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Original Investigation

Genetic Dimension of Intervertebral Disc Degeneration: Polymorphism of Matrix Metalloproteinase 1 and 3 in the North Iranian Population

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ABSTRACT

AIM: The etiology of intervertebral disc degeneration is still vague and both genetic and environmental factors are assumed as the main causes. One of the proposed genetic factors is the polymorphism of matrix metalloproteinases (MMPs) genes. The aim of this study was to explore the relationship between two polymorphisms (MMP-1-755 T/G [rs498186] and MMP-3 A/C [rs632478]) and disc degeneration.

MATERIAL and METHODS: We performed a case-control study on 130 cases with intervertebral disc degeneration confirmed by magnetic resonance imaging (MRI) and 210 healthy individuals. The Schneiderman criterion was used to determine the severity of the disc degeneration. Blood samples were collected from the participants. The genotyping was performed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Odds ratios and 95% CIs were determined as measures of the strength of association between genotypes and disc degeneration.

RESULTS: The most frequent intervertebral disc degeneration was observed in age range of 31-40 years (43.2%). A significant association was found between the MMP-3 polymorphism and disc degeneration ($p < 0.001$). The homozygote CC was associated with an increased risk of disc degeneration compared with the AA genotype (OR=5.25; 95%CI=2.82-9.77, $p < 0.001$). We did not find any significant association of the MMP-1 polymorphism with disc degeneration ($p = 0.95$).

CONCLUSION: The MMP-3 [rs632478] polymorphism may contribute to susceptibility to disc degeneration. To confirm our findings, additional well-designed studies in diverse ethnic populations are required.

KEYWORDS: Polymorphism, Matrix metalloproteinase-1, Matrix metalloproteinase-3, Intervertebral disc degeneration

INTRODUCTION

Intervertebral disc degeneration is a multifactorial disorder of the musculo-skeletal system and one of the main causes of backache and disability (1). Intervertebral disc degeneration is irreversible and progressive (28,32). It usually occurs at the third or fourth decades of life (31). Degeneration can lead to herniation, radiculopathy, myelopathy, spinal

stenosis, and degenerative spondylolisthesis (19). Although its etiology and pathogenesis are still vague, both genetic and environmental factors are assumed as the main causes (11). Age, mechanical pressure, genetic factors and biochemical and inflammatory changes are important factors (11,23,31,33). Studies have highlighted the strong family predisposition to disc degeneration and herniation (10,18,29). Findings of two



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studies a decade ago reported 60% impact of heredity on this process (5,25). Moreover, different genes have been blamed for disc degeneration development (2,6,14).

Intervertebral disc degeneration has a unique outer structure made of annulus fibrosus and an inner nucleus pulposus with elasticity that makes it resistant to compression and distribution of power in the spinal cord (30). Developing intervertebral disc degeneration generates a multi-step process, one major step of which is the deterioration of matrix (20). Today, the majority of research emphasizes the importance of discovering the mechanism of extracellular matrix deterioration, particularly regulation of gene expression. According to various reports, polymorphism in a number of genes such as Agrecan, interleukin-1 and 6, vitamin D receptor and matrix metalloproteinase genes is associated with an increased risk of intervertebral disc degeneration (7,30,31).

Studies have found a number of matrix metalloproteinase (MMPs) (including MMP type 1,28,32,23,33,10,8,25,7,16) associated with disc degeneration (33). These proteases are the most important catabolic enzymes in the destruction of intervertebral discs (4,21). In humans, there are 23 distinct MMPs comprising secreted and transmembrane proteins. MMPs are commonly classified based on the type of substrate, protein structure and cell localization into namely Collagenase, Gelatinase and Stromelysin and Matrilysin and membrane-type matrix metalloproteinases (30). MMP-mediated remodeling of the pericellular microenvironment is therefore essential for many physiological processes. In degraded tissue, there is an increase in many MMPs, including MMP-1, 2, 3, 7, 9, 13, and 28, often according to the severity of degeneration (19). Matrix metalloproteinase 1 (MMP-1=interstitial collagenase) has a specific ability to degrade type-I collagen, whereas MMP-3 (Stromelysin) degrades a broader range of substrates (e.g. fibronectin, laminin, collagens III, IV, IX, and X, and cartilage proteoglycans), and may also affect the expression of other MMPs (17).

Given the importance of genetic factors in patients with intervertebral degenerative disc, this study sought to find the relationship between polymorphism of matrix metalloproteinases-1 and -3 with intervertebral disc degeneration, for the first time in a population of the Guilan province in northern Iran.

■ MATERIAL and METHODS

This hospital-based case-control study included 130 patients with newly diagnosed lumbosacral disc degeneration referred to the spine clinic of Guilan University of Medical Sciences, Iran, between September 2013 and December 2015. The patients consisted of 68 men and 62 women, whereas the controls included 210 disease-free subjects recruited from the same institute with similar age and sex distribution (119 men, 91 women). The magnetic resonance imaging (MRI) findings of the healthy controls showed normal intervertebral disc tissue.

The participants' demographic data, history and imaging findings were entered in a designed checklist. Positive family history was considered as having any evidence of intervertebral disc degeneration in first- and/or second-degree family docu-

mented by whole spinal MRI or typical history. Positive history of smoking was defined according to the Standard National Survey on Drug Use and Health current cigarette smoking variable definition (NSDUH-S)(23). The level of occupation load was scaled based on US-National Institute for Occupational Safety and Health (NIOSH) as low/moderate and high (32). The ethical committee of Guilan University of Medical Sciences approved the study by code of IR. GUMS.REC.1394.56 and all study subjects supplied a written informed consent that was subsequently archived. This study was performed in accordance with the declaration of Helsinki (1989).

Imaging Procedure

All participants underwent an MRI examination using a non-contrast 1.5 Tesla MRI and T2-weighted fast spin-echo (2700/110, TR/TE) for 12 sagittal and axial scans of the whole spine with 0.5 mm slices. The severity of disc degeneration was assessed according to Schneiderman's classification in the most destructed disc (22).

Genotyping

Two ml of whole blood was collected in vacutainers containing ethylene diamine tetra acetic acid (EDTA) for deoxyribonucleic acid (DNA) isolation. Genomic DNAs were isolated using the GPP solution kit (Gen Pajooan Pouya, Iran) following standard procedures. DNA purity and concentration were determined spectrophotometrically at 260 to 280 nm. Each DNA sample was stored in TE buffer (5 mM Tris-HCl, 0.1 mM EDTA, pH 8.5) at -20°C until analysis.

The genotypes of MMP-1 and MMP-3 (rs498186 and rs632478, respectively) were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Amplifications were carried out using primers designed by Oligo primer analysis software (Version 7.54, Molecular Biology Insights, USA). Primer sequences for PCR amplification of each polymorphism were as follows: MMP-1 (rs498186), forward 5'-GTTATCTCATACTCCGCCTGT -3' and reverse 5'-CCTTCTGCCTTTGTTAAGCTG -3'; MMP-3 (rs632478), forward 5'- TTCACATCACTGCCACCACT-3' and reverse 5'- GCCCATTTTGCTTCCTCTCA -3'. Each reaction mixture (25 µl) contained 30 ng DNA template, 1x PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 0.5 µM each primer and 1.5 U Taq DNA polymerase (Bioflux, Japan). Similarly, the PCR conditions for both polymorphisms of the MMP-1 and MMP-3 were as follows: initial denaturation at 95 °C for 5 minutes, 35 cycles of denaturation at 95 °C for 45 seconds, annealing at 56 °C (MMP-1) and 57 °C (MMP-3) for 40 seconds, with a final step at 72 °C for 5 minutes to allow a complete extension of the PCR fragment.

The PCR amplicons generated for MMP-1 (462 bp) and MMP-3 (519 bp) were subjected to restriction digestion (Figures 1 and 3). TspRI and HphI restriction enzymes were used to detect polymorphisms in MMP1 and MMP3, respectively. PCR products with a T at the MMP-1 (rs498186) polymorphic site were digested into two 259 and 203 bp fragments, while those with G were not because of the absence of the TspRI restriction site (Figure 2). The MMP-3 (rs632478) genotypes were assessed as follows: a single 519 bp fragment for the AA

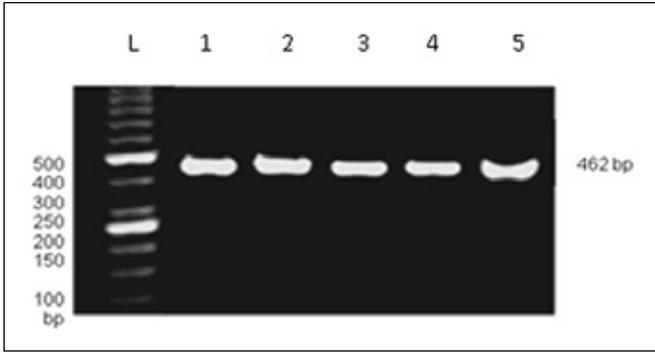


Figure 1: Agarose gel electrophoresis stained by ethidium bromide after PCR amplification of MMP-1. L ladder 1-5: amplified fragment.

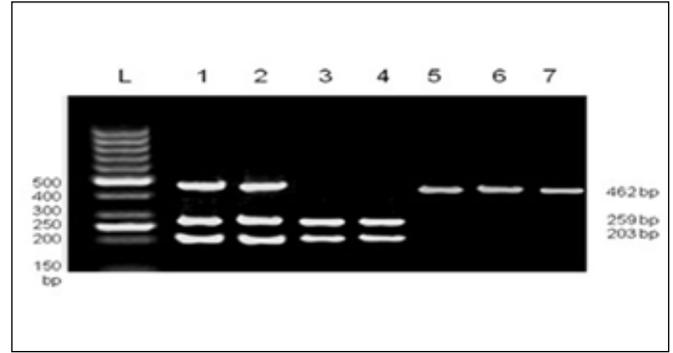


Figure 2: RFLP analysis of the MMP-1 polymorphism. L, DNA ladder, 1 and 2: GT heterozygote, 3 and 4: TT homozygote, 5, 6 and 7: GG homozygote.

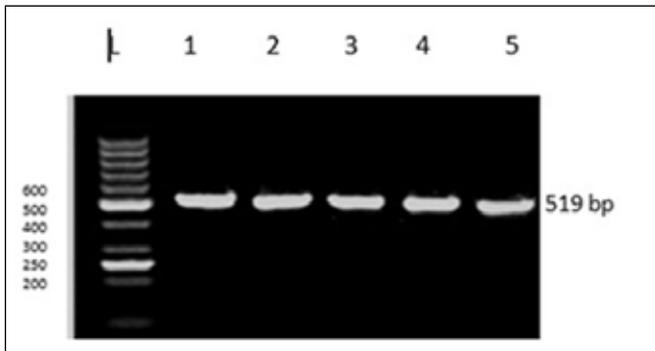


Figure 3: Agarose gel electrophoresis stained by ethidium bromide after PCR amplification of MMP-3. L ladder 1-5: amplified fragment.

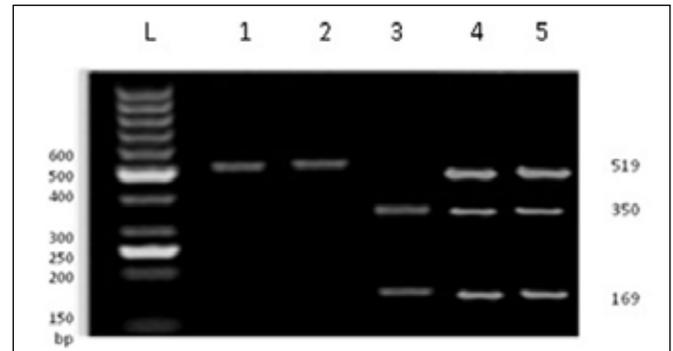


Figure 4: RFLP analysis of the MMP-3 polymorphism. L DNA ladder 1, 2: AA homozygote, 3: CC homozygote and 4, 5: AC heterozygote.

genotype; two fragments of 350 and 169 bp for the CC; and three fragments of 519, 350 and 169 bp for the AC genotype (Figure 4). The PCR-RFLP products were separated on 2% agarose gel and visualized by ethidium bromide staining. All the samples with ambiguous results were repeated to ensure quality control purposes.

Statistical Analysis

Statistical analyses were carried out using SPSS software version 19. Allelic frequencies were estimated by the “counting method” and differences in allele frequencies between case and control subjects were tested using the likelihood ratio χ^2 tests for 2 x 2 tables (two alleles, case vs. control subjects). The odds ratio (OR) and range with 95% confidence interval (CI) were calculated for the presence of the reference genotype and allele using a logistic regression model. Statistical significance was set at $p < 0.05$.

RESULTS

In the present study, a total of 340 subjects including 130 patients with disc degeneration and 210 disease-free control subjects were evaluated. The mean age was 37.9 ± 8.8 years for the subjects and 35.7 ± 7.4 years for the controls ($p = 0.157$). The main characteristics of the patients are summarized in Table I.

Among the 130 patients with intervertebral disc degeneration, 18.2% of patients had been diagnosed for less than six months, 38.6% six months to one year, 15.9% one to two years, 20.5% two to five years and 6.8% more than five years. Interestingly, the highest frequency belonged to patients with disc protrusion (59.1%), followed by disc bulging (25%), extrusion (11.4%) and migration (4.5%) based on Schneiderman’s classification.

The distribution of the MMP-1 (rs498186) and MMP-3 (rs632478) polymorphisms was compared using the Chi-square test. Genotype and allele frequencies of the MMP-1 and MMP-3 polymorphism in patients with disc degeneration and controls are shown in Tables II and III. The frequencies of the AA, AC and CC genotypes of MMP-3 were 52.38%, 35.71% and 11.9% in controls and 27.69%, 39.23% and 33.07% in the cases, respectively. Four genetic models (co-dominant, dominant, recessive and over-dominant) were applied for the analysis of the SNPs. In a recessive model (polymorphic homozygous genotype vs. heterozygous + wild-type homozygous genotypes), the CC genotype of MMP-3 was associated with susceptibility to disc degeneration compare to that of the AC+AA genotype (OR=3.65; 95% CI=2.09-6.37, $p < 0.001$). In a co-dominant model (polymorphic homozygous genotype vs. wild-type homozygous genotype), we found that the CC genotype of MMP-3 was correlated with

a higher risk of disc degeneration when compared with that of the AA genotype (OR=5.25; 95%CI = 2.82-9.77, p<0.001). Comparison with the control group MMP-3 allele C showed a significant difference in disc degeneration cases (OR=2.62; 95%CI=1.90-3.62, p<0.001). On the contrary, no significant difference was observed in the distribution of MMP-1 polymorphism in cases and controls both at the genotype and allele levels ($\chi^2=3.60$; P=0.16, $\chi^2=0.003$; p=0.95).

No significant relationship was found between the severity of intervertebral disc degeneration and genotypes of MMPs-1 and 3 (p=0.690, p=0.931, respectively), and there was no association with the MMP alleles (p>0.05).

DISCUSSION

Beside environmental factors, the genetic background plays an important role in the development of disc degeneration (21). One of the most important stages of disc degeneration is destruction of its matrix by enzymes such as matrix metalloproteinase (17,19,22,24). MMP is widely considered to play a role in disc degeneration by degrading collagen type II, IX, X and aggrecan found in the matrix (3). The result of a study conducted by Fang and Jiang demonstrated that IL-1 β and high mobility group box 1(HMGB1) additively promotes the release of inflammatory cytokines and the expression of MMPs in human IVD cells (9).

Matrix metalloproteinase-3 (Stromelysin 1) is an important member of the MMP family and has a critical role in the

regeneration of connective tissue (16). However, in high concentrations it is known as the most important cause of intervertebral disc degeneration. It has also been shown that MMP-1 expression is associated with the degradation of the matrix in disc degeneration (8). Promoter polymorphisms have been identified for both MMP-1 and MMP-3. Variants in the promoter region can function by modulating transcription and leading to unusual protein expression.

The results of present study revealed a significant association between the MMP-3 A/C [rs632478] polymorphism and intervertebral disc degeneration. Its homozygous variant [CC] manifested five-fold positive significant risk for disc degeneration relative to the AA variant. Moreover, subjects with the variant genotypes (CC+AC) showed an overall increased risk of disc degeneration more than two-fold relative to AA carriers. Also the presence of [C] allele increased the risk of disc degeneration near two-fold in relation to the A allele. However, the MMP-1G allele frequency in our study of mostly patients with disc degeneration was consistent with the frequencies in controls. Thus, the risk of disc degeneration does not appear to be influenced by the MMP-1 rs498186 genotype.

So far, there is no report concerning the relationship of MMP-1rs498186 and MMP-3 rs632478 genetic polymorphism with disc degeneration. Song et al. reported that individuals with the presence of D allele for the -1607 promoter polymorphism of MMP-1 (rs1799750) are about 1.5 times more susceptible to develop degenerative disc disease when compared with those

Table I: Demographic Characteristics of Subjects Included in Two Groups of Study Based on Having Inter-Vertebral Disc Degeneration

Variable	Status	Group with Intervertebral disc degeneration (n=130) n (%)	Control Group (n=210) n (%)	p value
Sex	Male	68 (52.3)	119 (56.7)	0.63
	Female	62 (47.7)	91 (43.3)	
Age (year)	20-30	30 (23.0)	65(31.0)	0.61
	31-40	56 (43.2)	80 (38.0)	
	41-50	44 (33.8)	65 (31.0)	
Age (year)		37.9 \pm 8.8	35.7 \pm 7.4	0.157
Smoking	Yes	21 (16.2)	31 (14.8)	0.87
	No	109 (83.8)	179 (85.2)	
BMI	19-25	68 (52.3)	82 (39.0)	0.38
	26-30	53 (40.8)	111 (52.9)	
	>30	9 (6.9)	17 (8.1)	
BMI		25.42 \pm 2.95	25.9 \pm 2.73	0.37
Family history	Yes	65 (50)	45 (21.4)	0.0001
	No	65 (50)	165 (78.6)	
Level of occupation load (NIOSH)	High	30 (23.1)	34 (16.2)	0.38
	Low/moderate	100 (76.9)	176 (83.8)	

having G allele only (26). However, Jacobsen et al. revealed no difference in the frequency of the MMP-1 rs1799750 2G allele in patients with disc herniation compared with controls. They also found that the patients homozygous for the 2G allele had more pain and reduced function compared with those carrying the 1G allele (13).

Takahashi et al. found that the MMP-3 5A5A and 5A6A genotype in the elderly was associated with a significantly larger number of degenerative discs than the 6A6A, but there was no significant difference in the youth (27). More closely, similar observations have been reported by Yuan et al. in the Chinese population and their findings suggested that people with the mutation genotypes 5A of MMP-3 and/or A of VDR-

Table II: Genotype Frequencies of MMP-1 and MMP-3 Polymorphisms in Patients with Disc Degeneration and Controls

Genetic mode	Genotypes	Patients n (%)	Controls n (%)	OR (95%CI)	p value
MMP-1(rs498186)					
Co-dominant	TT	31(23.84)	59(28.09)	1.00	
	TG	93(71.53)	132(62.85)	1.34(0.80-2.23)	0.25
	GG	6(4.61)	19(9.04)	0.60(0.21-1.65)	0.32
Dominant	TT	31(23.84)	59(28.09)	1.00	
	GG+TG	99(76.15)	151(71.90)	1.26(0.76-2.08)	0.36
Recessive	TT+TG	124(95.38)	191(90.95)	1.00	
	GG	6(4.61)	19(9.04)	0.48(0.18-1.25)	0.13
Over-dominant	GG+TT	37(28.46)	78(37.14)	1.00	
	TG	93(71.53)	132(62.85)	1.48(0.92-2.38)	0.10
MMP-3(rs632478)					
Co-dominant	AA	36(27.69)	110(52.38)	1.00	
	AC	51(39.23)	75(35.71)	2.07(1.23-3.48)	0.005
	CC	43(33.07)	25(11.9)	5.25(2.82-9.77)	< 0.001
Dominant	AA	36(27.69)	110(52.38)	1.00	
	AC+CC	94(72.3)	100(47.61)	2.87(1.79-4.59)	<0.001
Recessive	AA+AC	87(66.92)	185(88.09)	1.00	
	CC	43(33.07)	25(11.9)	3.65(2.09-6.37)	<0.001
Over-dominant	AA+CC	79(60.76)	135(64.28)	1.00	
	AC	51(39.23)	75(35.71)	1.16(0.74-1.82)	0.51

OR: Odds ratio, **95% CI:** 95% confidence interval. *Bold values indicate p < 0.05.*

Table III: Association Between MMP-1(rs498186) and MMP-3(rs632478) Alleles and Susceptibility of Disc Degeneration

Polymorphic site	Allele n (%)	Patients n (%)	Controls (95%CI)	OR	p value
MMP-1(rs498186)					
	T	155(59.61)	250(59.52)	1.00	
	G	105(40.38)	170(40.47)	0.99(0.72-1.36)	0.98
MMP-3(rs632478)					
	A	123(47.30)	295(70.23)	1.00	
	C	137(52.69)	125(29.79)	2.62(1.90-3.62)	<0.001

OR: Odds ratio; **95% CI:** 95% confidence interval. *Bold value indicates p < 0.05.*

Apa may have increased risk of developing lumbar disc degeneration if they are exposed to whole-body vibration and/or bending/twisting (31). In another study, Zawilla et al. investigated that mutant allele 5A of MMP-3 and mutant allele T of VDR were significantly associated with lumbar disc degeneration (32). The conflicting results of these reports may be due to differences in sample sizes, ethnic groups, genetic heterogeneity in the pathogenesis of disc degeneration and the impact of other genetic and environmental factors.

We have also found that there is no significant relationship between the severity of disc degeneration and MMP-1 and MMP-3 polymorphisms. However, another study by Zigouris et al. found a strong link between expression of MMP-1 and MMP-3 and the grade of disc degeneration (34). Efforts by Le Maitre et al. to seek the cells that express the enzymes MMP 1 and 3 in patients with disc degeneration unveiled an increased number of these cells along with the severity of degeneration (15). Also in the Japanese population, having either one or two copies of the MMP-3 5A allele in the elderly, but there was no significant difference in the young population (27).

In the present study, the number of men was a little more than women. Yet, no difference was observed between the groups in terms of gender. In our geographical setting which is full of active farm fields, women are vigorously involved in farming and so suffer from low back pain due to disc degeneration and other musculoskeletal disorders and this can explain the fair equality of genders in our study. Most of our study participants were young adults. As both groups were the same, we cannot blame environmental and genetic causes in youth. Apparently, geographical, physical and clinical conditions of the youngest persons facilitate their cooperation in the study. Yet, in a study by Williams et al., most participants were old women (67%) with mean age of 57.7 years old (1).

Our study showed no significant relationship between smoking and intervertebral disc degeneration, whereas some other similar studies concluded that smoking can be a risk factor exposing people to disc degeneration (12,31,32). The impact of this factor would be more apparent with bigger study sample size.

Finally, our study revealed that family history of intervertebral disc degeneration was significantly higher in the cases than the control group. Zawilla's study also has the same findings. To sum, the importance of genetic factors in exposing people to disc degeneration is remarkable and worthy of consideration (32), as our results confirm this fact.

Some limitations of our study are as follows; we only evaluated two SNPs in the MMP-1 and MMP-3 genes, which was inadequate to assess the disc degeneration risk for gene studies. However, numerous factors act individually and together to influence on the risk of disc degeneration disease. So, we should involve more factors in our future work.

■ CONCLUSION

MMP-3 rs632478 polymorphism may contribute to increased susceptibility to disc degeneration. However, MMP-1

rs498186 was not associated with disc degeneration in the Iranian population. Moreover, the mechanisms underlying this association need to be further explored. Further studies investigating the role of MMPs genetic variants in disc degeneration and replication of the present study in diverse ethnic groups are needed to better understand the pathobiology of disc degeneration.

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