International Journal of Latest Research in Science and Technology **ISSN (Online):2278-5299**
Volume 5, Issue1: Page No111-114, January-February 2016
https://www.mnkpublication.com/journal/ijlrst/index.php

ASSOCIATION STUDY BETWEEN IDIOPATHIC SCOLIOSIS AND FUNCTIONAL POLYMORPHISMS OF *ACE* AND *ACTN3* GENES

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Abstract: ***The most common type of scoliosis, idiopathic scoliosis (IS), has no specific identifiable cause. For the aims of the current case-control study we selected the functional polymorphisms: ACE (I/D) and ACTN3 (R577X) trying to investigate the association between these candidate-genes and susceptibility to and progression of IS among Bulgarian patients.***
The ACE polymorphism was genotyped by direct amplification and the ACTN3 polymorphism was genotyped by amplification followed by restriction. The statistical analysis was performed by Pearson's chi-squared test to make genotype and allele comparisons between cases and controls as well as test for Hardy-Weinberg equilibrium. A value of $p < 0.05$ was considered to be statistically significant for comparison between data sets.
The results of the statistical analysis in this study indicated that the ACE and ACTN3 polymorphisms were not associated with susceptibility to IS, curve severity, onset of the disease, familial history or gender. On the basis of these results the examined polymorphic variants could not be considered as genetic variants with predisposing or modifying effect in Bulgarian population. Replication case-control studies will be needed to examine the association between these candidate-genes and IS in different populations.
The identification of molecular markers for IS could be useful for early detection and prognosis of the risk for a rapid progression of the curve. That would permit early stage treatment of the patient with the least invasive procedures.

Keywords: Idiopathic Scoliosis, ACE, ACTN3, Susceptibility, Progression

I. INTRODUCTION

The most common type of scoliosis, idiopathic scoliosis (IS), has no specific identifiable cause. During the period from 1992 to 2015 the studies on molecular genetics of IS have indicated substantial genetic heterogeneity in the etiology of the disease.

IS may be caused by multiple genes segregating differently in various populations and ethnic groups [1]. First, there are predisposition genes that usually have low penetrance and are associated with a moderate increase of the risk of developing the disease. In addition to predisposition to the development of idiopathic scoliosis, genetic factors could also influence the severity of the disease. The concept of disease-modifier genes as an element of genetic heterogeneity has been widely accepted and reported [2].

The candidate genes have been selected by several methods, including prior knowledge of the biological pathway, linkage studies, expression studies, and genome wide association studies.

Angiotensin converting enzyme (ACE) plays an essential role in two physiological systems, one leading to the production of angiotensin II and the other to the degradation of bradykinin. The wide distribution and multifunctional properties of these peptides suggest that ACE could be involved in various pathophysiological conditions. The discovery that ACE levels are under genetic control ushered in a new era of investigation [3]. Most studies focused on an insertion/deletion (I/D) polymorphism in intron 16 of the *ACE* gene as a marker for a functional polymorphism. The *ACE* (I/D) polymorphism has been associated with improvements in performance and exercise duration in a variety of populations. The I allele has been consistently demonstrated to be associated with endurance-orientated events. Meanwhile, the D allele is associated with strength-

Publication History
Manuscript Received : 16 February 2016
Manuscript Accepted : 22 February 2016
Revision Received : 27 February 2016
Manuscript Published : 29 February 2016

and power-orientated performance, and has been found in significant excess among elite swimmers [4].

An important structural component of the Z disc is α-actinin-3, where it anchors actin thin filaments, helping to maintain the myofibrillar array only in fast twitch muscle fibers that are responsible for high velocity and force for power-generating contractions [5, 6]. *ACTN3*, the gene for encoding α-actinin-3 has a 1747C>T transition within exon 16, which results in the R577X polymorphism, giving three genotypes: the RR, RX and XX genotypes. The XX genotype is associated with a deficiency of α-actinin-3 due to the premature stop codon without a pathological phenotype, whereas the RR and RX genotypes express α-actinin-3. It has been suggested that the *ACTN3* variant for the expression or deficiency of α-actinin-3 in fast-twitch muscle fibers should influence the power-generating muscle function [5, 6, 7].

Wajchenberg et al. [2013] examined the possible association between the functional polymorphisms of the *ACE* gene and of the *ACTN3* gene with IS in a Brazilian family containing multiple affected individuals. Among the 25 family members, 22 had some degree of scoliosis and nine had curves with 15 or more degrees of deformity. It was found that 19 (76%) subjects had DD and 6 (24%) had ID genotype of the *ACE* polymorphism. The prevalence of the D allele was 88% while that of the I allele was 12%. Regarding the *ACTN3* polymorphism there were 6 subjects with RR genotype (24%), 11 with RX genotype (44%) and 8 with XX genotype (32%). The prevalence of the R allele was 23 (46%) and that of the X allele was 27 (54%). In conclusion, the authors found a difference between the distribution of the functional polymorphisms of *ACE* and *ACTN3* in the family. A higher prevalence of the D allele of *ACE* (I/D) was observed [8].

For the aims of the current case-control study we selected the functional polymorphisms: *ACE* (I/D) and *ACTN3* (R577X) trying to investigate the association between these candidate-genes and susceptibility to and progression of IS among Bulgarian patients.

II. MATERIALS AND METHODS

In this study, Bulgarian patients with IS (n=105) and healthy unrelated gender-matched controls (n=210) were included. All participants in the study were informed about its purpose and were included only after the subjects/families signed their informed consent. Peripheral blood samples were obtained from patients and control subjects. The study protocol was approved by the Ethics Committee of the Medical University-Sofia.

Patients. Patients with IS were recruited with the help of orthopaedic surgeons from Tokuda Hospital Sofia. The IS diagnosis was confirmed clinically and radiologically. Scoliosis as a phenotypic characteristic like Marfan's syndrome was excluded. The curves were measured by the Cobb method. The mean value of Cobb angles was $54,6\pm22,7^0$. The mean age at the beginning of the disease was 11.2±3.1 years. In this study, male (n=19) and female (n=86) patients were included.

Controls. The control group including healthy subjects without clinical signs of IS was recruited from a pool of unrelated gender-matched volunteers from other Units and Clinics of Tokuda Hospital Sofia, National Genetic Laboratory, hospital staff members and students. The controls were selected among adult patients with skeletal maturity with negative family history of IS. Radiological examination was not performed in the control group.

*Genotyping***.** Genomic DNA was extracted from the peripheral blood leucocytes using magnetic bead technology (chemagic DNA Blood Kit special, Chemagen) on automated high throughput nucleic acid isolation platform (chemagic Magnetic Separation Module I, Chemagen).

The *ACE* polymorphism was genotyped by direct amplification (Figure 1) and the *ACTN3* polymorphism was genotyped by amplification followed by restriction (Figure 2). The primer sets are listed in Table 1.

The polymerase chain reaction (PCR) was carried out in a a reaction mix of 20μl containing 100-ng DNA and 10X Prime Taq buffer (Genet Bio, Korea), 10 mM dNTPs Mixture (Genet Bio, Korea), 20 pmol Forward and Reverse primers (AlphaDNA, Canada), and 0.1 U Prime Taq DNA Polymerase (Genet Bio, Korea). PCR ampification was performed in an AB 2720 Thermocycler (Life Technologies, USA) with an initial denaturation at 94^0C for five minutes and a final extension of seven minutes at 72^0C. The following thermal cycle was repeated 30 times: denaturation at 94^0C for 30 seconds, annealing for 30 seconds at temperature presented in Table 2, and extension at 72^0C for 30 seconds.

The restriction fragment length polymorphism (RFLP) analysis was performed with the appropriate restriction enzyme (NEB, USA), according to the manufacturer's instructions, and the restriction fragments were separated on agarose 3% gel in VG-SYS Horizontal Electrophoresis System (Biochrom, USA). The restriction enzyme and the lengths of the fragments representing the genotypes are presented in Table 2.

Statistical analysis. The statistical analysis was performed by Pearson's chi-squared test to make genotype and allele comparisons between cases and controls as well as test for Hardy-Weinberg equilibrium. A value of $p < 0.05$ was considered to be statistically significant for comparison between data sets. Odds ratios (OR) were calculated with 95% confidence interval (95% CI). Statistical analysis was conducted with the IBM SPSS 19.0 (NY, USA) software package for Windows.

III. RESULTS AND DISCUSSIONS

This is the first case-control study between the *ACE* (I/D) and the *ACTN3* (R577X) functional polymorphisms and IS.

Genotypes were in Hardy-Weinberg equilibrium. The overall frequencies of the genotypes and alleles of *ACE* (I/D) in the patients with IS were comparable with the controls (DD vs. II, p=0.28, OR: 1.51; 95% CI: 0.71-3.21 and D vs. I, p=0.36; OR: 1.17; 95% CI: 0.83-1.63). The genotype and allele frequencies of *ACTN3* (R/X) were also comparable between cases and controls (RR vs. XX, p=0.2; OR: 0.65; 95% CI: 0.33-1.26 and R vs. X, p=0.28; OR: 0.83; 95% CI: 0.59-1.16). On the basis of these results the genetic variants of *ACE* and *ACTN3* alone could not be considered as predisposing factors for IS in Bulgarian population.

Adolescent idiopathic scoliosis (AIS) is the most common spinal deformity [9] and the most frequently studied idiopathic scoliosis. In the subgroup of the adolescents (n=78) the genotype and allele frequencies of the *ACE* and *ACTN3* polymorphisms were also comparable between cases and controls (p>0.05). These results showed the

polymorphisms are not associated with the susceptibility to AIS.

In the subgroup of surgical cases (n=84) where Cobb angle $>40^0$ the genotype and allele frequencies of the *ACE* and *ACTN3* polymorphisms were comparable between cases and controls ($p>0.05$). In conclusion, these polymorphisms could not be considered as modifying factors of IS associated with a rapid progression of the deformity in Bulgarian population.

Scoliosis is more common in females than males. In the subgroup of female patients (n=86) no statistically significant associations between the *ACE* and *ACTN3* polymorphisms and the clinical phenotype were observed ($p>0.05$). In the subgroup of male patients (n=19) no statistically significant associations were observed ($p>0.05$). In conclusion, the genotypes and alleles of the *ACE* and *ACTN3* polymorphisms could not be associated with gender.

In the subgroup of the familial cases (n=28) the genotype and allele frequencies of the *ACE* and *ACTN3* polymorphisms were comparable between cases and controls ($p>0.05$). In conclusion, the genotypes and alleles of these polymorphisms could not be associated with the familial history of IS among Bulgarian patients.

Odds ratios of genotypes and alleles in the subgroups are summarised in Table 3.

Wajchenberg et al. [2013] found prevalence of the D allele of the *ACE* gene [8] but their study included only patients with adolescent idiopathic scoliosis (AIS) among the members of one family. In our study we selected the participants between patients with infantile, juvenile and adolescent idiopathic scoliosis and gender-matched controls. We separated the cases in subgroups according to age, gender, Cobb angle and familial history and then investigated the associations in the general sample and in the different subgroups as well. First, the observed differences in the results between the different population groups could be explained with different selection criteria for the samples (pre-analytical), technical errors (analytical) and differences in the preferred statistical methods with or without corrections (post-analytical). Second, the genotype and allele frequencies could be different in the different population and even ethnical groups.

The results of the statistical analysis in this study indicate that the *ACE* and *ACTN3* polymorphisms were not associated with susceptibility to IS, curve severity, onset of the disease, familial history or gender. On the basis of these results the examined polymorphic variants could not be considered as genetic variants with predisposing or modifying effect in Bulgarian population. These results don't exclude a potential role of the same polymorphic markers in other population groups or impact of other polymorphisms of *ACE* and *ACTN3* on the etiology and pathogenesis of IS in Caucasian population. Replication case-control studies will be needed to examine the association between these candidate-genes and IS in different populations.

The identification of molecular markers for IS could be useful for early detection and prognosis of the risk for a rapid progression of the curve. That would permit early stage treatment of the patient with the least invasive procedures.

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TABLE 1. PCR primers

Gene, Polymorphism	***Primers***
ACE (I/D)	*Forward: 5'-CTGGAGACCACTCCCATCCTTTCT-3'*
	Reverse: 5'-GATCTGGCCATCACATTCGTCAGAT-3'
ACTN3 (R577X)	*Forward: 5'-CTGTTGCCTGTGGTAAGTGGG-3'*
	Reverse: 5'-TGGTCACAGTATGCAGGAGGG-3'

PCR indicates polymerase chain reaction; ACE, angiotensin converting enzyme; ACTN3, α-actinin-3.

TABLE 2. PCR-RFLP protocol

Gene, SNP	**Annealing, ^{0}C**	**PCR Product Size, bp**	**Restriction Enzyme**	**Restriction Fragments, bp**
ACE (I/D)	61	II: 490 ID: 490 + 190 DD: 190	No	No
ACTN3 (R577X)	60	205 + 86	DdeI	RR: 205 + 86 RX: 205 + 108 + 97 + 86 XX: 108 + 97 + 86

PCR indicates polymerase chain reaction; RFLP, restriction fragment length polymorphism; bp, base pair; ACE, angiotensin converting enzyme; ACTN3, α-actinin-3.

TABLE 3. Odds ratios of genotypes and alleles in different subgroups with IS

Subgroup	Gene, Polymorphism	Genotype, Allele	p-value	OR [95% CI]
General (n_1=105, n_2=210)	ACTN3 (R577X)	RR vs. XX	0.2	0.65 [0.33-1.26]
		R vs. X	0.28	0.83 [0.59-1.16]
	ACE (I/D)	D vs. I	0.36	1.17 [0.83-1.63]
		DD vs. II	0.28	1.51 [0.71-3.21]
AIS (n_1=78, n_2=210)	ACTN3 (R577X)	RR vs. XX	0.11	0.55 [0.27-1.14]
		R vs. X	0.15	0.76 [0.52-1.10]
	ACE (I/D)	D vs. I	0.47	1.15 [0.79-1.66]
		DD vs. II	0.39	1.43 [0.63-3.27]
Familial group (n_1=28, n_2=210)	ACTN3 (R577X)	RR vs. XX	0.76	0.73 [0.23-2.34]
		R vs. X	0.66	0.85 [0.48-1.49]
	ACE (I/D)	D vs. I	0.78	0.90 [0.52-1.58]
		DD vs. II	1	0.82 [0.23-2.88]
Non-familial group (n_1=77, n_2=210)	ACTN3 (R577X)	RR vs. XX	0.21	0.63 [0.31-1.30]
		R vs. X	0.31	0.82 [0.57-1.20]
	ACE (I/D)	D vs. I	0.19	1.28 [0.88-1.87]
		DD vs. II	0.14	1.90 [0.80-4.51]
Cobb angle >40^0 (n_1=84, n_2=210)	ACTN3 (R577X)	RR vs. XX	0.22	0.65 [0.32-1.30]
		R vs. X	0.37	0.85 [0.59-1.22]
	ACE (I/D)	DD vs. II	0.1	2.05 [0.87-4.84]
		D vs. I	0.16	1.30 [0.90-1.87]
Males (n_1=19, n_2=38)	ACTN3 (R577X)	RR vs. XX	0.23	0.32 [0.06-1.70]
		R vs. X	0.17	0.56 [0.25-1.22]
	ACE (I/D)	D vs. I	0.32	1.62 [0.73-3.56]
		DD vs. II	0.4	2.72 [0.48-15.5]
Females (n_1=86, n_2=172)	ACTN3 (R577X)	RR vs. XX	0.36	0.71 [0.34-1.48]
		R vs. X	0.61	0.91 [0.62-1.32]
	ACE (I/D)	D vs. I	0.66	1.09 [0.75-1.57]
		DD vs. II	0.46	1.37 [0.59-3.17]

All P values were not significant. IS indicates idiopathic scoliosis; AIS, adolescent idiopathic scoliosis; OR, odds ratio; CI, confidence interval; ACE, angiotensin converting enzyme; ACTN3, α-actinin-3; n_1, number of cases; n_2, number of controls.

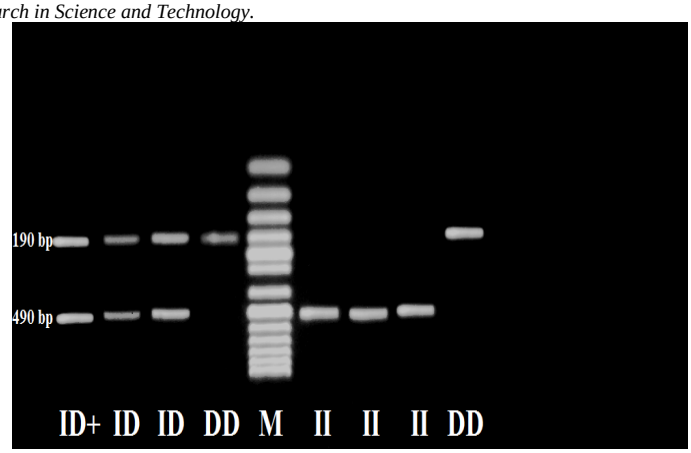

Figure 1. Results from the direct amplification of *ACE* (I/D): II – homozygotes (490 bp); DI – heterozygotes (490 + 190 bp); DD – homozygotes (190 bp); M – ladder 50 bp; First position (left) – sequenced control.

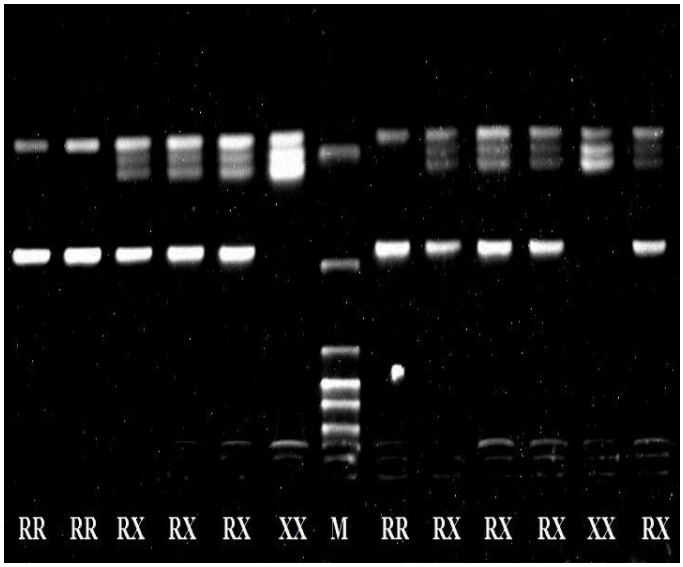

Figure 2. Results from the restriction analysis of *ACTN3* (R577X): RR – homozygotes (205+86 bp); RX – heterozygotes (205+108+97+86 bp); XX – homozygotes (108+97+86 bp); M – ladder 50 bp.