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Research Article

Genetic Polymorphisms in Turkish Patients with Lumbar Disk Herniation

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Lomber Disk Hernisi Tanısı Alan Türk Hastalarda Genetik Polimorfizim

Abstract

Lumbar disc herniation (LDH) defined in the early 1930s and has since been treated, is the most common disease group in which neurosurgeons have dealt with and performed operations. The pathogenesis of this disease is still unknown. The study aimed to evaluate any potential contribution of disease-associated single nucleotide gene polymorphisms (SNPs) in LDH etiology.
Male and female volunteers with LDH participated in the study. All variants (SOD1 IVS3+35 A>C); SOD2 c.*3409T>A; c.*441A>G; MTHFR p.Ala222Val (c.677C>T), and PAI-1-844G>A) were analysed using polymerase chain reaction and restriction fragment length polymorphism (PCR–RFLP) method.
In MTHFR 677C>T, "T" additive model and "T" dominant model, CC vs. CT (OR:5.69 Cl: 2.4-13.4 p<0.001) and CC vs. TC+TT(OR:5.69 Cl: 2.4-13.4 p<0.001) increased the risk of LDH almost 6-fold, "C" recessive model, CT+TT vs. CC (OR:5.68 Cl:2.4-13.5 p<0.001) reduced the risk of LDH almost 6-fold while others were not associated with LDH. In a paired analysis of MTHFRc.677C>T/PAI-1-844G>A genotypes, it was determined that AG genotype increased the protective effect of CC genotype (CC+AG vs others) of the MTHFR gene (OR: 8.4 Cl:2.2-3.1 P<0.001) while reducing the negative effect of the TC genotype of MTHFR gene(TC+AG vs others) in LDH risk(OR: 4.4 Cl: 1.5-12.1*P*=0.005). A similar result was not determined for the investigated SOD1 and SOD2 variants. For the first time, in our study groups, MTHFR677C>T polymorphism was found associated with an increased risk of LDH.

Keywords: Lumbar disk herniation; polymorphisms; risk assessment; gene(s); genetic model of inheritance

Öz

1930'ların başından itibaren tanımlanan ve tedavi edilen bel fıtığı (LDH), beyin cerrahlarının ilgilendiği ve en yaygın ameliyat nedeni olan hastalıktır. LDH patogenezi hala bilinmemektedir. Bu çalışma kaspsamında hastalıkla ilişkili olduğu belirlenen beş adet tek nükleotid gen polimorfizmlerinin(SNPs) LDH etiyolojisine olan katkısının araştırılması amaçlanmıştır.
Çalışmaya LDH tanısı alan erkek ve kadın gönüllüler dahil edimiştir. Polimorfizimlerin tamamı (SOD1 IVS3+35 A>C; SOD2 c.*3409T>A; c.*441A>G; MTHFR p.Ala222Val (c.677C>T) ve PAI-1-844 G>A

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polimeraz zincir reaksiyonu ve kesim parçası uzunluk polimorfizm (PCR– RFLP) yöntemi kullanılarak analiz edilmiştir.
MTHFR677C> T polimorfizminde, "T" editif modelde CC'ye göre CT (OR: 5.69 Cl: 2.4-13.4 p<0.001) ve "T" baskın modelde CC'ye göre TC + TT (OR: 5.69 Cl: 2.4-13.4 p<0.001) genotiplerine sahip olmanın LDH riskini yaklaşık 6 kat artırdığı, "C" resesif modellde ise CT + TT'ye karşı CC genotipine sahip olmanın LDH riskini yaklaşık 6 kat (OR: 5.68 Cl: 2.4-13.5 p<0.001) azalttığı belirlenmiştir. MTHFR c.677C>T/PAI-1-844G>A genotiplerinin ikili analizlerinde, CC genotipinin, koruyucu etkisinin AG genotipi ile birlikteliğinde arttırdığı (OR: 8.4 Cl:2.2-3.1 P<0.001), buna karşın TG genotipinin neden olduğu LDH riskini azalttığı (OR: 4.4 Cl: 1.5-12.1 P=0.005) belirlenmiştir. Benzer bir sonuç araştırılan SOD1 ve SOD2 varyantları için belirlenmemiştir. Çalışma grubumuzda MTHFR677C> T polimorfizmine bağlı olarak artan LDH riski ilk defa bu çalışma ile belirlenmiştir.

Anahtar Kelimeler: Lumbar disk herni, polimorfizim, risk değerlendirmesi, gen(ler), genetik kalıtım modelleri

1. Introduction

In the neurosurgical practice, one of the most common complaints doctors encounter is low back pain (LBP) and 70% of the population suffer from severe back pain at least once in their lifetime (Andersson 1999). The most common reasons for the wide variety of LBP is intervertebral degeneration leading to degenerative disc disease and lumbar disc herniation (LDH) (Martin et al. 2008).

Lumbar disc herniation, defined in the early 1930s and has since been treated, is the most common disease group in which neurosurgeons have dealt with and performed operations (Allan and Waddell 1989). Lumbar disc herniation may consider as an important socio-economic and public health problem from all these aspects. With all these aspects lumbar disc disease is an important socio-economic and public health problem. The pathogenesis of this disease is still unknown. Therefore, an effective understanding of LDH pathogenesis is essential to apply the appropriate treatment. The condition is estimated to have about 75% inheritance (Janeczko et al. 2014). Genes that increase the risk of LDH have been identified in previous studies include encoding structural proteins, matrix metalloproteinases (Mio et al. 2007), apoptosis factors, growth factors, and vitamin D receptor resulting in inflammatory cytokine imbalance (Martirosyan et al. 2016).

An adverse symptom of inflammation is related to stimulation of the nerve fibers, which eventually may lead to pain. Besides, inflammation has been identified as an important factor associated with LDH reduction. Inflammation is a protective defense mechanism used by tissues against endogenous and exogenous antigens. Inflammation and oxidative stress are closely related pathophysiological events and thus, the cellular side effects of chronic inflammation mainly occur as a result of increased free radical production and decreased antioxidants (Hold and El-Omar 2008). Exposure to certain chemical and physical agents causes excessive reactive oxygen species (ROS) production. On the other hand, ROS is eliminated from tissues and cells by enzymatic and non-enzymatic antioxidative protective mechanisms (Apel and Hirt 2004; Rodriguez and Redman 2005). Superoxide dismutase (SOD) is one of the most potent, the first enzymes of the antioxidant defense mechanism. In disc tissues, oxidative stress can cause or contribute to matrix degradation and cell apoptosis, ultimately causing disc degeneration (Nerlich et al. 2007).

Homocysteine (Hcy) has an important role in oxidative stress (Husemoen et al. 2006) and one of the factors affecting Hcy metabolisms is methylenetetrahydrofolate reductase (MTHFR)

gene mutations. The researchers also demonstrated that Hcy partially induces endothelial cell apoptosis by increasing ROS formation and decreasing membrane potential. Hcy is a risk factor for the development of coronary artery disease (Whincup et al. 1999). Laboratory studies suggest that an elevated homocysteine concentration is both atherogenic and thrombogenic(Hankey and Eikelboom 2000) and thus hyperhomocysteinemia impair angiogenesis. Usually, few blood vessels are found in the mature IVD. However, the proliferation of new vessels at the border of herniated tissue has already been demonstrated, and this is thought to be an important determinant of the spontaneous regression of LDH (Autio et al. 2006). Additionally, even if the Hcy level was not found to be high in the LDH patient group, the Hcy level was found to be high in a patient with osteoporotic fracture.

Impaired fibrinolytic activity was found in patients with chronic low back pain, and it was thought that pain may be associated with inflammation (Cooper et al. 1991; Jayson 1989). Plasminogen inhibitor 1 (PAI-1) is the main regulator of the fibrinolytic system and its elevation is related to the impaired fibrinolytic system. Moreover, PAI-1 and different components of the fibrinolytic system play a role in the development of inflammation, arteriosclerosis, cancer, and fibrosis as well (Iwaki, Urano, and Umemura 2012; Ghosh and Vaughan 2012). Impaired fibrinolytic activity determined by PAI-1 measurement has been found related to poor improvement in LDH operated patients.

Single nucleotide gene polymorphisms (SNPs) have enormous importance in an effective understanding of the genetic etiology of multifactorial diseases. Therefore, the study, for the first time, aimed to evaluate potential contribution of disease-associated SNPs, superoxide dismutase 1 (SOD1) IVS3 +35 A>C (rs2234694), superoxide dismutase 2 (SOD2) c.*3409T>A (rs2842980), and c.*441G>A (rs5746136), MTHFR p.Ala222Val (rs1801133) and PAI-1 -844 G>A (rs2227631) in LDH etiology.

2. Subjects and Methods

Study Population

The study was conducted as a case-control study. Fifty healthy control and fifty-one patients with lumbar disk herniation between 19-55 years old male and female volunteers participated in the study. All volunteers were examined in the department of Neurosurgery and Physical Therapy and Rehabilitation departments at Erzincan Binali Yıldırım University Mengücek Gazi Research and Training Hospital between June 2019-June 2020. Any volunteers with the radiologically detected presence of (protrude or extrude) L4-L5 or L5-S1 lumbar herniated disc using lumbar magnetic resonance imaging (MRI) and presence of symptoms clinically related to discopathy (lumbargia, sciatica, etc.) consisted of the LDH patients group. Any volunteers without discopathy in lumbar MRI examinations and/or without any complaint of low back pain were included in the study as a control group. Blood group, family history of LDH, and smoking habits were recorded of the volunteers. The study was approved by the Ethics Committee of Medical School at Erzincan Binali Yıldırım University. The ethics committee decision number and date were 33216249-804.01-E.30008 and 25th June 2019, respectively. Written informed consent was received from all the volunteers.

Genotyping Analyses

Genomic DNA was isolated from ethylenediaminetetraacetic acid (EDTA) treated peripheral blood leukocytes using a commercial genomic DNA isolation kit (Roche, Germany). All

variants (SOD1 rs2234694 (IVS3 +34 A>C; g.11931A>C; NM_000454.5; NG_008689.1), SOD2 rs2842980 (c.*3409T>A; NM_001322820.1), rs5746136 (c.*441G>A ;NM_001322815.2), MTHFR rs1801133(c.677 C>T; p.Ala222Val; NM_001330358.2;NP_005948.3) and PAI-1 rs2227631 (-844 G>A; g.4160A>G; NG_013213.1) were analyzed by using polymerase chain reaction and restriction fragment length polymorphism (PCR–RFLP) method. All PCR reactions were performed using Dream Taq DNA polymerase (Thermofisher, USA) according to manufacturer recommendations. Analyses of the SOD1 rs2234694), SOD2 rs2842980, and rs5746136 variants were done as described previously(Polat and Simsek 2020). To analyze the PAI-1 rs2227631variant, the genomic region was amplified using a forward primer 5'GGCTCCCACTGATTCTAC3' and reverse primer 5' GTTGACACAAGAGAGCCCTC 3'. PCR fragment was digested with Xho1 restriction enzyme (Takara Bio, Japan) according to manufacturer recommendations. In the GG genotype for homozygous wild, two fragments with a size of 364bp and 146 bp, the GA genotype for a heterozygous mutant, three fragments with a size of 510bp, 364bp, and 146 bp, and the AA genotype for a homozygous mutant, one fragment with a size of 510bp was obtained. The rs1801133 variant of the MTHFR gene was amplified using a forward primer 5'GCTCAAGGCAGGACAGTG 3' and reverse primer 5'CTGGGAAGAACTCAGCGAAC 3'. PCR fragment was digested with the Taq1 restriction enzyme (Thermofisher, USA) according to manufacturer recommendations. In the TT homozygous mutant genotype, two fragments with a size of 359bp and 226 bp, the CT heterozygous mutant genotype three fragments with a size of 585bp, 359bp, and 226bp, and the CC homozygous wild genotype one fragment with a size of 585bp was obtained.

Statistical Analyses

Results were reported as mean (standard deviation; STD) for the variables which were continuous and as n (%) for categorical variables. The normality of variables was confirmed using the Kolmogorov-Smirnov test. The comparisons were performed with the Student's t-test between study groups. For the variables which not normally distributed, the Mann-Whitney U test was used. For determining genotype frequencies or alleles between study groups the Chi-square test was used. Logistic regression analysis was applied to determine odds ratios (OR) and confidence intervals (95% CI) for testing the relative risk associated with risk allele for PCOS. The estimate of differences between study groups for variables analysis of covariance (ANCOVA) was used with age and body mass index correction. The *P* values less than 0.05 were accepted as significant for all tests. IBM SPSS 22 (IBM Corp. Released2010. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp) was used for all performed statistical analyses.

3. Results

Characteristics of the study groups

The characteristics of the study groups were presented in Table 1. The mean age (STD) was 28.3 (9.4) and 31.25 (10.5) in the control and LHD groups, respectively. There were 29 (58%) male, 21 (42%) female, and 29 (56.9%) male, 22 (43.1%) female in control, and LDH groups, respectively. Mean values of the age, height, weight were similar in each group but, BMI was found significantly higher (P=0.045) in the LDH group compare to the control.

The study group was evaluated in terms of the frequency of blood groups A (38.8% vs 54.8%), AB (14.3% vs. 11.9%), O (34.7% vs. 23.8%), and B (12.2% vs. 9.5%) there was no significant (P=0.491) difference between the control and LDH groups, respectively. On the

other hand, the frequency of A (38.8% vs. 54.8%), and O (34.7% vs. 23.8%) blood groups was higher in LDH and control groups, respectively. When the Rh factor was analyzed, the number (%) of individuals with Rh (-) and Rh (+) respectively was determined as 6 (12.2%), 1 (2.4%) and 43 (87.8%), 41 (97.6%) in the control and LDH group (P =0.083). Additionally, the number of those with a positive family history of LDH was higher (69% vs. 18.4%) than those without LDH family history (P<0.001) in the study groups. There was no significant difference in smoking between the groups (P=0.398).

Table 1: Characteristics of the LDH patients and control groups

	Control	**LDH**	**p**
Age(year)	28.3 (±9.4)	31.25 (±10.5)	0.154
Height (cm)	171.3 (±8.3)	170.3 (±9.8)	0.571
Weight (kg)	71.3 (±12.9)	75.5 (±14.9)	0.134
BMI	24.24 (±3.8)	26.01 (±4.8)	**0.045***
	Control n(%)	**LDH n(%)**	**p**
A	19(38.8)	23(54.8)	0.493
AB	7(14.3)	5(11.9)	
O	17(34.7)	10(23.8)	
B	6(12.2)	4(9.5)	
Rh (-)	6 (12.2)	1(2.4)	0.083
Rh (+)	43(87.8)	41(97.6)	
Family history of LHD (negative)	40(81.6)	9(31)	**<0.001***
Family history of LHD (positive)	9(18.4)	20(69)	
Smoking (negative)	34(68)	27(73)	0.398
Smoking (positive)	16(32)	10(27)	

Results were presented as mean±SD and n(%). BMI: body mass index; LDH: lumbar disk herniation

Genotype Distributions and Allele Frequency

Genotype distributions and allele frequency of the variants between each group were given in Tables 2 and 3. In the rs2234694 (IVS3 +34 A>C) polymorphism, only AA genotype (AA: 100%) was determined and other genotypes (CA, CC) did not exist. “A” allele frequency was determined as 100% (A: 1/100) and 100% (A: 1/102) in the control and patient groups.

In the rs2842980 (SOD2 c.*3409T>A) polymorphism, AA and AT genotypes were determined and there was no TT genotype in both control and LDH groups. The frequencies of the AA and AT genotypes were 70%, 30%, and 74.5%, 25.5% in the control and patient groups, respectively without significance (*P*=0.613). Additionally, A and T allele frequencies were 85% (A: 0.85/85), 15% (T: 0.15/15) and 87.3% (A: 0.87/89), 12.7% (T: 0.13/13) in control and LDH groups, respectively. In the rs5746136 (SOD2 c.*441G>A) polymorphism, all three genotypes (AA, AG, GG) were determined. The frequencies of the AA and AG genotypes were 18.4%, 81.6%, and 15.7%, 80.4% in the control and patient groups (*P*=0.362), respectively. The GG genotype was determined only in the LDH group, and its frequency was 3.9% with two individuals only. A and G allele frequencies were 59.2% (A: 0.6/58), 40.8% (G: 0.4/40) and 55.9% (A: 0.6/57), 44.5% (G: 0.4/45) in control and LDH groups, respectively.

In the rs1801133 (MTHFR c.677 C> T) polymorphism, CC and TC genotypes were determined but there was no TT genotype. The frequencies of the CC and TC genotypes were

74%, 26%, and 33.3%, 66.7% in the control and patient groups (P<0.001), respectively. C and T allele frequencies were 87% (C: 0.87/87), 13% (T: 0.13/13) and 66.7% (C: 0.67/68), 33.3% (T: 0.33/34) in control and LDH groups (P<0.001), respectively. In the rs2227631 (PAI-1 -844 G>A) polymorphism, all three genotypes (GG, GA, and AA) were determined in the study group. The frequencies of the GG, GA, and AA genotypes were 24%, 40%, 36%, and 19.6%, 56.9%, 23.5% in the control and patient groups (P=0.220), respectively. G and A allele frequencies were 44% (G: 0.44/44), 56% (A: 0.56/56) and 48% (G: 0.48/49), 52% (A: 0.52/53) in control and LDH groups, respectively.

Table 2: Genotype frequencies of the variants

	Genotype	Control n(%)	LDH n(%)	p
rs2234694 (SOD1 +35 A>C)	AA	50 (100)	51(100)	NA
	AC	0 (0)	0 (0)	
	CC	0 (0)	0 (0)	
rs2842980 (SOD2 3'UTR A>T)	AA	35 (70)	38 (74.5)	0.613
	AT	15 (30)	13 (25.5)	
	TT	0 (0)	0 (0)	
rs5746136 (SOD2 3' UTR A>G)	AA	9 (18.4)	8 (15.7)	0.362
	AG	40 (81.6)	41 (80.4)	
	GG	0 (0)	2 (3.9)	
rs1801133 **(MTHFR 677 C>T)**	CC	37 (74)	17 (33.3)	**<0.001***
	TC	13 (26)	34 (66.7)	
	TT	0 (0)	0 (0)	
rs2227631 (PAI -844 G>A)	GG	12 (24)	10 (19.6)	0.220
	GA	20 (40)	29 (56.9)	
	AA	18 (36)	12 (23.5)	

NA: not applicable

Table 3: Alleles frequencies of the variants

	Allele	Control (n)	LDH (n)	p
rs2234694 (SOD1 +35 A>C)	A	1 (100)	1 (102)	NA
	C	0 (0)	0 (0)	
rs2842980 (SOD2 3'UTR A>T)	A	0.85 (85)	0.873 (89)	0.643
	T	0.15 (15)	0.127 (13)	
rs5746136 (SOD2 3' UTR A>G)	A	0. 592 (58)	0.559 (57)	0.637
	G	0. 408 (40)	0.441 (45)	
rs1801133 **(MTHFR 677 C>T)**	C	0.87 (87)	0.67 (68)	**0.001***
	T	0.13 (13)	0.33 (34)	
rs2227631 (PAI -844 G>A)	G	0.44 (44)	0.48 (49)	0.565
	A	0.56 (56)	0.52 (53)	

NA: not applicable

A Genetic Model of Inheritance Analyses

The investigated variants were further analyzed using genotyping test models "dominant/recessive/additive" to explore the genotype-phenotype association of the genes and LHD risk. The data relating to genetic modeling are presented in Table 4.

Genotypic test modeling could not be applied to rs2234694 (SOD1 IVS3 +34 A> C) because "AA" was the only genotype we determined. In the rs2842980 (SOD2 c.*3409 T>A) polymorphism, the "TT" genotype was not determined in the complete study group, and for the rs5746136 (SOD2 c.*441G>A) polymorphism, the "GG" genotype was determined in two volunteers with LDH therefore, those two could not include in all genetic test model analyses (Table 4).

In the rs2842980 (SOD2 c.*3409 T>A), "T" additive model, AA vs AT (OR: 1.2 95% Cl: 0.5-3.0, *P* =0.613), "A" recessive model TT + AT vs AA (OR : 1.25 95% Cl: 0.5-3.0 *P*=0.613) and "T" dominant model, AA vs AT + TT (OR: 0.8 95% Cl: 0.33-1.9 *P*=0.613) were not associated with increased risk of LDH.

In rs5746136 (SOD2 c.*441G>A), "G" additive model AA vs AG, (OR:0.9 95% Cl 0.3-3.0, *P*=.790), "G" dominant model AA vs AG+GG, (OR:1.2 95% Cl: 0. 4-3.4, *P*=0.721) and "A" recessive model AG+GG vs AA (OR:0.95 95% Cl: 0.34-2.6, *P*=0.925) were not associated with increased risk of LDH.

In rs1801133 (MTHFR c.677C>T), "T" additive model and "T" dominant model, CC vs CT (OR:5.7 95% Cl: 2.4-13.4 *P*<.001) and CC vs TC+TT (OR:5.7 95% Cl: 2.4-13.4 *P*<0.001) increased the risk of LDH almost 6-fold, "C" recessive model, CT+TT vs CC (OR:5.7 95% Cl:2.4-13.5 *P*<0.001) reduced the risk of LDH almost 6-fold.

In rs2227631(PAI-1 -844 G>A) “G” additive model AA vs AG (OR:0.6 95% Cl: 0.2-1.6 *P*=0.284), or AA vs GG (OR: 1.2 95% Cl: 0.4-3.8 *P*=0.284) and “A” additive model GG vs AG (OR:0.5 95% Cl: 0.2-1.2 *P*=0.694) or GG vs AA (OR:0.8 95% Cl: 0.26-2.4 *P*=0.694) were not associated with increased risk of LDH. In “G” dominant and “G” recessive models AA vs AG + GG (OR: 1.3 95% Cl: 0.5-3.3 *P*= 0.593) and AG + GG vs GG (OR: 1.2 95% Cl: 0.2-1.3 *P*=0.173) did not increase the risk of LDH, respectively. In "A" dominant and "A" recessive models GG vs AG + AA (OR: 1.8 95% Cl: 0.8-4.4 *P* =0.173) and AG + GG vs AA (OR: 0.8 95% Cl: 0.3-2.0 *P*=0.593) did not increase the risk of LHD, respectively.

Investigated SNPs were analyzed in combination in itself. Binary analyses of SOD2 c.*3409T>A/SOD2 c.*441G>A revealed that AA + AG (OR: 1.04 95% Cl: 0.5-2.3 *P*=0.925) and AT + AG (OR: 0.9 95% Cl: 0.4-2.1 *P*=0.776) was found to have no effect on LDH risk.

In a binary analyses of the **MTHFR c.677 C>T/ PAI-1 -844 G>A** genotypes TC + AA (OR: 1.3 95% Cl: 0.3-6.3 *P*=0.716) and TC + GG (OR: 2.5 95% Cl: 0.8-7.7 *P* =0.119) were found to have no effect on LDH risk. On the other hand, TC + AG (OR: 4.4 95% Cl: 1.5-12.1 *P*=0.005) increased the risk of LDH, while CC + AG (OR: 8.4 95% Cl: 2.2-3.1 *P*=0.001) was found to be protective against LDH (Table 5).

Table 4: Genetic test model analyses of the investigated genotypes

PAI -844 G>A		**OR (95% CI)**	**p**
“G” Additive Model	AA	Ref.	0.284
	AG	0.6 (0.2-1.6)	
	GG	1.2 (0.4-3.8)	
“A” Additive Model	GG	Ref.	0.694
	AG	0.5 (0.18-1.16)	
	AA	0.8 (0.26-2.4)	
“G” Dominant Model	AA	Ref.	0.593
	AG+GG	1.3 (0.5-3.3)	
“G” Recessive Model	AG+AA	Ref.	0.173
	GG	0.5 (0.23-1.3)	
“A” Dominant Model	GG	Ref.	0.173
	AG+AA	1.8 (0.77-4.4)	
“A” Recessive Model	AG+GG	Ref.	0.593

	AA	0.8 (0.3-1.99)	
MTHFR 677 C>T	**OR (95% CI)**		**p**
"T" Additive Model	CC	Ref.	**<0.001***
	CT	**5.69 (2.4-13.4)**	
	TT	NA	
"C" Additive Model	TT	Ref.	NA
	CT	NA	
	CC	NA	
"C" Dominant Model	TT	Ref.	NA
	CT+CC	NA	
"C" Recessive Model	TC+TT	Ref	**<0.001***
	CC	**5.68 (2.4-13.5)**	
"T" Recessive Model	CC+CT	Ref.	**NA**
	TT	NA	
"T" Dominant Model	CC	Ref.	**<0.001***
	TC+TT	**5.69 (2.4-13.4)**	
SOD2 3'UTR A>T		**OR (95% CI)**	**p**
"T" Additive Model	AA	Ref.	0.613
	AT	1.2 (0.5-3.0)	
	TT	NA	
"A" Additive Model	TT	Ref	NA
	AT	NA	
	AA	NA	
"A" Recessive Model	TT+AT	Ref.	0.613

	AA	1.25 (0.52-3.0)	
"A" Dominant Model	TT	Ref.	NA
	AT+AA	NA	
"T" Recessive Model	AT+AA	Ref.	NA
	TT	NA	
"T" Dominant Model	AA	Ref.	0.613
	AT+TT	0.8 (0.33-1.9)	
SOD2 3' UTR A>G		**OR (95% CI)**	**p**
"G" Additive Model	AA	Ref.	0.790
	AG	0.9 (0. 3-2.4)	
	GG	NA	
"A" Additive Model	GG	Ref.	NA
	AG	NA	
	AA	NA	
"G" Dominant Model	AA	Ref.	0.721
	AG+GG	1.2 (0. 4-3.4)	
"G" Recessive Model	AG+AA	Ref.	NA
	GG	NA	
"A" Dominant Model	GG	Ref.	-
	AG+AA	NA	
"A" Recessive Model	AG+GG	Ref.	0.925
	AA	0.95 (0.34-2.6)	

* shows significant difference NA: not applicable

Table 5: Combinatorial genetic test model analyses of the investigated genotypes

SOD2 3'UTR A>T/ SOD2 3' UTR A>G	**OR (95% CI)**	**p**
AA+AG vs. others	1.04 (0.5-2.3)	.925
AT+AG vs. others	0.9 (0.4-2.1)	.776
MTHFR 677 C>T/ PAI -844 G>A	**OR (95% CI)**	**p**
TC+AA vs. others	1.3 (0.3-6.3)	.716
TC+AG vs. others	4.4 (1.5-12.1)	.005*
TC+GG vs. others	2.5 (0.8-7.7)	.119
CC+GG vs others	NA	NA
CC+AG vs others	8.4 (2.2-3.1)	<.001*

NA: not applicable

4. Discussion

LDH is a condition in which degeneration and herniation of the nucleus pulposus of the intervertebral disc (IVD) of the lumbar spine exist. Today, disc herniation is the most common diagnosis among the degenerative abnormalities of the lumbar spine, and it is the condition that causes spinal surgery (Skovron 1992; Andersson 1999). Although disc herniation has been diagnosed in different age groups, it is determined that LDH occurs mainly in the range of the fourth and fifth decades of life (mean age 37 years) (Obukhov et al. 1996; Mayer, Mellerowicz, and Dihlmann 1996; Garrido 1993). It is determined that 2- 3% of the population may be affected. It has been recognized as a worldwide health problem because of its higher incidence and unknown etiology and pathogenesis (Matsui et al. 1998; Sambrook, MacGregor, and Spector 1999; Long et al. 1996) Genetic factors were involved in the etiology of lumbar disc degeneration and the condition is estimated to have about 75% inheritance (Janeczko et al. 2014). In our study group, the frequency of individuals with a family history of LDH was statistically higher (69% vs. 18.4%) compared to the control group (P<0.001).

Smoking affects endothelial function by modulating oxidative stress, inflammation, and platelet activation. The leading cause of death worldwide is tobacco consumption. A meta-analysis indicated that smoking promoted LDH development and was found significantly associated with increased risk of LDH (Huang et al. 2016). In our study group, no difference was found between LDH and control groups in smoking (P=0.083). Besides, no relationship was found between blood groups and LDH susceptibility (P=0.493), similarly with Rh groups

(*P*=0.083), but only one individual in the LDH group was determined as Rh (-). ABO antigens are thought to be an evolutionary advantage in conferring resistance against pathogens. However, the ABO blood group has been linked with susceptibility to various diseases, such as cancer, cardiovascular diseases, infections, and hematologic disorders (Anstee 2010; Franchini and Lippi 2015). Additionaly, a study determined that non-OO carriers positive for MTHFR c.677 C>T had a 5.7 times greater risk of thrombosis than that recorded in OO carriers negative for MTHFR c.677 C>T (Jukic et al. 2009). As far as we know, there is no similar study in the literature to compare blood groups and LDH risk or susceptibility.

Inflammation is an internal defense mechanism that protects from infection and other insults. Failure in inflammatory responses to regulate its mechanism can lead to becoming chronic, and contribute to the continuation of the disease. LDH tissue is characterized by the existence of inflammatory cells (IC). The IC exited in LDH is mainly macrophages, T-cells, and mast cells. Inflammatory cells liberate several reactive oxygen species at the site of inflammation, causing increased oxidative stress (OR). On the other hand, several reactive oxygen species can initiate an intracellular signaling cascade that enhances the expression of the pro-inflammatory genes (Anderson et al. 1994; Flohe et al. 1997). Thus, inflammation and oxidative stress are closely connected and tightly linked pathophysiological events.

ROS is any species with one or more unpaired electrons with a strong tendency to form pairs to become stable. Therefore, free radical tends to donate its unpaired electron to another molecule or to steal it to became stable(Halliwell 1989). Increasing evidence determined that oxidative interaction between the biomolecules including lipids, proteins, and DNA, contributes to diseases pathogenesis. On the other hand, the removal of ROS is operated by both enzymatic and non-enzymatic protective antioxidant mechanisms (Apel and Hirt 2004; Rodriguez and Redman 2005). In disk tissues, oxidative stress may initiate matrix destruction, cell apoptosis, and eventually disk degeneration (Nerlich et al. 2007). There is evidence showing that ROS has a contribution to progressive degenerative diseases of different skeletal tissues (Kousteni 2011; Ziskoven et al. 2011). Hydrogen peroxide (H_2O_2) induced oxidative stress caused apoptosis of rat central nucleus pulposus (NP) cells and human annulus fibrosus (AF) cells (Kim et al. 2007; Gruber et al. 2008).

Superoxide dismutase is one of the most potent, the first enzymes of antioxidant defense mechanism. It catalyzes the conversion of superoxide anions to dioxygen(O_2) and H_2O_2, which is then removed by catalase (CAT) or glutathione peroxidase (GPx) and maintaining homeostasis of intracellular ROS. The SOD family of antioxidant enzymes grouped as SOD types 1, 2, and 3 also referred to as intracellular (Cu/Zn-SOD), mitochondrial (Mn-SOD), and extracellular (EC-SOD) enzymes, respectively(Leopold and Loscalzo 2009). While MnSOD does not have similar characteristics with the other SODs family members, similarities are put forth as a result of genetic comparisons between Cu/ZnSOD and ECSOD genes in certain homology levels of amino acids (Parge, Hallewell, and Tainer 1992). SOD1 and SOD2 genes are located on chromosomes 21q22.11 and 6q25.3 and are comprised of five and ten exons, respectively. Both are widely expressed almost in all human tissues (Fagerberg et al. 2014). Since it accounts for approximately 50-80% of the total SOD activity, SOD1 is considered an excellent tool against oxidative insults (Didion et al. 2002; Fukai et al. 2000).

The rs2234694 (IVS3 +35 A/C) polymorphism in SOD1 is adjacent to the splicing point (exon3/intron3) and AA genotype with greater SOD1 activity. c.*3409T>A (rs2842980) is a variant located in the 3409th base in the 3’ UTR region of the SOD2 gene. c.*441G>A (rs5746136) is located in the 441st base in the 3’ UTR region and 65bp downstream of the poly-A site. It is also located in <1kb upstream from specificity protein 1 (SP1) and *nuclear*

factor kappa B (NF-kB) transcription element sequences (Wan, Devalaraja, and St Clair 1994). There were no significant differences between LDH and control groups in genotype distribution and allele frequencies of the SOD1 rs2234694 (IVS3 +35A>C), SOD2 rs5746136 (c.*441G>A), and rs2842980 (c.*3409T>A) variants although these variants were found to be associated with an increased risk of nephropathy in patients with type 1 diabetes mellitus (T1DM), polycystic ovary syndrome (PCOS), positive outcome in breast cancer patients receiving radiotherapy in addition to an increased risk of primary open-angle glaucoma (POAG), respectively in different studies (Martin et al. 2006; Panduru et al. 2010; Zhang et al. 2017). Some studies report the variants are not disease-associated(Nithya et al. 2016). There was no association between SOD1 and SOD2 genotype and LDH risk in our study groups when the genetic model of inheritance analyses was performed. As far as we know, there are no studies in the literature to compare our findings in LDH groups but significantly lower glutathione reductase (GR) and malondialdehyde (MDA) levels were reported in patients with lumbar disc degeneration(Bakirezer et al. 2019).

MTHFR gene rs1801133 (c.677 C> T) polymorphism encodes a variant of cytosine (C) to thymine (T) transition at nucleotide 677, which leads the alanine (A) to valine (V) substitution at codon 222 and 30% and 70% reduction in enzyme activity in heterozygous and homozygous genotypes, respectively (Frosst et al. 1995). In the general population, hyperhomocysteinemia (HHcy) is related to reduce the activity of the MTHFR resulted from single nucleotide polymorphism (Frosst et al. 1995). The other factor can be an insufficient dietary intake of folate. In humans, the MTHFR is located on chromosome 1 and consisted of 12 exons. The human MTHFR gene has three transcripts with a size of 2.2 kb, 7.5 kb, and 9.5 kb, respectively (Tran et al. 2002). The gene has fourteen nucleotide polymorphisms associated with enzymatic deficiency (Liew and Gupta 2015). Among them, c.677C>T and c.1298A>C (rs1801131) are the most commonly reported variants that reduce the MTHFR activity in various degrees. As opposed to the c. 677C>T variant, though the c. 1298 A>C reduces the MTHFR activity, it is not related to HHcy or a lowered plasma folate concentration neither homozygous nor heterozygous states(van der Put et al. 1998).

Affected Hcy metabolism by MTHFR gene mutations stimulates HHcy. In MTHFR mutant patients, reduction in enzymatic activity and decreased remethylation of Hcy to methionine lead to elevated total Hcy. The increase of the Hcy levels causes increased cytokine activity, lipid peroxidation accompanied by vascular endothelial damage, prothrombotic process, aterothrombogenesis, thromboembolism, hypercoagulability, systemic vascular occlusive diseases, and acts as a risk factor in vascular stiffness (Frosst et al. 1995). When added to plasma, Hcy is automatically oxidized and produces ROS (free radicals) such as superoxide and hydrogen peroxide that causes direct damage to endothelial cells (Pierdomenico et al. 2003; van Guldener, Nanayakkara, and Stehouwer 2003). ROS production due to HHcy may contribute to further impairment of angiogenesis (Loscalzo 1996). Circulating HHcy can be seen not only in genetic deficiency of enzymatic pathways involved in Hcy catabolism such as MTHFR polymorphisms but also in environmental factors that may cause HHcy including nutritional deficiencies, drugs, and some diseases mainly induce a deficiency of folic acid, vitamin B12, and B6(Motulsky 1996). In our study groups, it was determined that in the rs1801133 (MTHFR c.677C>T), the CT genotype increased the risk of LDH almost 6-fold while the CC genotype showed a protective effect against LDH with a significantly higher frequency of “T” allele in LDH group. As far as we know, there are no studies in the literature to compare our findings in LDH groups. Moreover, regardless of the cause of the inflammation, the response includes events such as increased blood flow to the inflammation site and increased capillary permeability resulting from the withdrawal of endothelial cells (Calder et al. 2009). The metabolic environment of disc cells is governed by the avascular

nature of the tissue and disc cells depend on the blood supply at the margins of the discs for their nutrients. Oxygen is compulsory for cellular activity as well(Grunhagen et al. 2006). Factors that impair blood flow to the disc (e.g., sickle cell anemia or other disorders that affect the blood supply) can cause nutrients to drop to levels that can no longer support cell activity or vitality. Since high Hcy concentration is both atherogenic and thrombogenic, it may contribute to the pathology of LDH by preventing the blood and nutrient intake to the inflamed area in both cases.

Impaired fibrinolytic activity was reported in patients with chronic back pain (Cooper et al. 1991; Jayson 1989). Plasminogen inhibitor 1 (PAI-1) is the main regulator of the fibrinolytic system and its elevation is related to the impaired fibrinolytic system. Complete PAI-1 deficiency is caused by mutations in the SERPINE1 gene. This gene is responsible for encoding a protein called plasminogen activator inhibitor 1 (PAI-1) that function in normal blood clotting. The PAI-1gene is located on chromosome 7 (7q21.3-22) and consisting of 9 exons(Klinger et al. 1987). There are two known promoter polymorphisms, the −675 4G>5G (rs1799768) and the −844 G>A (rs2227631), which are associated with increased transcriptional activity and PAI-1 protein levels (Morange et al. 2007; Torres-Carrillo et al. 2008).

Impaired fibrinolytic activity determined by PAI-1 measurement has been found related to poor improvement in LDH operated patients (Haaland et al. 1992). Increased fibrinogen levels were reported in patients with pain in the shoulder, neck, upper, or lower back (Schell et al. 2008). PAI-1 may play important roles in vascular homeostasis and pathological vascular remodeling (Luo et al. 2017). In our study group, no association was found between the PAI-1 -844 G>A polymorphism and the LDH risk in a genetic model of inheritance analyses. Additionally, there were no significant differences between LDH and control groups in genotype distribution and allele frequencies of the variant. As far as we know, there are no studies in the literature to compare our findings in LDH groups. On the other, in paired analysis, it was determined that AG genotype increased the protective effect of CC genotype of MTHFR gene from 5.7 to 8.4 fold while reducing the risk effect of the TC genotype of MTHFR gene from 5.7 to 4.4 fold in LDH risk.

There are two limitations to our study. Firstly, it is a case-control study with a relatively small study cohort. Secondly, the lack of information about Hcy, folic acid, PAI, and superoxide levels in blood circulation, although biochemical analyses in the blood sample may be insufficient to provide information about herniated tissue(s).

5. Conclusion

While MTHFR rs1801133 polymorphism was found to be associated with increased risk of LDH, PAI-1 rs2227631 showed a protective effect and SOD1 rs2234694, SOD2 rs2842980, and rs5746136 polymorphisms were not associated with LDH risk in our study groups. Additional studies are needed on gene polymorphisms responsible for antioxidant mechanisms and vascular homeostasis to better understanding the role of both mechanisms in LDH pathogenesis in larger study groups.

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Conflict of interest

The authors declare that there is no conflict of interest relevant to the subject matter or materials included in this work.

Ethical Approval

The study was approved by the Ethics Committee of Medical School at Erzincan Binali Yıldırım University. 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.

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