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Case Report

Association between an *ACAN* gene variable number tandem repeat polymorphism and lumbar disc herniation: a case control study

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ABSTRACT. We investigated the association between an aggrecan gene (*ACAN*) polymorphism and lumbar disc herniation (LDH). This was a case-control study with quinquennial age and gender groups. The study comprised 119 men and women aged between 20 and 60 from Goiânia (Brazil). Of these, 39 were allocated to the case group (Ca) and 80 to the control group (Ct). We gathered sociodemographic and clinical data, and peripheral blood samples. DNA was isolated for genotyping the *ACAN* variable number tandem repeat (VNTR) via conventional polymerase chain reaction (PCR). Data were statistically

analyzed using the chi-square test, multiple comparison analysis, the Student *t*-test, and odds ratios, with a level of significance set at 5% ($P \leq 0.05$). The groups were homogenous in terms of sociodemographic, anthropometric, and life style variables. The allele score for the *ACAN* VNTR was significantly lower in volunteers with LDH; the *A22* allele was significantly more prevalent in this same group; the Ca group presented greater frequency of short alleles *A13*-*A25*, whereas the Ct group presented a higher frequency of long alleles. However, this difference was not statistically significant. In both groups, the most common alleles were *A28*, *A27*, and *A29*, and the *A26/A26* genotype was significantly more common in the Ca group. The results showed an association between short alleles and LDH among the investigated adults (Ca), corroborating the hypothesis that aggrecan with shorter repeat lengths can lead to a reduction in the physiological proteoglycan function of intervertebral disc hydration and, consequently, increased individual susceptibility to LDH.

Key words: Lumbar disc herniation; Variable number tandem repeat; Intervertebral disc degeneration; Genetic polymorphism; Proteoglycans; *ACAN*

INTRODUCTION

Lumber disc herniation (LDH) is a common diagnosis among degenerative alterations of the lumbar spine, and is the leading cause of spinal surgery in the adult population. Although it is a benign condition, it is considered a global health problem because it leads to debilitation, negative impacts on physical and work activities, decreased quality of life, and psychological distress in affected individuals (Vialle et al., 2010; Karppinen et al., 2011; Eskola et al., 2012).

The disease is intimately related to intervertebral disc degeneration (Falavigna et al., 2010). However, the etiology of degenerative disc disease (DDD) is complex. Although environmental, ergonomic, and anthropometric factors have traditionally been determined as causes, several studies have suggested that genetic factors or familial predisposition contribute to the degeneration and consequent herniation of intervertebral discs (Battié et al., 2004; Brioni Nunes et al., 2007; Zhang et al., 2008; Eser et al., 2010; Paz Aparicio et al., 2011).

According to Videman et al. (2009), the process of disc degeneration involves desiccation, collagen fragmentation, and failures in the annulus fibrosus, resulting in disc height narrowing. Zhang et al. (2008), Eser et al. (2010), and Mayer et al. (2013) indicate several genes that have been associated with DDD, including the human aggrecan gene (*ACAN*). Cong et al. (2010) reported that this gene is responsible for encoding aggrecan, the largest proteoglycan in the hyaline cartilage and fibrocartilaginous disc, which is responsible for creating an osmotic pressure gradient and preserving the water content of intervertebral discs, contributing to hydration.

Studies have demonstrated a strong association between disc degeneration and genetic factors, indicating that individuals who present with a significant degenerative process usually have a family history of DDD (Chan et al., 2011; Kao et al., 2011). The first genetic polymorphisms associated with DDD were two variations in the vitamin D receptor gene

(*VDR*). Later, polymorphisms in the *COL9A2*, *COL9A3*, and *ACAN* genes were identified (Kelempisioti et al., 2011). Although some studies have shown a positive association between LDH and *ACAN* polymorphisms, this is the first study conducted among Brazilian individuals.

Considering that LDH is a frequent disorder among adults, understanding its relationship to genetic factors can provide new prevention and intervention goals for individuals affected by the disease. Furthermore, given that genes can act independently but are also susceptible to environmental interactions, people with a family history of LDH must be given more effective guidance regarding external risk factors, regardless of exposure to environmental factors, to prevent the emergence of the disease.

Thus, the aim of this study was to investigate the association between the *ACAN* gene polymorphism and LDH in men and women residing in the metropolitan region of Goiânia (Goiás, Brazil).

MATERIAL AND METHODS

This was a case-control study with quinquennial age and gender groups. The research was carried out in the Replicon Research Center, part of Pontifícia Universidade Católica de Goiás (PUC Goiás), and in the Human Cytogenetics and Molecular Genetics Laboratory (LaGene/Dr. Giovanni Sysneiro Public Health) of the Health Secretariat of the State of Goiás. Patients who had been treated or were still undergoing treatment at the physical therapy teaching clinic and in rehabilitation reference centers in the city of Goiânia were invited to participate in the study. Individuals with no history of LDH or lumbar pain living in the city of Goiânia were recruited to the control group.

This study abided by resolution No. 466/12 of the Brazilian National Health Council and was approved by the research Ethics Committee of PUC Goiás, under resolution No. 563.672. All participants were selected using convenience sampling, and comprised 39 individuals with LDH [the case group (Ca)] and 80 individuals without LDH for pairing [the control group (Ct)], totaling 119. The statistical power was set at 85% with a confidence level of 95% (0.05).

All participants voluntarily donated peripheral blood samples for genetic analysis. Sociodemographic, anthropometric, and clinical data were also gathered using an assessment form. All clinical data were related to LDH levels and type of herniation in the Ca group, in addition to family history of LDH, life habits, physical exercise, and concomitant diseases. All participants were duly informed about the research and signed informed consent forms.

The following criteria were adopted for the inclusion of participants: men and women between 20 and 60; for the Ca group, a confirmed history of LDH via imaging tests [magnetic resonance imaging (MRI) or computerized tomography]; and individuals with LDH, treated or untreated, either with conservative or surgical treatment. The inclusion criterion for the Ct group was the absence of LDH.

Exclusion criteria were: trauma-related LDH, previous diagnosis of rheumatic and neurological diseases, LDH together with cervical and/or thoracic herniation, absence of LDH, but presenting lower back pain, lumbosciatica or any suggestive signs or symptoms of the disease, cognitive limitations and/or any discomfort that could prevent individuals from providing blood samples and personal data.

The first step consisted of approaching potential research participants and explaining the objectives and importance of the study. After expressing consent, participants were

asked about specific issues that could exclude them from the study, and after ascertaining that they met the inclusion criteria they read and signed the consent form. Next, an anamnesis was carried out to collect personal data on the assessment form, which was developed by the researcher responsible for this study. The next phase consisted of gathering 4-mL peripheral blood samples from participants using ethylenediaminetetraacetic acid anticoagulant. The DNA was isolated to genotype the variable number tandem repeat (VNTR) of *ACAN* via conventional polymerase chain reaction (PCR). Two 2-mL aliquots were prepared in conical tubes and preserved in a freezer at -20°C for subsequent DNA extraction, which was isolated using the commercial kit Ilustra Blood GenomicPrep Mini Spin® (GE Healthcare, UK), according to the manufacturer instructions. The isolated DNA was identified and stored at -20°C, and then used to assess polymorphic variants in the *ACAN* VNTR region.

The sense and antisense primer sequences used for PCR were 5'-TAGAGGGCTCTGC CTCTGGAGTTG-3' and 5'-AGGTCCCCTACCGCAGAGGTAGAA-3', respectively.

A PCR assay was carried out using Platinum TAQ DNA Polymerase. A 50-µL solution was used, containing 10 pmol sense and antisense sequence, 5 µL genomic DNA, 27.5 mM dNTP (deoxynucleotide triphosphates), 0.3 µL Taq DNA polymerase, 25 mM $MgCl_2$, and 10X PCR buffer and water. Amplification was conducted over 38 cycles, with denaturation at 95°C for 5 min, followed by annealing at 66°C for 50 s and extension at 72°C for 50 s. The PCR products were separated on 1.5% agarose gel and visualized using a photodetector (BioRad, USA) after staining with ethidium bromide gel (Eser et al., 2010, 2011). Alleles were measured using a standard molecular weight of 100 bp. The number of repeats in the region was determined according to procedures set forth by Roughley et al. (2006).

Alleles were categorized according to size: short (13 to 25 repeats), medium (26 to 27 repeats), or long (28 to 32 repeats), as described by Doege et al. (1997), Kawaguchi et al. (1999), and Solovieva et al. (2007). In this study, each allele was represented using the letter A followed by the number of repeats; e.g., *A13* indicates an allele with 13 repeats, and so forth.

The homogeneity of the sociodemographic data between case and control groups was tested using the chi-square test (χ^2) using contingency tables. The comparison of frequency distribution for each allele and genotypes of these same groups was conducted using χ^2, including a multiple comparison analysis (*post hoc*), with Bonferroni correction. The Student *t*-test was chosen to compare Ca and Ct group allele scores. All analyses were conducted with the help of the Statistical Package for the Social Sciences (SPSS) software, version 23.0, with a significance level of 5% ($P \leq 0.05$).

RESULTS

The sample comprised 119 individuals, of which 42% were women and 58% were men. Most participants were under 45 years of age, corresponding to 58.8% of volunteers. Regarding family history of LDH, 38.5% of individuals in the Ca group responded affirmatively, whereas in the Ct group this percentage was 28.8%. However, the analysis of the total study sample showed that most participants (68.1%) did not have a family history of LDH.

According to the results of the χ^2 test, there were no statistically significant differences between the groups, demonstrating that they were homogenous concerning the presented variables (Table 1).

Table 1. Distribution of sociodemographic, anthropometric, life style and family history data in case group and control group to assess the impact of aggrecan gene polymorphism on susceptibility to lumbar disc hemiation in Goiânia, Goiás, Brazil, 2015.

Independent variables	Case [N (%)]	Control [N (%)]	Total [N (%)]	P
Age				0.98
=45 years	23 (59.0)	47 (58.8)	70 (58.8)	
>45 years	16 (41.0)	33 (41.2)	49 (41.2)	
Gender				0.87
Female	16 (41.0)	34 (42.5)	50 (42.0)	
Male	23 (59.0)	46 (57.5)	69 (58.0)	
BMI				0.43
=24	20 (51.3)	31 (38.8)	51 (42.9)	
25-29	15 (38.5)	39 (48.8)	54 (45.4)	
=30	4 (10.3)	10 (12.5)	14 (11.8)	
Smoking				0.92
No	31 (79.5)	63 (78.8)	94 (79.0)	
Yes	8 (20.5)	17 (21.3)	25 (21.0)	
Physical exercise				0.71
No	27 (69.2)	58 (72.5)	85 (71.4)	
Yes	12 (30.8)	22 (27.5)	34 (28.6)	
Family history				0.39
No	24 (61.5)	57 (71.3)	81 (68.1)	
Yes	15 (38.5)	23 (28.8)	38 (31.9)	
Occupation				0.32
Student	0 (0.0)	6 (7.5)	6 (5.0)	
Science professional	10 (25.6)	20 (25.0)	30 (25.2)	
Administrative services	10 (25.6)	12 (15.0)	22 (18.5)	
General maintenance and repair services	10 (25.6)	25 (31.3)	35 (29.4)	
Commercial workers	9 (23.1)	17 (21.3)	26 (21.8)	
Time at profession				0.79
=10 years	18 (46.2)	39 (48.8)	57 (47.9)	
>10 years	21 (53.8)	41 (51.3)	62 (52.1)	

The distribution of allele frequency in the *ACAN* gene VNTR region among the Ca and Ct groups was calculated using the χ^2 test. The results show that the most common alleles in both groups were *A28*, *A27*, and *A29*. In the Ca group, these frequencies corresponded to 32.1, 17.9, and 12.8%, respectively. In the Ct group, the frequency of allele *A28* was 37.5%, whereas alleles *A27* and *A29* represented 15.6%. The frequency of allele *A22* was also higher in the Ca group (7.7%) than the Ct group (1.9%). These data are presented in Figures 1 and 2.

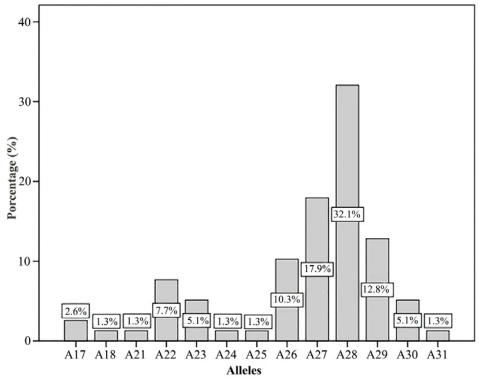

Figure 1. Distribution of allele frequencies in the aggrecan gene (*ACAN*) VNTR region in the case group to assess the impact of *ACAN* polymorphism on susceptibility to lumbar disc herniation in Goiânia, Goiás, Brazil, 2015.

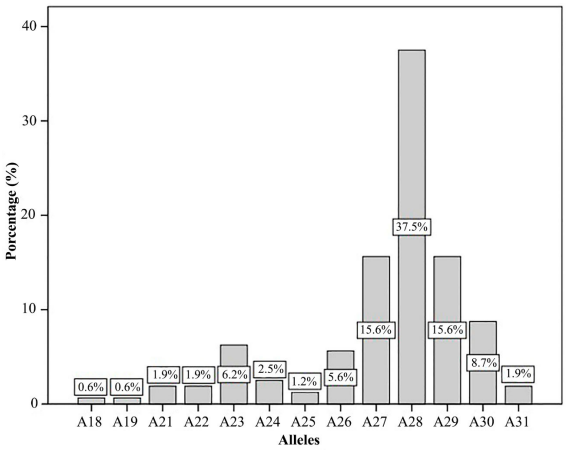

Figure 2. Distribution of allele frequencies in the aggrecan gene (*ACAN*) VNTR region in the control group to assess the impact of *ACAN* polymorphism on susceptibility to lumbar disc herniation in Goiânia, Goiás, Brazil, 2015.

Alleles *A17* to *A31* were present in the study sample. In accordance with the cited methodology, we used the following categories: short (13-25), medium (26-27), and long (28-32) alleles. Comparison analysis of the distribution of allele frequency between the Ca and Ct groups revealed that the frequency of the short alleles was higher in the Ca group; however, only *A22* presented a statistically significant difference (P = 0.03) between the two groups (Table 2). This finding indicates that this allele may be a risk factor for LDH.

Table 2. Comparison of allele frequency in the aggrecan gene (*ACAN*) VNTR region in case and control groups to assess the impact of *ACAN* polymorphism on susceptibility to lumbar disc herniation in Goiânia, Goiás, Brazil, 2015.

Alleles	Case [N (%)]	Control [N (%)]	P
A17	2 (2.6)	0 (0.0)	0.06
A18	1 (1.3)	1 (0.6)	0.61
A19	0 (0.0)	1 (0.6)	0.49
A21	1 (1.3)	3 (1.9)	0.74
A22	6 (7.7)	3 (1.9)	0.03*
A23	4 (5.1)	10 (6.3)	0.73
A24	1 (1.3)	4 (2.5)	0.54
A25	1 (1.3)	2 (1.3)	0.99
A26	8 (10.3)	9 (5.6)	0.20
A27	14 (17.9)	25 (15.6)	0.65
A28	25 (32.1)	60 (37.5)	0.41
A29	10 (12.8)	25 (15.6)	0.57
A30	4 (5.1)	14 (8.8)	0.32
A31	1 (1.3)	3 (1.9)	0.74

*P < 0.05.

The distribution of allele size was compared between the Ca and Ct group participants. The findings showed that individuals with LDH (the Ca group) presented a higher frequency of short alleles *A13-25* (20.5%), whereas those without LDH (the Ct group) presented a higher frequency of longer alleles (63.8%). However, no statistically significant differences were observed (P = 0.181) (Table 3).

Table 3. Comparison of the distribution of allele size in the aggrecan gene (*ACAN*) VNTR region among case and control groups to assess the impact of *ACAN* polymorphism on susceptibility to lumbar disc herniation in Goiânia, Goiás, Brazil, 2015.

Alleles	Case [N (%)]	Control [N (%)]	Total	P
A13-25	16 (20.5)	24 (15.0)	40 (16.8)	0.181
A26-27	22 (28.2)	34 (21.3)	56 (23.5)	
A28-33	40 (51.3)	102 (63.8)	142 (59.7)	

The genotype frequency distribution observed for the *ACAN* gene indicates that in the Ca group, the most common genotypes were *A27/A28* (12.8%) and *A28/A29* (15.4%), whereas in the Ct group, the most prevalent were *A28/A28* (13.8%) and *A28/A29* (13.8%). There was no statistically significant difference between the Ca and Ct groups, except for genotype *A26/A26*, whose frequency in the Ca was 7.7% and in the Ct group was 0.0% (P = 0.01).

Following the analysis of the distribution of allele and genotype frequency, the Student *t*-test was used to compare allele scores of individuals in the Ca and Ct groups. This score was defined as the sum of the number of allele repeats for each individual (Roughley et al., 2006), e.g., the score for genotype *A26/A26* was 52, and so forth. The mean allele score in the Ca group was 53.1 and in the Ct group it was 54.6, which was a statistically significant difference (P = 0.05) (Table 4). These data corroborate the results of allele frequencies presented in previous studies and allow us to infer that the Ca group participants presented a lower frequency of short repeat alleles, indicating a possible association of this finding with disc degeneration and LDH.

Table 4. Comparison of allele scores between case and control groups in Goiânia, Goiás, Brazil, 2015.

Groups	N	Means ± SD	P
Case	39	53.1 ± 4.6	0.05*
Control	80	54.6 ± 3.3	

*P < 0.05 is statistically significant.

DISCUSSION

To date, studies have been conducted to assess the association between genetic factors, disc generation, and LDH. A systematic review carried out by Eskola et al. (2012) demonstrated that the *ACAN* gene is among the most studied as a possible candidate for intervertebral disc degeneration, together with *VDR* and collagen IX (*COL9A2* and *COL9A3*) genes. Even though the review included 52 articles, the authors concluded that the variation between study designs, populations, sampling methods, and definition of phenotypes weakened the level of evidence of the association between genetic polymorphism and disc degeneration, suggesting the need for further studies. This is the first case-control study investigating the *ACAN* gene VNTR region polymorphisms associated with LDH in the Brazilian population.

The first study that showed a correlation between *ACAN* gene polymorphisms and intervertebral disc degeneration involved 64 young women with a mean age of 21.3 years, of which 32 presented with disc degeneration and 32 did not, as confirmed by MRI. The most common allele was *A27*. Alleles with 18 and 21 repeats (short) were more prevalent among individuals with severe or multiple levels of disc degeneration. Based on these data, the researchers concluded that individuals with short repeat alleles had short chondroitin sulfate-1 (CS1) domains, and consequently presented a greater risk of developing early disc degeneration (Kawaguchi et al., 1999).

The present study identified 13 different alleles in the *ACAN* gene polymorphism region (VNTR), with repeats ranging from 17 to 31; the exception was allele *A21*. The most prevalent allele, both among participants with LDH and in the control group, was allele *A28*, followed by alleles *A27* and *A29* (28, 27, and 29 repeats). However, the frequency of allele *A28* in the researched population was greater when compared with Korean (Kim et al., 2011), Chinese (Cong et al., 2010; Cong et al., 2014), Turkish, (Eser et al., 2010), and Iranian (Mashayekhi et al., 2010) populations. One factor that influences genetic variability is the ethnic difference among these populations. In this sense, Vieira et al. (2013) emphasized that the Center-West region of Brazil, similar to other Brazilian populations, derives from the mixture of three main parental groups: Amerindian, European (particularly Portuguese), and Africans from sub-Saharan Africa.

The findings of this study are similar to those of Eser et al. (2011), who investigated 100 Turkish individuals with and without LDH. The most prevalent alleles were *A28*, *A27*, and *A29*. Kim et al. (2011) researched 104 Korean subjects (66 men and 38 women) with disc degeneration, of which 89 had disc herniation. They found a greater prevalence of allele *A27*, followed by alleles *A26* and *A28.*

Eser et al. (2010) and Mashayekhi et al. (2010) also found similar results to those of our study. The study investigated 300 young Turkish individuals between the ages of 20 and 30, divided into a case group (with disc degeneration and/or LDH) and a control group (without disc degeneration), with 150 individuals in each group. The researchers identified alleles *A14* to *A33*, with a greater prevalence of *A27*, *A28*, and *A26* in the research groups. In the study by Mashayekhi et al. (2010), 71 subjects with lumbar disc degeneration and 108 controls were studied, all from Iran. The most frequent alleles were *A27* and *A28*, and alleles ranging from 18 to 29 repeats were detected.

When comparing the distribution of allele frequencies in the *ACAN* gene VNTR region in the Ca and Ct groups in the present study with individuals from Goiânia, we observed that allele *A22* was significantly more prevalent in the Ca group (P = 0.03), allele *A17* was present only in the Ca group (P = 0.06), and allele *A18* was also more prevalent in the Ca group; however, these differences were not statistically significant (P = 0.61). These data are confirmed when comparing allele sizes, in which Ca also presented a higher frequency of short repeat alleles (20.5%) in comparison with Ct (15.0%), even though the result was not statistically significant (P = 0.181). Similarly, participants with LDH had a lower mean allele score in comparison with those without the disease (P = 0.05).

According to Roughley et al. (2002, 2006), *ACAN* has been identified as a gene that presents VNTR polymorphism in the portion of exon 12 responsible for encoding the CS1 domain. This domain is composed of multiple repeats of 19 amino acids, and each repeat contains a potential location for chondroitin sulfate (CS) chains to attach. Individuals with short alleles, i.e., with a lower number of repeats, have the lowest number of CS chains on intervertebral disc aggrecan molecules, resulting in impaired aggrecan function.

The consequence of such polymorphism is more pronounced in adults owing to the cumulative effects of proteolytic processing of the aggrecan core protein. Such processing is effected by the action of metalloproteinases, especially aggrecanases, resulting in the removal of part or most of the CS2 domain region, to which CS chains also attach, contributing to increased tissue degeneration with age (Roughley et al., 2006).

A study conducted by Cong et al. (2014) investigated the interaction between polymorphisms in the *ACAN* gene VNTR region and obesity, and found that the studied

individuals presented susceptibility to LDH. They researched 259 Chinese men (61 cases and 198 controls) and found a high frequency of the researched polymorphism. The findings demonstrated that alleles with 25 or less repeats (short alleles) were associated with increased risk for LDH. The research also suggested an interaction between *ACAN* VNTR, obesity, and LDH.

The relationship between a predominance of short repeat alleles and disc degeneration is also cited in research by Roughley et al. (2006) and Xu et al. (2012), and the relationship between a predominance of short repeat alleles and LDH is cited by Mashayekhi et al. (2010), Kim et al. (2011), Eser et al. (2010, 2011), and Cong et al. (2010, 2014).

A study by Solovieva et al. (2007), conducted in Finland to verify the association between VNTR polymorphisms and disc degeneration, found that individuals with longer alleles were less prone to intervertebral disc degeneration. A comparison of the distribution of genotype frequencies in the present study showed a significant difference in genotype *A26/A26* ($P = 0.01$) only among Ca individuals (7.7%). According to Solovieva et al. (2007) and Cong et al. (2014), individuals with genotype *A26/A26* had a higher risk of dark nucleus pulposus, a factor associated with degenerated discs.

Based on the results of this study, it is concluded that the allele score for the *ACAN* gene VNTR region was significantly lower among volunteers with LDH; allele *A22* was significantly more prevalent among the Ca group; individuals with LDH demonstrated a higher frequency of short alleles from *A13* to *A25* (20.5%); and genotype *A26/A26* was significantly more common in the case group. Therefore, there was an association between short alleles and LDH among the studied adults, compatible with the international literature. Individuals with short alleles produce aggrecan with fewer repeats, and consequently fewer chondroitin sulfate chains attached to the proteoglycan in the intervertebral disc; this results in hindered hydration and increased susceptibility to LDH.

Considering that genes can exert an independent effect but are also susceptible to environmental interactions, people with a family history of LDH must be given more effective guidance regarding external risk factors, regardless of exposure to environmental factors, to prevent the emergence of the disease. Thus, the results of our study can help health professionals when formulating preventive measures.

This study had some limitations, the most important being the cost of nuclear magnetic resonance examination. Candidates for the case group should take this examination because LDH diagnosis can only be confirmed by NMR. Moreover, individuals with no LDH in the control group were considered through clinical examination, but without confirmation by NMR. Thus, although suitable for this kind of study, the sample size was relatively small. These aspects must be considered in further studies to improve the reliability of the results.

Conflicts of interest

The authors declare no conflict of interest.

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