [3]. The underlying immune response in RA seems to be influenced by sex hormones, which have been shown to modulate the onset and progression of connective tissue diseases, including RA, in clinical and *in vivo* studies [4]. In addition, some studies have suggested that female sex hormones and pregnancy are factors possibly associated with RA symptoms. For example, amelioration of RA occurs in approximately three quarters of pregnancies. In these cases, most women who improve experience initial relief in the first trimester, but RA almost invariably recurs within 3 or 4 months after delivery [5]. Another investigation concluded that the menopausal state could be responsible for the major part of the differences in outcome of RA between men and women [6].

To understand the functional role of estrogens in RA development, it is important to study not only estrogens but also the estrogen receptor (ER). Two types of ERs have been identified and cloned: ER*α* and ER*β* [7]. ER*α* and ER*β* are both expressed in synovial tissue, but ER*β* has been identified as the predominant ER subtype in normal human synovial tissue [8]. The relative ER*α*/ER*β* messenger RNA expression ratio was reported to be significantly lower in RA than in non-inflamed synovial tissue [9].

Several studies have investigated the potential roles of

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ERβ (14q22 - 24) genetic polymorphisms in disease. For example, a cytosine-adenine (CA) dinucleotide repeat in intron 6 and the single nucleotide polymorphism (SNP) rs1256049 (Rsa polymorphism) have been studied in the context of various human phenotypes, including osteoarthritis [10], bone mineral density [11], and climacteric disorder [12]. In RA, we previously reported that longer CA repeats in the intron 6 of ERβ and the GG genotype at SNP rs1256049 were potential risk factors for RA [13, 14].

In this study, we investigated the association between the CA repeat polymorphism in the intron 6 of the ERβ gene and RA in men and women and compared the results obtained with those found in OA patients. In addition, we investigated the relationship between RA severity and CA repeat polymorphism.

2. SUBJECTS AND METHODS

2.1. Subjects and Study Design

The protocols and procedures for this experiment were approved by the ethics committee of the Graduate School of Pharmaceutical Sciences of Chiba University. All the genetic information used in this study remains confidential.

A total of 270 Japanese patients (222 women and 48 men) from the Chiba University Hospital, Japan, were recruited to participate in this study. These patients belong to 2 groups of subjects: those with rheumatoid arthritis (n = 193; 150 women and 43 men) and those recruited from the same geographical area and who had osteoarthritis (n = 77; 72 women and 5 men), designated as the control subjects. Informed consent was obtained from all subjects who participated in this study.

2.2. Analysis of the CA Repeat Polymorphisms

Genomic DNA was extracted from human peripheral blood leukocytes using the QIAamp DNA Mini Kit (Qiagen, Inc., Hilden, Germany) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was performed in 75 μL of reaction mixture with the following components: 150 ng of human genomic DNA, oligonucleotide primers designed to amplify polymorphic CA repeats in the intron 6 of the human ERβ gene (forward: 5'-CAA TTC CCA ATT CTA AGC CT-3' and reverse: 5'-ATT CTT CTT TAG GCC AGG CA-3') at 0.4 μM, dNTP mixture (TaKaRa Bio, Inc., Otsu, Japan) at 200 μM, 7.5 μL of 10× Reaction Buffer (containing 15 mM $MgSO_4$) (Transgenomic, Inc., Omaha, USA), and 2.5 U of Optimase Polymerase (Transgenomic, Inc.). The reactions were brought to a total volume of 75 μL by adding MilliQ water. The amplification profiles were as follows: 35 cycles of denaturing at 94˚C for 30 seconds, annealing at 60˚C for 30 seconds, and extension at 72˚C for 30 seconds. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and used in the following analysis.

The analysis of the CA repeat polymorphisms was conducted by dye-terminator cycle sequencing using the Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter, Inc. Fullerton, USA) and the CEQ2000 DNA Analysis System (Beckman Coulter, Inc.) according to the manufacturers' protocols.

2.3. Statistical Analysis

The allele and genotype frequencies were compared between the patient groups using the Fisher's exact probability test, except for 2 groups (age and the mean numbers of CA repeats), which were compared using the student's t test. $P < 0.05$ was considered to represent statistically significant differences for all the analyses.

3. RESULTS

The characteristics of the RA and OA patients are shown in **Table 1**. Significant differences were detected between the RA and OA patients with respect to sex, although in both groups of patients, the occurrence of disease in the women was higher than that in men. In addition, in the female and male groups, the RA patients were significantly younger than the OA patients. We used the same

Table 1. Characteristics of the RA and OA patients.

	RA	OA	*P* value
Characteristic			
All patients (n)	193	77	
Sex (n (%))			
Women	150 (77.7)	72 (93.5)	0.001[a]
Men	43 (22.3)	5 (6.5)	
Age (mean (SD, range))			
Women	61.0 ± 10.8 (24 - 86)	70.0 ± 9.33 (50 - 91)	0.000[b]
Men	61.9 ± 11.00 (38 - 83)	73.4 ± 14.15 (51 - 89)	0.037[b]
Total	61.2 ± 10.83 (24 - 86)	70.2 ± 9.62 (50 - 91)	0.000[b]
Severity (n (%))			
LES	48 (24.9)	-	
MES	135 (69.9)	-	
MUD	10 (5.2)	-	

P values are shown for comparisons between RA and OA by [a]the Fisher's exact probability test and [b]student's t test. Differences were considered significant at $P < 0.05$. LES, least erosive subset; MES, more erosive subset; MUD, mutilating disease.

criteria as those used by Ochi *et al.* [15] to classify our patients into 3 severity types (least erosive disease, LES; more erosive disease, MES; and mutilating disease, MUD). These severity classifications were determined by orthopedic specialists based on long-term observation data. Most of the patients in the study were classified as MES, similar to those in our previous study [14].

Figure 1 shows the frequency distribution of the CA repeats in the RA and OA patients. In the RA patients, the number of CA repeats ranged in length from 15 to 28, with a mean value of 21.47 and a median of 22. In the OA patients, the number of CA repeats ranged in length from 15 repeats to 29 repeats, with a mean value of 21.23 and a median of 22. The mean numbers of CA repeats were not statistically different between the RA and OA patients ($P = 0.324$). In addition, we categorized 2 allelic types according to the median number of CA repeats: ≦21 repeats (short allele, S), and ≧22 repeats (long allele, L). The cutoff value was based on that used in previous studies [10,12,13]. Based on these alleles, 3 genotypes (SS, SL and LL) were identified.

The allele and genotype frequency distributions for the ERβ CA repeat polymorphism in the RA and OA patients are shown in **Table 2**. There were no significant differences observed between the RA and OA patients in this respect. The RA patients were also classified and divided according to sex and RA severity: mild (LES) and severe (MES and MUD). No significant differences in genotype frequency between the RA severity classes were observed before or after stratifying these groups by sex (**Table 3**).

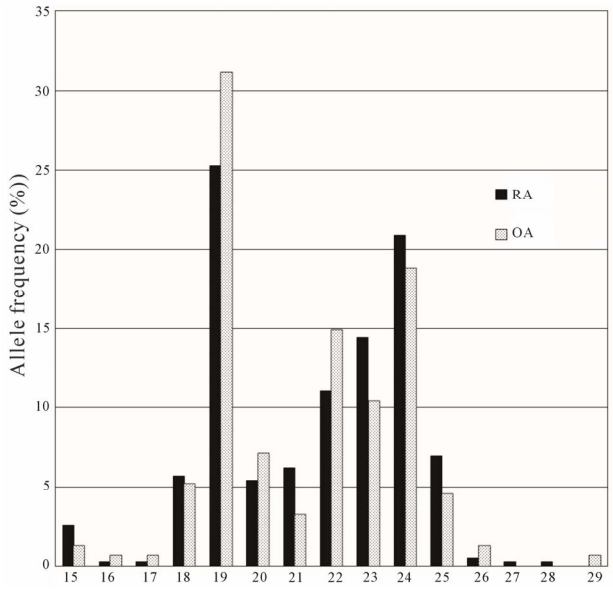

Figure 1. Frequency distribution of alleles at the CA repeat polymorphism in the estrogen receptor β gene for the RA and OA patients.

Table 2. Comparison of genotype and allele frequencies for the ERβ CA repeat polymorphism in the RA and OA patients.

Genotype	RA	OA	Genotype distribution	Allele S vs allele L	
	n (%)	n (%)	*P* value	OR (95% CI)	*P* value
SS	48 (24.9)	22 (28.6)	0.703	0.801	0.446
SL	78 (40.4)	32 (41.5)		(0.45 - 1.42)	
LL	67 (34.7)	23 (29.9)			

Differences were considered significant at $P < 0.05$ using the Fisher's exact test. OR: Odds ratio; 95% CI: 95% confidence interval.

Table 3. Comparison of the genotype and allele frequencies for the ERβ CA repeat polymorphism between mild (LES) RA, severe (MUD + MES) RA, and OA patients.

		RA (LES)	RA (MES + MUD)	OA	Genotype distribution
		n (%)	n (%)	n (%)	*P* value
Female	SS	7 (19.4)	30 (26.3)	20 (27.8)	0.455
	SL	21 (58.3)	46 (40.4)	31 (43.0)	
	LL	8 (22.2)	38 (33.3)	21 (29.2)	
Male	SS	3 (25.0)	8 (25.8)	2 (40.0)	0.985
	SL	3 (25.0)	8 (25.8)	1 (20.0)	
	LL	6 (50.0)	15 (48.4)	2 (40.0)	

Differences were considered significant at $P < 0.05$ using the Fisher's exact test. LES: Least erosive subset; MES: More erosive subset; MUD: Mutilating disease.

4. DISCUSSION

We investigated the association of the CA repeat polymorphism in the intron 6 of the ERβ gene with RA and OA to follow up on the findings from a previous report [13]; however, our results did not reveal significant differences between RA and OA patients with respect to allele and genotype frequencies identified at this locus.

Based on the patient characteristics (**Table 1**), we evaluated the degree of severity of RA in the patients according to Ochi *et al.* [15]. Because this evaluation is primarily dependent on the stages and progress of joint deterioration (e.g., mild or rapid, and oligo arthritis or multi joint), classifications of disease severity are not influenced by differences in sensitivity to drug treatment. Most of the patients enrolled in this study were classified as MES RA, which was consistent with the findings of the previous study by Sato *et al*. investigating the effects of the ERβ Rsa polymorphism on RA [14]. Furthermore, in this study, we found that the OA patients were older than the RA patients, which is perhaps not surprising given that OA pathologic features is closely associated with aging. These findings were also similar to those reported by Sato *et al.* [14]. In the present study, we ana-

lyzed the CA repeat polymorphism by direct sequencing, a method typically used for the analysis of microsatellite polymorphisms. The mean CA repeat length observed for the RA patients was the same as that reported in a previous study [13]. Thus, neither the patient background nor the methods used for the analyses in the present study are likely to have resulted in the lack of association observed here. Similar patient cohorts were recently used to identify the association between the Rsa polymorphism in the ERβ gene and RA risk [14]. Considering this, it could be suggested that the Rsa and CA repeat polymorphisms are functionally different, a finding that would not be surprising given that the Rsa polymorphism resides within an exon and the CA repeat polymorphism resides within an intron; however, additional studies are needed to investigate this further.

To date, only a single study has investigated the potential association between the ERβ CA repeat polymerphism and RA [13]. Wang *et al.* [16] recently reported that shorter alleles (S, <23 repeats) were marginally associated with increased risk of systemic lupus eryhtematosus (SLE) compared with longer alleles (L, ≧23 repeats). While it is well known that, like RA, SLE is an autoimmune disease, a link between this polymorphism and RA should not be inferred based solely on the association to SLE [16] given that many differences are known to exist between SLE and RA, such as age at onset and sex differences [17]. Furthermore, Dziedziejko *et al*. [18] examined the association of SNPs in ERα and ERβ gene with RA but did not observe any significant differences in the distributions of genotypes and alleles between RA patients and controls and no significant association of the genotypes with rheumatoid factor (RF), erosive disease, extra-articular manifestations, or anticyclic citrullinated peptide (anti-CCP) antibodies [18]. In a subsequent study, Dziedziejko *et al.* [19] also examined potential associations of these SNPs with response to treatment with leflunomide in RA patients but did not reveal a significant association in ERβ SNPs [19]. Engdahl *et al.* [20] reported that ERα, but not ERβ or GPR-30 signaling, was shown to ameliorate disease and associated development of osteoporosis in a well-established model of postmenopausal RA. These results indicate that the role of ERβ in the pathogenesis of RA should be reconsidered.

Furthermore, the effects of estrogen on the development and severity of RA remain controversial, as pro-inflammatory and anti-inflammatory effects have been reported. For example, some selective ERβ agonists like ERB-041 are reported to have potent anti-inflammatory effects in animal arthritis models [21], but in a clinical trial, ERB-041 failed to demonstrate anti-inflammatory efficacy in RA patients despite the evidence of strong activity in a preclinical study [22]. Randomized controlled trials conducted by the Women's Health Initiative revealed that there was no significant evidence for a difference in the hazard rate of RA incidence or symptom severity between postmenopausal hormone therapy and placebo groups [23]. Ganesan *et al.* [24] recently reported protective effects of estrogen and testosterone. This study showed that estrogen had a higher potency in rat arthritic synovial fibroblasts, whereas progesterone was not observed to inhibit TNF-α-induced change [24].

In addition to estrogen, other sex hormones have also been implicated in the etiology of RA. For example, the level of dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS), the major androgen in women, were associated with the onset of RA [1]. Karlson *et al.* [25] investigated potential association between androgen levels; polymorphisms in ERβ, PGR, and CYP19 genes; and risk of RA, but no significant associations were observed [25]. To the best of our knowledge, there have been no reported associations between the ERβ CA repeat polymerphism and sex hormones. Thus, studies assessing potential combinatorial effects of multiple genetic polymorphisms and hormone levels on the risk of RA are warranted.

5. CONCLUSION

In conclusion, the results of the present study did not reveal a significant association between alleles at the CA repeat polymorphism in the intron 6 of the ERβ gene and RA severity. Additional studies are needed to further clarify potential roles of this polymorphism, as well as roles for estrogen and ER in the pathogenesis and severity of autoimmune diseases.

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