Research Article

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Estrogen receptor alpha gene (*ESR1*) variant is associated with rheumatoid arthritis susceptibility and to particular clinical manifestations of the disease

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Objective: Several lines of evidence suggest a pivotal role of the estrogen pathway in several autoimmune-related responses and disease. The aim of this study was to investigate the association of a variant in *ESR1* gene with RA susceptibility and expand the analysis to specific clinical manifestations of RA in a sample of Brazilian patients.

Methods: 449 individuals (268 healthy controls and 181 RA patients) were genotyped for the polymorphism rs2234693 in the *ESR1* gene with Taqman SNP probes. The patient's group was composed by 153 female and 28 men. Association analysis were performed by logistic regression using SPSS software.

Results**:** The rs2234693 variant is associated with RA in males and females (rs2234693-CC genotype OR = 2.21, p = 0.0032). The rs2234693-CC genotype was also associated with the presence of cardiopathy (OR = 2.40, p = 0.025), high blood pressure (OR = 2.50, p = 0.019) and osteoporosis (OR = 1.94, p = 0.031), and also with the presence of anti-CCP antibodies (rs2234693-C allele OR= 1.91, p = 0.024).

Conclusion**:** *ESR1* is associated with RA, as well as with the presence of anti-CCP antibodies and particular RA-associated clinical manifestations (cardiopathy, high blood pressure and osteoporosis). Our study presents the first evidence of association between *ESR1* variant and RA.

Keywords: autoimmune disease ● estrogen receptor ● genetic association ● genetic polymorphism

Introduction

Rheumatoid Arthritis (RA), as most Autoimmune Diseases (AD), has an unclear and complex etiology. And importantly, displays a significant sex bias, with incidence approximately three times higher in women [1]. The etiological pathways underlying this noticeable female predisposition are unclear; however probable mechanisms include the influence of sex hormones and their receptors, in immune-related pathways [2].

Estrogen is the main female hormone, produced endogenously mostly during reproductive years and acting through the estrogen receptors ER-α and ER-β, codified by *ESR1* and *ESR2* genes, respectively. These nuclear receptors are ligand-dependent transcription factors that directly influence the expression of numerous groups of target genes. Today several lines of evidence suggest a pivotal role of this pathway in several immune-related responses, including T helper cell polarization, B cell differentiation, activation, function and survival, neutrophil numbers and functions, macrophage function, dendritic cell maturation, expansion and frequency of regulatory T cells (Tregs), among several other mechanisms [3]. Thus, it is not a surprise that estrogens and their receptors are closely involved in pathways correlated with AD development.In fact, for Systemic Lupus Erythematosus (SLE), estrogens seem to be a risk or triggering factor for disease development [4]. ERα deficiency in lupus prone murine strains prolongs survival, reduces proteinuria and the renal pathology score, compared to wild-type mice [5]. Interestingly, man-to-woman transgender individuals upon estrogenic treatment are

practically as susceptible as women to develop SLE. In addition, analysis of peripheral blood mononuclear cells from patients with SLE has shown increased levels of ERα mRNA expression compared to healthy controls, although there are some controversies between studies [6]. *ESR1* polymorphisms have already been associated with SLE and specific clinical manifestations [7] that might partially explain the overexpression of the receptor. This higher expression can cause different sensitivity to estrogens, leading to an overactive estrogen-signaling pathway, with a possible etiological role.

Interestingly, for Multiple Sclerosis (MS) and in MS experimental models estrogen seems to be protective, with anti-inflammatory, anti-demyelination and neuroprotective effects [8].

On the other hand, higher concentrations of estrogens, like during pregnancy, leads in some cases, like RA, to remission (Ostensen, 1999), probably because rheumatoid inflammation is T helper cell type 1 (Th1)-mediated and there is evidence that estrogen in higher levels induces a Th2 cytokine profile [8-10]. However, the influence of estrogen levels in disease activity is not clear and there is great variability between patients.

In the context of AD not only the endogenous estrogens are relevant but its exogenous administration is another important issue. Although there are some divergences, the use of estrogen-based oral contraceptive and hormonal replacement therapy is considered safe for AD patients [11,12]. However, the results of the studies in this field are controversial, probably because ADs are in general very heterogeneous. And this great variation in response to treatments, including hormones, is probably partially due to individual genetic variability.

A putative functional variant in the *ESR1* gene, SNP rs2234693, which might affect binding of the myb family of transcription factors and might be correlated with *ESR1* expression levels, has been identified [13]. The association of this variant with AD has been controversial [14]. No association has been found with myasthenia gravis, Hashimoto's thyroiditis, Graves' disease or Rheumatoid Arthritis in Caucasians [15, 16]. One study found association with age of onset only, in female RA patients [17]. It was also weakly correlated with SLE in a small study in North American female SLE patients [18]. Other studies found association with age of onset of SLE in Asian patients [19], juvenile form of the disease in a Polish study [2], and with specific SLE clinical manifestations in a Brazilian population [7] as well as skin involvement and milder forms of SLE in a Swedish population [20]. Since it is the only variant in this gene that has been related to AD and that it had not been analyzed regarding its correlation to RA in a Latin American population in this work we analyzed the association of this variant in Brazilian RA patients for susceptibility and with detailed clinical manifestations of the disease.

Methods

This study enrolled 449 individuals (181 RA patients and 268 controls). The patients, 153 female and 28 men, were recruited from the (University Hospital of Santa Catarina) and all fulfilled the American College of Rheumatology (ACR) classification criteria for RA. The characterization of the patients is shown in **Table 1**. Controls were healthy blood donor volunteers from the same hospital, with no known personal or family history of AD or inflammatory joint disorder. The average age of the patients grup was 43.15 ± 12.85 and the control group was 42.6 ± 14.36. The age did not statistically differ between patients and control group (p = 0.709). The present study has been approved by the ethics committee of the University of Santa Catarina and the University Hospital. All the enrolled patients and healthy volunteers provided written informed consent.

Genomic DNA was extracted from peripheral white blood cells by standard phenol-chlorophorm protocol. SNP rs2234693 (T/C) was genotyped by pre-designed TaqMan genotyping assay (Life Technologies, cat nr 4351379: C_3163590) and using the HT7900 real-time PCR system by Life Technologies (Foster City, CA, USA).

Association analysis were performed by logistic regression using SPSS software. Association was tested for all main criteria for diagnosis of RA as well as other relevant manifestations and family history. A χ^2 test was used to test for Hardy-Weinberg Equilibrium (HWE) in the control group. For genetic association ORs (Odds-ratio) and 95% CIs were used to assess the strength of the correlation between the *ESR1* polymorphism and RA susceptibility and specific clinical manifestations. Additive (CC vs TT), recessive (CC vs CT/TT) and dominant model (CC/CT vs TT) as well as allelic association (C vs T) were tested. A p value of <0.05 (two-tailed) was considered statistically significant. When analyzing multiple clinical features, Bonferroni correction for multiple testing was used and both corrected and uncorrected p values are presented.

Results

The description of the clinical manifestations of the RA patients enrolled in the study is shown in **Table 1**. The

Table 1. Clinical characterization of the 181 RA patients analyzed in the study.

Characteristics	**n *(%)***
Synovitis	133 (73.5)
Rheumatoid nodules	31 (17.1)
Rheumatoid vasculitis	10 (5.5)
Cardiopathy	37 (20.4)
Dislipidemia	109 (60.2)
Depression	38 (21.0)
High blood pressure	121 (66.9)
Osteoporosis	63 (34.8)
Anemia	36 (19.9)
Leukopenia	6 (3.3)
Thromocytopenia	10 (5.5)
Elevated ESR	121 (70.7)
Elevated C-reactive protein	88 (48.6)
Autoantibodies	
Anti-RF +	132 (73.0)
Anti-CCP +	119 (65.4)
DAS 28 (mean, ± SD)	3.69 ± 0.92
Age of onset (mean, ± SD)	43.15 ± 12.85
Other ADs present	19 (10.5)
RA in the family	18 (9.9)
Other ADs in the family	37 (20.4)

Anti-RF: Anti-Rheumatoid Factor Antibody; Anti-CCP: Anti-Cyclic Citrullinated Peptide Antibody; ESR: Erythrocyte Sedimentation Rate; DAS28: Disease Activity Score Based on 28 Joints

patient's data revealed a similar profile as in European studies. The autoantibodies play an essential role in the pathogenesis of many ADs and mediate both tissue injury and systemic inflammation. Our results showed that Rheumatoid Factors (RFs) were present in about 73% of RA patients. ANA was also frequent (about 66%) in RA patients (data not shown). Also, 72% of the patients presented elevated Erythrocyte Sedimentation Rate (ESR). The high frequency of these autoantibodies and elevated ESR suggest that most patients may be in the more advanced stages of RA.

The control group were in Hardy-Weinberg equilibrium (p=0.29) for the variant tested. The association analysis revealed that the rs2234693-C allele was a risk factor for RA and related clinical manifestations. rs2234693-CC genotype was significantly associated with RA itself (OR=1.40, p = 0.0014 for allelic association and OR=2.21, p=0.0032 by genotypic association with a dominant model) (**Table 2**).

Analyzing the clinical features of the patients, the C allele was also associated with the presence of a number of sub-phenotypes; cardiopathy (OR=2.40, p=0.025), high blood pressure (OR=2.50, p=0.019) and osteoporosis (OR=1.94, p= 0.031). The variant was also slightly

Table 2. Multivariate logistic regression analyses of *ESR1*- rs2234693 with RA development and specific clinical features. Distribution of allele and genotype frequencies of epidemiological and clinical data for the case group (+) (RA patients who present the manifestation) and control group (-) (patients who do not have the manifestation), measure of association (OR), confidence interval (CI 95%) and *p* value.

	MAF		**OR (CI)**	***p-value***	**CC genotype**		**OR (CI)**	***p-value***
Characteristic	+	-			+	-		
RA	0.57	0.43	1.40 (1.02-1.72)	0.0014	0.29	0.19	2.21 (1.31-3.75)	**0.**0032
Synovitis	0.48	0.46	1.08 (0.67-1.74)	0.736	0.24	0.13	1.02 (0.61-1.77)	0.891
Rheumatoid nodules	0.43	0.47	0.87 (0.49-1.53)	0.628	0.1	0.22	0.32 (0.49-1.24)	0.222
Rheumatoid vasculitis	0.35	0.47	0,61 (0.23-1.57)	0.308	0.12	0.2	0.37 (0.19-2.41)	0.213
Cardiopathy	0.57	0.43	1.74 (1.04-2.91)	0.036[a]	0.24	0.18	2.40 (1.06-5.43)	0.025[e]
High blood pressure	0.58	0.42	1.79 (1.08-3.11)	0.032[b]	0.25	0.16	2.51 (1.12-5.79)	0.019[f]
Osteoporosis	0.59	0.42	1.99 (1.13-3.49)	0.016[c]	0.21	0.14	1.94 (1.06-3.46)	0.031[g]
Hematologic disorders*	0.46	0.47	0.88 (0.69-1.58)	0.747	0.19	0.17	1.01 (0.65-1.77)	0.876
Elevated ESR	0.57	0.43	1.41 (1.16-1.93)	0.039[d]	0.26	0.17	1.98 (1.16-3.01)	0.033[h]
Elevated CRP	0.47	0.45	1.02 (0.51-1.37)	0.807	0.19	0.25	0.73 (0.38-1.36)	0.276
Antibodies								
Anti-CCP +	0.55	0.39	1.91 (1.09-3.36)	0.024[i]	0.15	0.23	0.75 (0.36-1.48)	0.713
Rheumatoid Factor +	0.46	0.49	0.90 (0.56-1.43)	0.648	0.19	0.2	0.94 (0.54-1.60)	0.972

	TT	**CT**	**CC**	***p*-value**
Number of ACR criteria met (mean ± SD)	4.72 ± 1.66	4.18 ± 1.60	3.97 ± 1.73	0.051
DAS28 (mean ± SD)	3.58 ± 0.91	3,77 ± 0,86	3,60 ± 1,08	0.104
Age of onset (mean ± SD)	42.0 ± 12.5	44.6 ± 12.7	40.4 ± 13.6	0.39

MAF: Minor Allele Frequency; CI: Confidence Interval 95%; significant values are noted in bold – p value ≤0.05. ***Hematological disorders included anemia, leukopenia and thrombocytopenia*** [a]Bonferroni corrected p value = 0.43; [b]Bonferroni corrected p value = 0.38; [c]Bonferroni corrected p value = 0.192; [d]Bonferroni corrected p value = 0.46 ; [e]Bonferroni corrected p value = 0.30; [f]Bonferroni corrected p value = 0.29 ; [g]Bonferroni corrected p value = 0.37; [h]Bonferroni corrected p value = 0.40 ; ns = Not Significant in Any Model. ESR = Erythrocyte Sedimentation Rate; CRP = C-Reactive Protein; Anti-CCP = Anti-Cyclic Citrullinated Peptide

associated with elevated ESR (rs2234693-CC: OR = 1.98, p = 0.033),

Considering the serological parameters, no association was found with the presence of rheumatoid factor antibody. However, the rs2234693-C allele was associated with the presence of anti-CCP (Cyclic Citrullinated Peptide) antibodies (OR= 1.91, p = 0.024), an important prognostic tool for RA. We could not detect any association with the age of onset, number of ACR met or DAS28 (Disease Activity Score) index (**Table 2**).

Discussion

Despite the increasing evidence revealing the importance of sex hormones in autoimmune pathways, the etiological role and potential effects of estrogen and variations in this pathway in the development and/or triggering of AD are still unclear. It is known that this hormone stimulates B cell growth and antibody production, helper T cells differentiation and autoimmune-associated cytokine production, all of which could contribute to RA susceptibility [21,22]. Since estrogen´s effect is mediated mainly through the estrogen receptors and its downstream gene activation, polymorphisms that influences the expression of these receptors might have a role in individual responsiveness to estrogens and potentially influence a person's susceptibility to develop AD. Interestingly, the *ESR1* gene has not been identified by any of the genome-wide association studies for predisposition to rheumatoid arthritis. However, rs2234693-C allele has been associated with RA in a Japanese population, however not with the disease itself or any clinical finding, only with the age of onset and in female patients only [23]. Discording with the previous report, in our study, we could not detect a correlation with the age of onset of the disease however this variant was associated with RA *per se*. Also, another Asian study found association of the rs2234693-T allele with knee osteoarthritis in Chinese [24]. However, we found slightly different frequencies of several clinical manifestations described between our and the Asian patient group analyzed. These divergences might reflect simple sample heterogeneities or represent in fact true variances as a result of genetic and environmental dissimilarities between these populations. Other studies did not provide or analyze detailed clinical features [6,18]. Exploring individual clinical manifestations, interestingly the C allele was also correlated with the presence of cardiopathy, high blood pressure and osteoporosis, all of which have been previously independently associated with *ESR1* variants. [25] described an association of *ESR1* polymorphism with major arteriosclerotic cardiovascular disease. Ionnidis JP (2004) found that rs2234693 is a susceptibility variant for fractures, but in a mechanism independent of bone mineral density [26]. The SNP was has also been correlated with bone loss in early postmenopausal women [27] and with family history of osteoporosis and hip fracture [28]. In addition, a GWAS study revealed the involvement of *ESR1* variants with bone mineral density [29,30]. However, it had never been analyzed in the context of RA, where the SNP could represent an independent risk factor for these features or act as a modifying factor within the RA pathology.

We could not find association with the occurrence of other ADs in the same patient or presence of relatives with RA or other AD in the family, which had not been analyzed in the former studies.

But interestingly we detected an association between the variant and the presence of anti-CCP antibodies. Its association with disease activity and functional capacity is still not fully understood, although many studies suggest that these antibodies are associated with more severe disease [31,32]. The previous studies did not consider or explore in much detail the correlation of the *ESR1* variant with the presence of autoantibodies, so to our knowledge it is the first study to report an association between a genetic variant in the estrogen pathway and the presence of an autoantibody in RA patients; further studies might enlighten this connection.

The function of rs2234693 polymorphisms is unknown however is believed to affect expression levels of the gene. The SNPs is located within the first intron of the gene and the C allele is predicted to create a binding site for the transcription factor B-myb. Using luciferase assays it was found that the presence of C allele was correlated with a four times higher expression of a downstream reporter construct when compared to the T allele when co-transfected with B-myb [13,33]. Thus, the C allele could contribute to the upregulation of the *ESR1* expression and therefore explaining the higher expression of the receptor in a sub-group of AD patients [34,35]. This higher expression could lead to a potential higher responsiveness to estrogens. On the other hand, other studies have found a lower expression of *ESR1* in breast tissues of individuals with this allele [36]. Therefore, there is no clear picture of the effect of this variant on gene expression, which may vary depending of the tissue analyzed.

In the context of estrogens and AD, several studies have addressed the safety of oral contraceptives (estrogen and/

or progestin-based) and hormone replacement therapy for these patients, with conflicting results [11,12]. As the majority of complex diseases, the heterogeneity in RA patients is notable, and it is likely that the divergence observed between these studies reflect the heterogeneity between patient groups analyzed in each study and the explanation probably includes specific individual genetic variants. If the risk allele associated with RA in this study in fact leads to altered *ESR1* expression, it might result in altered responsiveness to endogenous as well as exogenous estrogens, influencing the expression of the disease.

Concerning treatment, [37] demonstrated by gene expression profiling that Methotrexate therapy results in overexpression of *ESR1* in patients. This information, combined to the here presented association of a genetic variant that might contribute to RA development and that possibly alters the *ESR1* expression, deserves further consideration. It could be that for some patients, the effect of this treatment on estrogen receptor expression might be potentialized or inhibited depending on their *ESR1* genotype and possibly influence the outcome and/or side effects of the therapy.

In addition, the correlation of genetic markers with particular clinical manifestations within the disease is of great importance in the understanding of the disease pathways and to more personalized diagnostic and therapeutic means in the future.

Conclusions

ESR1 is associated with RA, as well as with the presence of anti-CCP antibodies and particular RA-associated clinical manifestations (cardiopathy, high blood pressure and osteoporosis). To the best of our knowledge this is the first study describing an association of this polymorphism with RA in a Latin-American population. Our results are suggestive and as a small study has its limitations. Thus, the result must be considered preliminary and further validation in independent patient groups is warranted and might together contribute to the understanding of the mechanism behind the effect of genetic polymorphisms in the estrogen pathway and the development of RA as well as other AD.

Data Availability

The data that support the findings of this study are available from the corresponding author upon request.

Conflict of Interest

The authors declare no conflict of interest.

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Compliance with Ethical Standards

The present study has been approved by the ethics committee of the University of Santa Catarina and the University Hospital. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All the enrolled patients and healthy volunteers provided written informed consent.

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