Genetic *ACE* I/D and *ACTN3* R577X polymorphisms and adolescent idiopathic scoliosis

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ABSTRACT. The etiology of adolescent idiopathic scoliosis remains unknown. Angiotensin-converting enzyme and α-actinin-3 polymorphisms influence the characteristics of muscle fibers. The aim of this study was to examine the association between idiopathic scoliosis and genetic polymorphism of angiotensin-converting enzyme and α-actinin-3. Ninety-seven females with scoliosis, and 137 healthy, age-matched control females were studied. The presence of polymorphisms was determined by PCR. A χ^2 test was used to analyze differences, and odds ratios were estimated. The frequencies of *ACE* genotypes in the scoliotic group were 46.4% DD, 45.4% ID, and 8.2% II, while in the control group they were 40.1% DD, 43.8% ID, and 16.1% II (P = 0.197). The D allele had a frequency of 69.1% in patients with idiopathic scoliosis and 62% in the control group (P = 0.116). The

frequencies of *ACTN3* genotypes in females with scoliosis were 31.8% RR, 49.4% RX, and 18.8% XX, while in the control group they were 35% RR, 49% RX, and 16% XX (P = 0.810). The frequency of the R allele was 56.4% in the scoliotic group and 59.6% in the control group (P = 0.518). There was no statistically significant association between angiotensin-converting enzyme or α-actinin-3 polymorphisms and the presence of adolescent idiopathic scoliosis in females.

Key words: Scoliosis; Polymorphism; Angiotensin-converting enzyme; Alpha-actinin 3; Muscle fiber.

INTRODUCTION

The Scoliosis Research Society defines adolescent idiopathic scoliosis (AIS) as a lateral spinal curvature greater than or equal to 10° on plane radiographs, associated with a rotational deviation, without any identifiable underlying secondary cause (Ocaka et al., 2008). The etiology of idiopathic scoliosis remains unknown, and currently, the multifactorial theory is favored by a majority (Alden et al., 2006). Some studies show that genetic predisposition is a determinant in the etiology of this disease (Ogilvie et al., 2006). Several chromosomal regions have been linked to AIS (Wise et al., 2000; Alden et al., 2006; Ogilvie et al., 2006; Ocaka et al., 2008). Currently, studies of genetic polymorphisms are carried out on patients with scoliosis in an attempt to identify protein variations that might be related to this disease (Aulisa et al., 2007; Takahashi et al., 2011; Jiang et al., 2013; Chen et al., 2014).

Since the 19th century, theories have associated AIS with muscular changes, suggesting that the distortion of the spine could be caused by a paravertebral muscle imbalance (Meier et al., 1997; Mannion et al., 1998; Wajchenberg et al., 2015). The histochemical pattern of multifidus and longissimus muscles in the lumbar region of normal subjects (20-30 years-old), defined by Thorstensson and Carlson (1987), showed that type I muscle fibers were predominant (57-62%). Johnson et al. (1973) observed that these muscles, including the soleus, tibialis anterior, adductor pollicis, diaphragm, and extraocular, have a higher energy demand than others, and primarily use aerobic metabolism, which is characteristic of type-I fibers.

The human genome contains at least 214 variant gene sequences and genetic markers associated with physical performance and health-related fitness phenotypes (Macarthur and North, 2005). Wajchenberg et al. (2013) evaluated polymorphisms in two of these genes, angiotensin-converting enzyme (*ACE*) and α-actinin-3 (*ACTN3*), in a family with multiple members suffering from AIS. This family had a higher prevalence of the *ACE* deletion (D) allele, compared with the insertion (I) allele. The *ACE* polymorphism (I/D variant) is the most widely studied genetic variant in the context of elite athlete status and performance-related traits, involving the deletion or insertion of 287 bp within intron 16. Previous studies by our group have shown that the DD genotype leads to greater plasmatic ACE activity than the II or ID genotypes (Almeida et al., 2010).

Expression of the *ACTN3* gene is also associated with muscular efficiency. *ACTN3* is a specific isoform expressed only in fast-twitch, type-II muscle fibers. The *ACTN3* polymorphism at position 1747 is located in exon 16, and leads to the following possible genotypes: homozygous null (XX), heterozygous (XR), and homozygous (RR), which leads to maximum expression of the ACTN3 protein. The homozygous null genotype (XX) causes no noticeable phenotype or histological changes, suggesting that the presence of this ACTN3

protein variant is not essential to muscle fiber function. However, this variant plays a role in muscle structure and alters the function of the type-II muscle fibers (North et al., 1999; Bray et al., 2009). The aim of this study was to examine the association between AIS and the genetic polymorphisms of *ACE* and *ACTN3*.

MATERIAL AND METHODS

Subjects

This study received ethics approval of Ethics Committee of the Federal University of São Paulo / São Paulo Hospital (ERC 1256/10). A total of 234 females, aged 10-35 years, were studied between 2010 and 2013. Ninety-seven patients with AIS, with a Cobb angle above 20° were recruited from the spine group of Hospital of Universidade Federal de São Paulo (Brazil). Scoliosis curve severity was measured by the Cobb method on posteroanterior radiographs of the whole spine. Cases of secondary scoliosis with known etiology were excluded from the current study, including congenital scoliosis, neuromuscular scoliosis, or scoliosis with connective tissue abnormalities. A total of 137 healthy female blood donors were used as controls. Control subjects were clinically examined by experienced orthopedic surgeons to rule out any potential spinal deformity. All subjects and/or their parents authorized their participation in the study using the Informed Consent Form that was filed and approved by the Ethics and Research Committee of University Hospital of São Paulo (ERC 1256/10).

DNA extraction

Genomic DNA was extracted from circulating leukocytes using a blood DNA extraction kit (Charge Switch gDNA Blood Kit, Invitrogen, Waltham, MA, USA).

ACE genotyping

The *ACE* I/D polymorphism was identified by polymerase chain reaction (PCR). Briefly, primers (ECAS: 5'-CTGGAGACCACTCCCATCCTTTCT-3' and ECAR: 5'-GATGTGGCCATCACATTCGTCAGAT-3') flanking the polymorphic region outside of the Alu insert in intron 16 were used to amplify a portion of the *ACE* gene. The amplified product was analyzed by gel electrophoresis to determine the presence or absence of I and D alleles. Because the D allele is preferentially amplified in heterozygotes, each DD genotype was confirmed by a second independent PCR with another primer pair (ECAint: 5'-GTCTCGATCTCCTGACCTCGTG-3' and ECAS: 5'-CTGGAGACCACTCCCATCCTTTCT-3') that amplified the sequence specific to the I allele (Charbonneau et al., 2008; Almeida et al., 2010; Amorim et al., 2013).

ACTN3 R577X genotyping

Genotyping of the *ACTN3* R577X polymorphism was performed using two specific primers (Actn3f: 5'-CAG CTGGAGGATGGCCTGG-3' and Actn3r: 5'-GTC CAG GTATTT CTC TGCCAC C-3') that amplified the polymorphic region; these primers were used to classify individuals as RR, RX, or XX. The following reagents were used for genotyping by PCR: 10 µL DNA, 2.5 µL PCR buffer (10X), 1.25 µL $MgCl_2$ (50 mM), 1.0 µL dNTPs (10 mM), 0.5 µL

each primer (Actn3f and Actn3r), 0.5 µL Taq DNA polymerase (5 U/µL), Invitrogen, and 5 µL autoclaved MilliQ H_2O for a total reaction volume of 25 µL. The following conditions were used for PCR: 95°C for 7 min, followed by 35 cycles of 95°C for 45 s, 60°C for 45 s, 72°C for 45 s, and 95°C for 45 s, with a final step at 72°C for 10 min followed by 4°C indefinite hold. The amplified fragments were analyzed by electrophoresis on a 4% agarose gel containing SYBR® Safe DNA gel stain (0.2 mg/mL, Invitrogen). After checking PCR products for DNA integrity and amplification, digestion was performed at 37°C for 2 h using a mixture of 8 µL DNA (approximately 6 mg) from the PCR, 1 µL REACT 3 buffer (10X), and 1 µL *Dde*I restriction enzyme (10 U/µL). The result was analyzed by electrophoresis on a 3% agarose gel containing SYBR® Safe DNA gel stain, as before (North et al., 1999; Clarkson et al., 2005).

Statistical analysis

Genotyping and allelic frequency were analyzed in each group, and odds ratios were estimated with 95% confidence intervals using simple logistic regression. The association between groups was verified using a c^2 test. Statistical tests were performed with a significance level of 5%; results were analyzed using SPSS version 17.0 for Windows.

RESULTS

Patient characterization

The average age of patients with AIS was 18.5 years, and the average age of menarche was 12 years. Of the patients with AIS, 88.7% were Caucasian, and 17.5% had family members with AIS; 55.7% underwent surgical treatment, and 56.7% had a Cobb angle greater than 50°. Most patients with AIS had a right thoracic curve (80.5%), and 44.3% were type III, according to the King classification system (King et al., 1983). The average age of control subjects was 20.7 years.

ACE genotyping

The frequency of each *ACE* genotype in the AIS group was determined to be 46.4% DD, 45.4% ID, and 8.2% II; in the control group, 40.1% were DD, 43.8% ID, and 16.1% II. Although the frequency of the DD genotype was higher in patients with AIS, it was not significant (P = 0.197). In the isolated allele analysis, the frequency of the D allele was 69.1% in patients with AIS and 62% in the control group (P = 0.116).

The sample odds ratio revealed that subjects with the DD genotype were 2.25 times more likely to develop the disease than those with the II genotype. The risk of developing AIS was two-fold higher for heterozygotes than for those homozygous for the II genotype. Although the risk of developing AIS was 37% higher in those with the D allele, these results were not statistically significant (Table 1).

ACTN3 genotyping

We genotyped the *ACTN3* polymorphism in 85 of the patients with AIS; the DNA yield from the other 13 patients was too low to genotype both *ACE* and *ACTN3* genes. The frequency of each *ACTN3* genotype was determined to be 31.8% RR, 49.4% RX, and 18.8%

XX. In the control group, 130 volunteers were genotyped; 35% were found to be RR, 49% RX, and 16% XX. There was no statistically significant difference between the groups in this analysis (P = 0.810). In the isolated allele analysis, there was no statistically significant difference between patients with AIS, where the R allele made up 56.5% of the total allele distribution, and the control group, with 59.6% (P = 0.518). Odds ratio analysis of the *ACTN3* gene in both groups did not demonstrate a genetic profile of susceptibility (Table 2).

Table 1. ACE genotyping results and odds ratio.

	Group				OR	95%CI		P*
	Control		AIS					
	N	%	N	%				
ACE genotyping								0.197
II	22	16.1	8	8.2	1.00			
ID	60	43.8	44	45.4	2.02	0.82	4.95	
DD	55	40.1	45	46.4	2.25	0.92	5.53	
Allele								0.116
I	104	38.0	60	30.9	1.00			
D	170	62.0	134	69.1	1.37	0.93	2.02	

*Bivariate logistic regression. AIS = adolescent idiopathic scoliosis; ACE = angiotensin I converting enzyme; OR = odds ratio; CI = confidence interval; N = absolute number; II = insertion homozygous; ID = heterozygous; DD = deletion homozygous.

Table 2. *ACTN3* genotyping results and odds ratio.

	Group				OR	CI (95%)		P*
	Control		AIS					
	N	%	N	%				
ACTN3 genotyping								0.810
RR	46	35	27	31.8	1.00			
RX	63	49	42	49.4	1.14	0.61	2.10	
XX	21	16	16	18.8	1.30	0.58	2.91	
ACTN3 allele								0.518
R	155	59.6	96	56.5	1.00			
X	105	40.4	74	43.5	1.14	0.77	1.68	

*Bivariate logistic regression. AIS = adolescent idiopathic scoliosis; ACTN3 = Actinin 3; OR = odds ratio; CI = confidence interval; N = absolute number; RR = homozygous; RX = heterozygous; XX = null homozygous.

DISCUSSION

Studies suggest that genetic factors are critical in the pathogenesis of AIS; however, the inheritance pattern is not yet fully known. This disease is marked by its phenotypic complexity, and the range of possible prognoses includes an increase in spinal curve magnitude, stabilization, and resolution with growth (Kouwenhoven et al., 2006; Jiang et al., 2012). A wide phenotypic variation is characteristic of multifactorial diseases. Most consist of polygenic factors, delineating an individual's susceptibility to developing disease (Meier et al., 1997; Ogilvie et al., 2006).

In the current etiological study, we examined two genetic factors that may influence changes in the paraspinal muscles described in patients with AIS (Chagas et al., 1993). We investigated chromosomal loci that may facilitate the development and progression of AIS, in agreement with the polygenic disease hypothesis. In a genealogical study, Wynne-Davies (1968) concluded AIS was a multifactorial disease with a polygenic inheritance pattern, by identifying probands and characterizing their families.

Wise et al. (2000) reflected on the difficulty of mapping DNA changes in polygenic diseases using binding studies. The authors agreed that AIS has a pattern of autosomal dominant inheritance. They maintained the same study design, and mapped several chromosomes (6, 10q, 18q) in the families of AIS probands.

Polymorphisms related to AIS already identified include those encoding structural proteins of the extracellular matrix, including matrilin-1 (Chen et al., 2009), matrix metalloproteinases and their inhibitors (Jiang et al., 2012), and dipeptidyl peptidase (Qiu et al., 2008), and those related to bone metabolism, including calmodulin (Zhao et al., 2009) and the vitamin D receptor gene (Suh et al., 2010). Recently, somatotrophic polymorphisms and the androgen axis were considered potential candidates in the pathogenesis of AIS, including the estrogen receptor, estrogen receptor-coupled G-proteins (growth factors and insulin), and the growth hormone receptor gene (Inoue et al., 2002; Yang et al., 2009; Zhao et al., 2009; Peng et al., 2012). Moreover, a genome-wide association study in Japan identified three single nucleotide polymorphisms on chromosome 10q24.31, likely associated with AIS. These polymorphisms are located in the region containing the LBX1 gene, expressed in the central nervous system and skeletal muscle (Takahashi et al., 2011; Jiang et al., 2013).

Our investigation of DNA sequencing related to AIS susceptibility examined the muscular imbalance theory as a primary factor in AIS pathophysiology, since no previous studies have been conducted that relate genes involved in muscle fiber traits to this disease. The paraspinal musculature plays a role in trunk support and movement, and undergoes significant changes in patients with a trunk deformity. The multifidus muscles are spine rotators with a histochemical pattern of predominantly type-I fibers (57-62%). These fibers are resistant to fatigue and have a predominantly aerobic metabolism (Thorstensson and Carlson, 1987).

Previous histopathological studies of patients with AIS demonstrated that all muscular biopsies showed changes to the paraspinal muscles. The decrease in the prevalence of type-I muscle fibers on the concave side of the scoliotic curve is a major change described in the literature. In addition, atrophy, and core presence, also occur in these muscles in patients with AIS. However, there is disparity in the literature as to whether these changes are the cause or an effect of the deformity (Chagas et al., 1993*;* Meier et al., 1997; Luciano et al., 2015; Wajchenberg et al., 2015).

Our analysis of genotypic frequencies showed the DD genotype and D allele were highest in patients with AIS, in agreement with results reported by Wajchenberg et al. (2013) in a family with multiple members with AIS. We also observed that the incidence of the II genotype in healthy individuals was twice that in patients with AIS. According to the literature, the I/D polymorphism not only affects plasmatic ACE activity but also determines its concentration in tissues (e.g., skeletal muscle). Apparently, the interaction between local and serum renin-angiotensin systems, and metabolic adaptation of the muscle fiber type, occurs according to the individual's genotype (Reneland et al., 1999). In a previous study, the DD genotype and presence of the D allele were associated with high concentrations of ACE and reduced levels of bradykinin, which lowered glucose uptake and muscle blood flow, impairing muscle groups made predominantly of type I muscle fibers, such as the spinal erector and multifidus (Zhang et al., 2003). On the other hand, the aerobic metabolism requirement of these muscles favors the presence of the I allele, which behaves as a protective factor, and may be most frequently found in healthy individuals. Furthermore, odds ratio analysis of our sample showed that individuals with the DD genotype were 2.25 times more likely to develop AIS. Therefore, physicians should be cautious when interpreting a patient's risk of disease

development, considering the polygenic character of AIS, as well as the genetic and epigenetic factors that can alter the risk of developing a deformity.

The genetic profile analysis of *ACTN3* in both groups studied herein did not show statistically significant differences. *ACTN3* is a fast-twitch-specific isoform expressed only in type-II muscle fibers. A common *ACTN3* polymorphism that has been identified in humans leads to the loss of a detectable protein product in the muscles of individuals homozygous for the X allele (R577X). The *ACTN3* null genotype (XX) causes no discernable phenotype or histological abnormality in the muscles.

Our results were limited by the number of patients, and in future, multicenter studies will be necessary to prove this hypothesis. Furthermore, these results also require validation in other populations, since the current results may be specific to the ethnic-racial group studied. The study of gene expression of the renin-angiotensin system in the paraspinal musculature of patients with AIS could clarify the possible adaptive processes of skeletal muscle fiber in the scoliotic spine.

Overall, results of this study showed no statistically significant association between the *ACE* and *ACTN3* polymorphisms studied, and the presence of AIS in females.

Conflicts of interest

The authors declare no conflict of interest.

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