Association Between the Aggrecan Gene and Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** Genetic and environmental factors seem to be involved in the onset of rheumatoid arthritis (RA). We analyzed whether a variable number of tandem repeats (VNTR) polymorphism in the aggrecan gene was associated to RA.

Methods. The study population comprised 170 European-derived Brazilian patients with diagnosis of RA. The control group comprised 148 European-derived Brazilian healthy blood donors. The aggrecan VNTR polymorphism was genotyped by DNA amplification by polymerase chain reaction, followed by electrophoresis in polyacrylamide gel.

Results. There was a statistically significant higher frequency of alleles of shorter length in the patient group compared to controls ($p = 0.001$), suggesting that individuals carrying short alleles are more likely to develop RA. There was no association between short alleles and clinical characteristics of RA.

Conclusion. Our results provide evidence of an association between the aggrecan gene VNTR polymorphism and RA. (First Release Nov 1 2008; J Rheumatol 2008;35:2325–8; doi:10.3899/jrheum.071326)

Key Indexing Terms:
RHEUMATOID ARTHRITIS AGGRECAN POLYMORPHISM
VARIABLE NUMBER OF TANDEM REPEATS
ARTICULAR CARTILAGE CHONDROITIN SULFATE

Rheumatoid arthritis (RA) is an autoimmune disease with chronic inflammation primarily of synovial joints. The etiology of RA remains elusive, although it appears that genetic and environmental factors are involved[1]. There is inflammation and cellular proliferation in the synovium, a delicate lining of the joints. In RA, inflammatory pathology of joints involves the activation and hyperactivity of proinflammatory T cells[2]. These T cells produce mediators (cytokines) that initiate the inflammatory process, attracting other immune cells, and causing an excess of synovial fluid production[1]. This process leads to joint deformities that are characteristic of the disease. To date, there is no cure or prevention for RA and 0.5% to 1.0% of the adult population worldwide is affected[3].

Articular cartilage is predominantly composed of extracellular matrix (ECM), whose constituents are synthesized by the resident chondrocytes that are also responsible for its maintenance[4]. ECM molecules in cartilage include proteoglycans, hyaluronan, type II collagen, glycoproteins, and mixtures of elastic fibers[5]. The articular cartilage is based on hyaline cartilage, a thin, smooth, stiff, wear-resistant layer that provides a low-friction weight-bearing joint surface that allows the joint to move smoothly and without pain[5].

Aggrecan is the major proteoglycan of the hyaline cartilage, where it is present at very high concentrations in the form of aggregates, which create osmotic swelling pressure and draw water into the tissue. The aggrecan has 3 globular domains (G1, G2, and G3), a short interglobular domain (IGD) between domains G1 and G2, and a long glycosaminoglycan (GAG) attachment region between domains G2 and G3 that consists of adjacent domains of keratan sulfate (KS) and chondroitin sulfate (CS)[6]. The major function of aggrecan is intimately related to the KS and the CS domains since the negatively charged KS and CS attract Na^+ and consequently attract water.

Exon 12 of the aggrecan gene encodes the CS domain. This domain exhibits a variable number of tandem repeats (VNTR) polymorphism. The polymorphism presents repeats of 57 nucleotides, encoding each 19-amino acid unit. The described alleles range from 13 to 34 repeats[6,7]. Each repeat contains 2 possible attachment points for CS, so that the extreme range of alleles might vary by as many as 42 CS chains per core protein monomer. The length of the core

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protein varies directly with repeat numbers, and this length variation may lead to changes in cartilage function[8].

Our aim was to ascertain if the aggrecan VNTR polymorphism is associated with RA in a Brazilian population.

MATERIALS AND METHODS

Study population. The study population comprised 170 Caucasian patients with diagnosis of RA satisfying the American College of Rheumatology criteria[9], under the care of the Division of Rheumatology of the Hospital de Clínicas de Porto Alegre. Patients had their medical records reviewed or underwent a medical interview for documentation of clinical, laboratory and radiographic data. The extraarticular manifestations evaluated were rheumatoid nodules, amyloidosis, vasculitis, and episcleritis. The pattern of joint involvement and the presence of rheumatoid nodules were evaluated by physical examination. Radiographs of the hands and feet were obtained for differentiation between erosive and nonerosive disease by a radiologist and a rheumatologist. Rheumatoid factor was identified by nephelometry, latex agglutination, or Waaler-Rose test.

The control group consisted of 148 healthy Caucasian donors recruited from a blood bank of the urban population of Porto Alegre (the capital of the southernmost state of Brazil, Rio Grande do Sul), the same geographic area as the patients. The study was approved by the Institutional Ethics Committee of the Universidade Luterana do Brasil. All subjects gave written informed consent.

DNA analysis. Peripheral blood was collected and genomic DNA was extracted from the samples. The aggrecan gene (*AGC1*) VNTR polymorphism was genotyped from each sample by polymerase chain reaction (PCR) amplification. Fragments were amplified using the forward primer 5'-ATT GAG TGG CCC AGC ACT CCT ACG-3', as described[8], and the reverse primer 5'-AGG TCC CCT ACC GCA GAG GTA GAA-3'. The amplification reaction was carried out in 25 μl containing genomic DNA, 1 μM of each primer, 1.5 mM of $MgCl_2$, 200 μM dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), and 1 U Taq DNA polymerase. The amplification conditions were: 35 cycles, each cycle consisting of denaturation at 94°C for 15 s, and annealing and extension at 72°C for 2.5 min. The amplified fragments can vary from 742 base-pair (bp) for the 13-repeat allele to 1939 bp for the 34-repeat allele. PCR products were separated on 6% polyacrylamide gels stained with silver nitrate.

Statistical analysis. Data were analyzed using SPSS for Windows version 11.5 (SPSS, Chicago, IL, USA) and WINPEPI[10]. The association between categorical variables was tested using chi-square (with Yates' correction when necessary) or Fisher's exact tests. The comparisons of allele frequencies were done by the nonparametric Mann-Whitney U test. The quantitative variables with normal distribution were tested by Student's T test. A p value $\leq$ 0.05 was considered statistically significant. All P values presented are 2-tailed.

RESULTS

The allele frequencies in patients and controls are shown in Table 1. In the total sample, 15 alleles and 31 genotypes were observed, and the 26, 27, and 28 alleles were the most frequent. In the control group, 11 alleles and 26 genotypes were observed, with 26% of the individuals being homozygous. In the patient group, 13 alleles and 27 genotypes were observed, with 27% of the individuals being homozygous. In both groups, homozygous individuals were observed for the 26, 27, and 28 alleles. In the RA patient group, homozygous individuals were also observed for the 21, 22, and 23 alleles. The allele frequencies we observed were similar to those previously described for other populations, in which the most common alleles also possessed 26, 27, or 28 repeats[6].

Table 1. Allele frequencies of aggrecan gene VNTR polymorphism.

Allele	RA Patients, n = 170		Controls, n = 148	
	n	%	n	%
13	2	0.6	0	0
18	4	1.2	1	0.3
19	1	0.3	1	0.3
20	2	0.6	2	0.7
21	4	1.2	4	1.5
22	16	4.7	6	2.0
23	10	2.9	6	2.0
24	2	0.6	0	0
25	7	2.0	7	2.4
26	57	16.8	38	12.8
27	151	44.4	125	42.2
28	80	23.5	98	33.1
29	4	1.2	5	1.7
30	0	0	2	0.7
31	0	0	1	0.3

The allele score, defined as the sum of the VNTR allele number found in the 2 aggrecan alleles of each subject, was significantly different between RA patients and controls (mean RA patients' score 52.7; mean control group score 53.7; p = 0.004). The patients presented alleles of lower size significantly more frequently than controls (p = 0.001). When we compared demographic and clinical characteristics of patients carrying alleles with less than 23 repeats (divided arbitrarily, as suggested by Roughley, *et al*[6], in the mid-point of the range of CS repeats in the population), no statistically significant differences were observed (Table 2).

DISCUSSION

Aggrecan provides cartilage and intervertebral discs with the ability to resist compressive loads. The localized high concentrations of aggrecan provide the osmotic properties necessary for normal tissue function. This functional ability is dependent on a high GAG concentration in the ECM. The formation of large proteoglycan aggregates is essential to restrict the movement of aggrecan in the cartilage ECM and so prevent diffusion away from the site under compression.

In recent years, several RA susceptibility genes have been identified[11]. However, this is the first study regarding aggrecan polymorphism and RA. Previous studies analyzed the aggrecan VNTR polymorphism in diseases that also involve ECM degradation, such as osteoarthritis (OA), lumbar disc degeneration, and idiopathic scoliosis, with controversial results[6,7,12-16]. Kawaguchi, *et al*[15] found an association between the shorter alleles of the polymorphism and multilevel disc degeneration. The shorter alleles have not always been associated with disease. Association between the allele 27 and OA was described[12], but another study observed a protective effect of allele 27 in patients with

Table 2. Demographic and clinical characteristics of the RA patients and genotype distribution of the aggrecan VNTR polymorphism.

Characteristics	RA Patients, n = 170	Genotype Distribution in RA Patients	
		1 or 2 Alleles with < 23 Repeats, n = 35	2 Alleles with ≥ 23 Repeats, n = 135
Female, %	80	71	74
Age, mean ± SD, yrs	55.2 ± 11.9	55.1 ± 12.0	55.3 ± 11.5
Age at diagnosis, yrs	45.3 ± 13.1	45.0 ± 13.5	46.2 ± 12.2
Age at onset of symptoms, yrs	40.6 ± 13.0	40.7 ± 12.7	39.5 ± 14.2
Rheumatoid factor positivity, %	89	83	92
Erosions, %	86	83	87
Extraarticular manifestations, %	25	20	26
Rheumatoid nodules, %	21	23	21

OA[7]. We found an association between alleles of shorter length and RA. Subjects who carried the alleles with a smaller number of repeats have the potential to produce aggrecan molecules with a smaller number of CS chains. How this polymorphism could affect aggrecan function and be related to RA is an intriguing question. A possible explanation is related to autoimmunity, a feature present at several levels in RA. There is increasing evidence that aggrecan may be a potential autoantigen in humans with rheumatoid joint diseases[17–24]. A role for aggrecan in joint pathology was strengthened by the observation in an animal model that immunization with human aggrecan can lead to the development of progressive polyarthritis[25,26]. The G1 globular domain is the main target of immunity[27,28], but critical roles of GAG side chains of the aggrecan in antigen recognition and presentation were also described in BALB/c mice. Both GAG side chains (CS and KS) interfered with the immune response to T cell epitopes of the core protein and inhibited the development of proteoglycan-induced arthritis[26]. In addition, antigens containing repetitive epitopes, such as those present in aggrecan, could induce an immune response, even in cells with lower affinity for the epitope[29]. This response is predominantly primary (IgM), involves T cell-independent B cell activation, and there is no B cell maturation. The B cell activation depends on the affinity between antigen and B cell receptor. Previous studies have shown that depletion of the CS side chains increases the B cell responses to aggrecan[26], and that there is a decrease in the chain length and in the content of CS in aggrecan with increasing age[30]. Since aggrecan molecules coded by smaller alleles potentially have less CS, it is possible that they could present a higher degree of immunogenicity as compared to aggrecan coded by larger alleles.

Besides the link with autoimmunity, the presence of shorter alleles of the aggrecan can have consequences in the context of enhanced articular cartilage degradation observed in RA. During normal turnover, the aggrecan protein undergoes selective proteolytic degradation of the GAG-rich region of the molecule, resulting in accumulation of heterogeneous partially degraded aggrecan molecules in the ECM. Degradation of the articular cartilage is one of the early features of RA and it is mediated by increased activity of proteolytic systems. The aggrecan degradation products are free to diffuse into the synovial fluid. One could suggest that aggrecan molecules encoded by the smaller alleles could diffuse into the synovial fluid more easily than larger alleles, resulting in more pronounced effects of ECM degradation. Short CS1 domains also could be more susceptible to cleavage by proteolytic enzymes. There is little information on cleavage within the CS1 domain, but studies have shown that covalently bound CS regulates cleavage by proteolytic enzymes, and the removal of CS increases cleavage in the interglobular domain[31,32]. Moreover, studies indicate that some proteolytic enzymes that cleave aggrecan are upregulated by proinflammatory cytokines such as interleukin 1, which is frequently found in elevated concentrations in RA joints[33,34].

It is important to note that in association studies there are concerns about spurious associations secondary to ethnic variability in allele frequencies. Given the ethnic heterogeneity in the Brazilian population, it is possible that our results were influenced by population stratification. In order to limit the population admixture, our sample comprised individuals of European descent as ascertained by skin color and morphological characteristics[35] of our urban study population. Different from Brazil as a whole, the population of Rio Grande do Sul consists mainly of people with European ancestry (82%). For this reason, the possible bias due to population stratification was very small[36].

Our results provide evidence of an association between the aggrecan gene VNTR polymorphism and RA. These results give new insight into the pathogenesis of RA, suggesting that short aggrecan molecules may exhibit a different degree of immunogenicity or impaired function in the context of degradation of articular cartilage in patients with RA. Although our data do not provide definitive evidence of causality between aggrecan polymorphism and RA, they may assist in formulating testable hypotheses in future stud-

ies. Further investigations in different settings are needed to confirm the association and to elucidate the role of this polymorphism in the pathogenesis of RA.

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