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Increased Expression of Matrix Metalloproteinases in Ligamentum Flavum Hypertrophy of the Patients with Lumbar Spinal Stenosis

Betul Eser[1], Olcay Eser[2], Nejdet Simsek[3], Erdogan Bulbul[4] and Mustafa Guven[5]

[1]Department of Medical Genetics, Faculty of Medicine, Balikesir University, 10145 Balikesir, Turkey
E-mail: drbetuleser@gmail.com
[2]Department of Neurosurgery, Faculty of Medicine, Balikesir University, 10145 Balikesir, Turkey
E-mail: drolcayeser@hotmail.com
[3]Department of Histology and Embryology, Faculty of Medicine, Balikesir University, 10145 Balikesir, Turkey,
E-mail: nsimsek-58@hotmail.com
[4]Department of Radiology, Faculty of Medicine, Balikesir University, 10145 Balikesir, Turkey
E-mail: drerdoganbulbul@yahoo.com
[5]Department of Neurosurgery, Faculty of Medicine, Canakkale 18 March University, 17020 Canakkale, Turkey
E-mail: guven2340@hotmail.com

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ABSTRACT This paper shows an investigation of the expressions of MMP-3,-13 and their polymorphisms in patients with lumbar spinal stenosis (LSS). Hypertrophied LF tissues and peripheral bloods were obtained from 50 patients with LSS. The expressions of MMP-3,-13 and their polymorphisms were analyzed. No relationship was found between thickness of LFs and MMP-3,-13 genotypes. LF tissues were divided to three groups as grade 1, 2 and 3. Rich elastic fibrils were observed in grade 1. Elastic fibers and elastin/collagen rates decreased in grade 2-3 and 4, and collagen fibers increased and presented a cystic degeneration. MMP-3 immunopositive cells were higher than MMP-13. A correlation between LF thicknesses and MMP-3 was detected. Both MMP-3, -13 were expressed (MMP-3 in higher quantities) in high grade hypertrophied LF. The researchers expect that this paper would provide a better understanding of the pathogenesis of LF hypertrophy and lead to therapeutic alternatives for LSS patients.

INTRODUCTION

Hypertrophy of ligamentum flavum (LF) is one of the most important factors narrowing the lumbar spinal canal. Although hypertrophy of LF is a natural process of aging, genetic, metabolic and biological factors have been blamed lately (Uchida et al. 2011). Recent studies revealed the role of genetic factors in the development of pathologies of LF, which causes canal stenosis, through pedigree, twin studies and human leukocyte antigen haplotype studies (Sakou et al. 2000). Case reports associated with hypertrophy of LF on different populations (Caucasian, Afro-American and Asian populations) were reported (Van-Oostenbrugge et al. 1999; Pascal-Moussellardet al. 2005). However, the strongest relation between the disease process, symptom development and genetic predisposition was reported in the Japanese population (Shiraishi et al. 1995).

Ligamentum flavum is an elastic structure consisting of elastin (80%) and collagen (20%) (Viejo-Fuertes et al. 1998). In hypertrophic LF tissue, elastic fibrils degenerate and decrease while the number of collagen fibrils increases (Schrader et al. 1999; Kosaka et al. 2007). Many molecules, such as matrix metalloproteases (MMPs) (Cui et al. 2011; Oh and Ha 2009; Park et al. 2009), tissue inhibitors of matrix metalloproteases (Park et al. 2005), platelet-derived growth

Author for correspondence:
Betul Eser
Department of Medical Genetics
Faculty of Medicine
Balikesir University,
10145 Balikesir,Turkey
Telephone: +90266 6121010
Fax: +90266 6121294
E-mail: drbetuleser@gmail.com

factor-BB (Zhang et al. 2010), connective tissue growth factor (Zhong et al. 2011), bone morphogenetic protein (Shafaq et al. 2012) and inflammatory cytokines (Sairyo et al. 2007; Lohr et al. 2011) increase during LF hypertrophy.

Matrix metalloproteinases are a family created by more than 20 enzymes acting for degradation of the extracellular matrix (Visse and Nagase 2003). MMPs participate in connective tissue metabolism during normal physiological process. MMP-3 is an enzyme causing degradation of the cartilage and exists in etiology of rheumatoid arthritis and ankylosing spondylitis (Yoshihara et al. 1995; Ye et al. 2007; Zhang et al. 2016) and lumbar disc degeneration (Nemoto et al. 1997; Bachmeier et al. 2009). MMP-13 degrades elastic microfibrils as well as Type I, II and III collagens and acts in the metabolism of the extracellular matrix (Ashworth et al. 1999).

Objective

The study investigated the association of MMP-3,-13 gene polymorphisms and LF hypertrophy, and the correlation of expression of these genes and LF hypertrophy.

MATERIAL AND METHODS

Study Population

The study population consisted of the patients of the department of neurosurgery who had neurological claudication, diagnosed with LSS and waiting to be operated. A total of 50 patients took part in the study. Patients were informed about the study before the operation and their written consent forms were obtained. LF samples were obtained from 50 patients (38 females, 12 males) who had decompressive laminectomy. The LF samples were treated with ten percent formalin and embedded into paraffin blocks for histological and immune histochemical analysis. For polymorphism study, venous blood samples were collected from same patients into the tubes containing ethylenediaminetetra acetic acid and DNA extraction was performed. The DNAs were stored at -20°C until the analysis.

Ligamentum Flavum Thickness Measurement

Thickness of LF was measured by using preoperative magnetic resonance imaging (MRI), which was performed on each of the 50 patients. Maximum LF thickness was drawn by a specialist surgeon through manual cursor technique along facet joint in axial T1 weighted image and measured automatically by PACS system. This procedure was repeated at least three times for each ligament and the average values were recorded as the thickness of the LF. Patients were divided into two groups according to LF thickness of <4 mm LF (n=24) and >4 mm LF (n=26) (Honsawek et al. 2013).

Genetic Analysis

Genomic DNA extraction was performed from a 200µl peripheral blood sample by using GeneJet DNA Purification Kit (Thermo Scientific, San Diego, CA, USA) for each patient. MMP-3 (-1171 5A/6A) (rs 3025058) and MMP-13 (-77 A/G) (rs 2252070) gene polymorphisms were analyzed from the DNAs. PCR amplification was performed by using the LightCycler® Nano Real-Time polymerase chain reaction (PCR) Systems. Real Time PCR was applied according to the following parameters through FastStart DNA Master HybProbe (Roche Diagnostics, Germany) and specific primers at 95°C for 10 minutes, followed by 45 cycles with at 95°C for 10 seconds and at 60°C for 10 seconds and at 72°C for 15 seconds.

Histological Analysis

Subsequent three slices (6-µm thick) from the surgical samples embedded into the paraffin blocks were cut by microtome and stained by Hematoxylin-Eosin, Masson's trichrome and Gomori's aldehyde fuchsin, respectively. Elastin degradation by Hematoxylin-Eosin stain, the grade of fibrosis, calcification and collagen fibrils by Masson's trichrome staining were assessed. Gomori's aldehyde fuchsin staining was used to determine the grade of elastic fibril disorganization. Histological analysis was performed on 10 randomly selected fields of each sample. The average of fibrosis and elastin degradation was used as the final grade. The relevant parameters to be examined were the ratio of elastic/collagen fibrils and the perimeter of the elastic fibers. The semi-quantitative analysis of the MMP-3 and MMP-13 immunopositive cells was also evaluated by using the image-analysis system.

The severity of ligamentum flavum fibrosis was graded according to the guidelines present-

ed by Sairyo et al (2007). Grade 0 indicates normal tissue showing no fibrotic and calcification regions, Grade 1 indicates fibrosis at less than twenty percent of the entire area, Grade 2 indicates between twenty-five percent and fifty percent fibrosis, small amount elastin degradation, Grade 3 indicates between fifty percent and seventy-five percent fibrosis, intermediately elastin degradation and small cystic degeneration and calcification regions, and Grade 4 indicates more than seventy-five percent fibrosis and abundant elastin degradation, large cystic degeneration and calcification regions.

Immunohistochemical Analysis

Streptavidin-biotin-peroxidase method was used to determine the location of MMP-3 and MMP-13 proteins in the LF tissue. Subsequent two slices (6-µm thick) were taken from the paraffin-embedded tissue. Slices were deparaffinized in xylene, then they were rehydrated in a graded series of alcohol solutions. Tissue samples were treated with 0.3 percent H_2O_2 to inhibit the endogenous peroxidase activity. The samples were incubated with five percent normal bovine serum to reduce nonspecific staining. Mouse monoclonal antibody for MMP-3 (Merck Millipore, USA) and purified rabbit polyclonal antibody for MMP-13 (Novus Biologicals, USA) were used at an optimal dilution recommended by the manufacturing company. Results were evaluated by using the contrast staining method through Mayer's hematoxylin. Antibody binding rate was analyzed using a high-power light microscope (Olympus Corporation, Japan). The intensity of staining with antibodies was subjectively scored according to Zigorius et al. (2011) and Simsek et al. (2012) as follows: A = Non-reactivity, B = Weak, individualized cell reactivity in more than or equal to twenty-five percent of fibroblast and fibrocyte, C = Mild to moderate reactivity in less than or equal to fifty percent of fibroblast and fibrocyte, D = Strong reactivity in less than or equal to seventy-five percent of fibroblast and fibrocyte, E = Very strong reactivity in more than seventy-five percent of fibroblast and fibrocyte. The average staining intensity was calculated using the formula:

$$[(A \times 1) + (B \times 2) + (C \times 3) + (D \times 4) + (E \times 5)]/(A + B + C + D + E)$$

This was reported, as follows: absent (–) = 0.00, rarely present (+) = 0.01–1.00, present in small amount (++) = 1.01–2.00, intermediately present (+++) = 2.01–3.00, abundantly present (++++) = 3.01–4.00.

Statistical Analysis

The SPSS 20.0 software program was used for statistical analysis. All data was assessed within the ninety-five percent confidence interval. Comparison MMP-3 and MMP-13 gene polymorphisms of the groups created according to LF thickness with histopathological results was performed by chi-square test. ANOVA and TUKEY HSD test was used to compare immunohistochemical results with LF thicknesses. The correlation between MMP-3 and MMP-13 expression and LF thickness was analyzed through Spearman's correlation coefficient. Significance level was accepted as P value less than 0.05.

RESULTS

Genetic Analysis

As a result of real time PCR analysis, twelve percent of the patients had 5A/5A, forty-two percent had 5A/6A and thirty-six percent had 6A/6A genotype in terms of MMP-3 gene polymorphism. The allele frequencies of MMP-3 and MMP-13 gene polymorphisms were determined as thirty-three percent (5A) and sixty-seven percent (6A) for MMP-3, sixty-one percent (A) and thirty-nine percent (G) for MMP-13. In terms of MMP-13 gene polymorphism, thirty-eight percent had AA, forty-six percent had AG and sixteen percent had the GG genotype. Genotypes of MMP-3 and MMP-13 gene polymorphism of the 50 patients enrolled into the study and the groups created according to LF thicknesses were compared. No association was found between LF thicknesses and genotypes for both genes (p=0.155 for MMP-3 and p=0.295 for MMP-13) (Table 1).

Table 1: The association between MMP-3 and MMP-13 gene polymorphisms and LF thicknesses

Gene	*Genotypes*	*<4 mm (n=24)*	*>4 mm (n=26)*	*P value*
MMP-3	5A/5A	5	1	0.155
	5A/6A	10	11	
	6A/6A	9	14	
MMP-13	AA	11	8	0.295
	AG	11	12	
	GG	2	6	

Histopathological and Immunohistochemical Analysis

In the histopathological examination, rich elastic fibrils, located parallel to one another, were observed in grade 1 patients whereas elastic fibrils were observed to decrease (Figs. 1a-1b), collagen fibrils increased and elastin/collagen rate decreased in grade 2-3 and 4 patients (Figs. 1c-1d). Furthermore, disorganization and focal defects in the elastic fibrils, proliferation in collagen fibrils, and small and large cystic de-

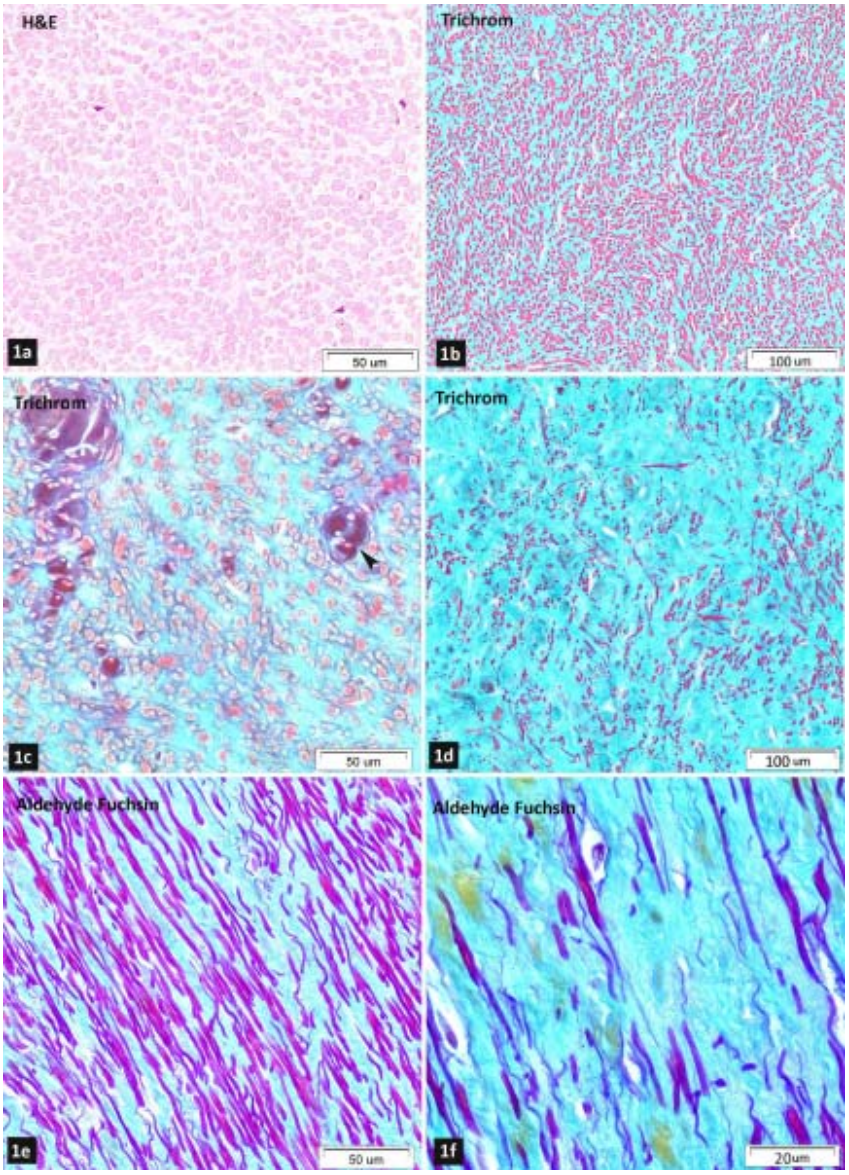

Fig. 1. Ligamentum flavum elastin degradation. 1a - 1b: Grade 1, parallel organized elastin fibers 1c: Grade 3, disorganized elastic fibers, collagen proliferation, and cystic degeneration (arrow head), 1d: Grade 4, the elastic fibers were fragmented, low volumes and uneven diameters. 1e: Grade 3 1f: Grade 4, most of the area was stained a green color, indicating the presence of fibrosis.

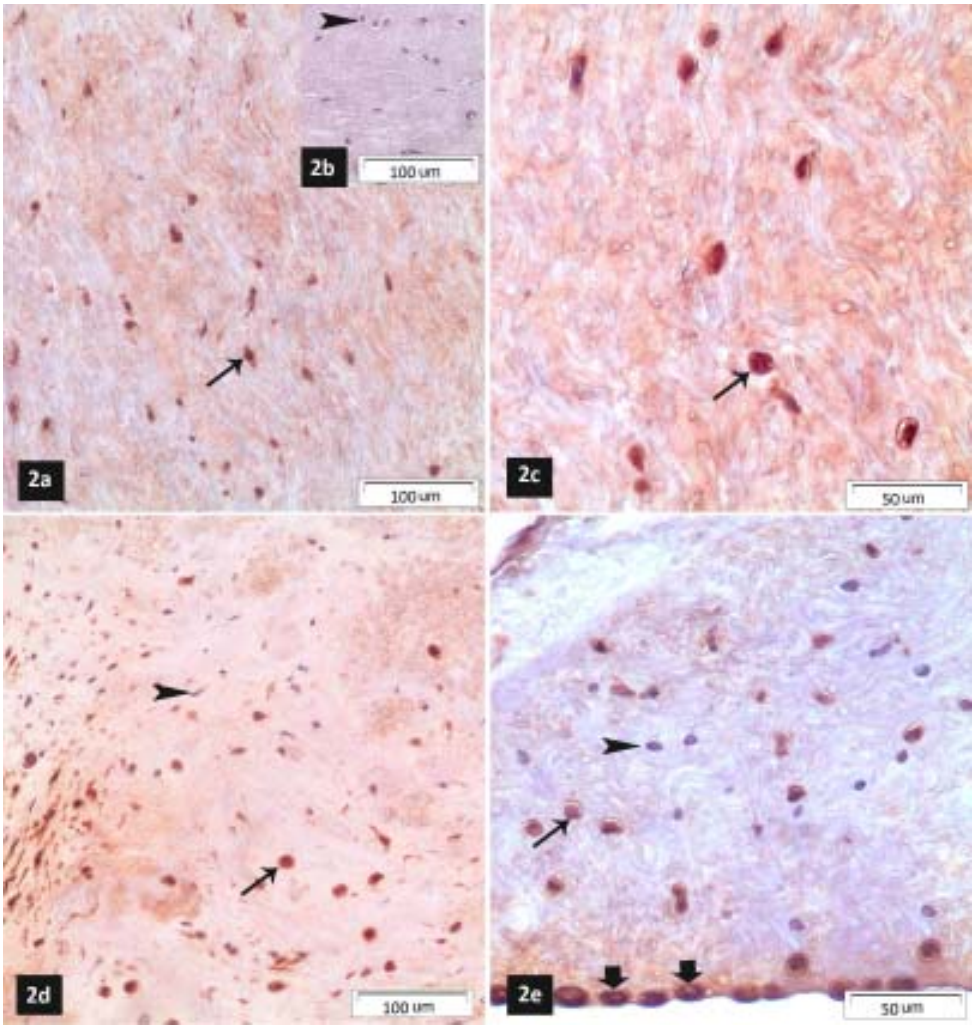

Fig. 2. MMP-3 photomicrographs showing immunohistochemical expression of in ligamentum flavum. Arrows: MMP -3 immunopositive cells, arrow heads: MMP-3 immunonegative cells, bold arrows: MMP -3 immunopositive chondrocytes. 2a- 2c: Grade 3, 2b: Negative control sample, 2d and 2e: Fibrocartilage areas, proliferated immunopositive fibroblasts and chondroblasts had increased in the area of the ligaments that connect bone, MMP-3 positive cells were abundantly on the ligamentum flavum fibroblasts of the patients with spinal stenosis grade -3 and -4. Streptavidin biotin peroxidase staining.

generations were detected in grade 3 and grade 4 patients. Masson's trichrome and Gomori's aldehyde fuchsin stains showed that a large area was stained pink and purple-violet, indicating in grade 1 patients, respectively. However, a large area in the grade 3 and 4 patients was stained green, indicating the presence of massive fibrosis and collagen proliferation (Figs. 1c-1f).

Immunopositive staining in cytoplasms of the fibroblast/fibrocysts in LF tissues with MMP-3 (Figs. 2a-2c-2e) and MMP-13 was determined (Figs. 3a-3b). When MMP-3 and MMP-13 immunopositive cell rates were compared, MMP-3 immunopositive cell density was observed in higher quantities than MMP-13 (Table 2).

Furthermore, it was also detected that chondrogenic cells increased in the areas where the

Table 2: Evaluations and semi-quantitatively scoring of histopathological changes, and MMP-3 and MMP-13 immunopositive cells in LF of the patients with lumbar spinal stenosis

Groups	*Histopathological changes*	*Ratio of elastic/collagen fibrils (%)*	*MMP-3 immunopositivity*	*MMP-13 immunopositivity*
Grade 1 (n=7)	+	82/18[a]	++	+
Grade 2 (n=21)	++	56/44[b]	+++	++
Grade 3 (n=16)	+++	38/62[c]	++++	++
Grade 4 (n=6)	++++	21/79[d]	++++	+++

- = absent, + = rarely present, ++ = present of small amount, +++ = intermediately present, ++++ = abundantly present. [a,b,c,d]: $P < 0.05$ (according to the grade 1)

ligament adheres onto the bones, the stains was denser and a fibro-cartilaginous tissue also appeared in these areas (Figs. 2d-2e). When LF thicknesses measured by preoperative MRI and histopathological groups were compared, radiological and histopathological evaluations were consistent (r=0.831, P<0.001) (Table 3). Moreover, a stronger correlation was detected between LF thicknesses and immunohistochemical expression levels in MMP-3 than MMP-13 (r=0.817, P<0.001 for MMP-3 and r= 0.522, P<0.001 for MMP13).

Table 3: The association between histopathological groups and LF thicknesses

Histopathological groups	*<4 mm (n=24)*	*>4 mm (n=26)*	*p value*
Grade 1 (n=7)	7	0	<0.001
Grade 2 (n=21)	16	5	
Grade 3 (n=16)	1	15	
Grade 4 (n=6)	0	6	

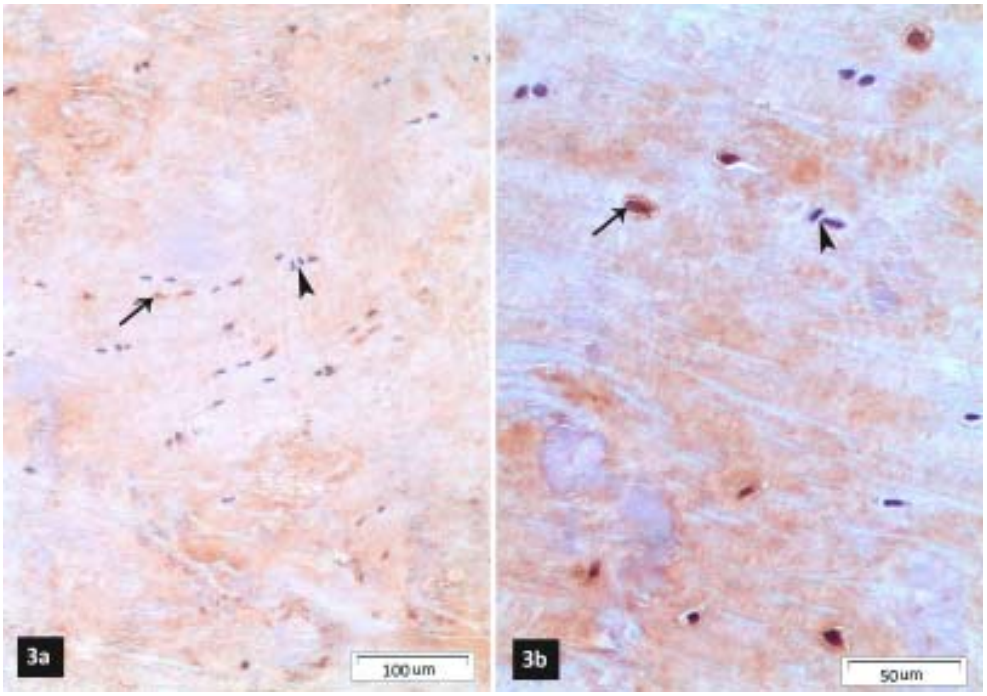

Fig. 3. MMP -13 photomicrographs showing immunohistochemical expression of in ligamentum flavum. Arrows: MMP -13 immunopositive cells, in the LF of the LSS patients were a significantly lower expression of MMP -13, arrow heads: MMP-13 immunonegative cells. Streptavidin biotin peroxidase staininig.

DISCUSSION

LSS is a process which progresses with LF hypertrophy and is detected in elder patients in particular. In the etiology of LF hypertrophy, aging as well as degenerative changes, mechanical stress, genetic and biological factors are blamed (Fukuyama et al. 1995; Schrader et al. 1999; Uchida et al. 2011). LF includes elastin fibrils in high concentration, which allows contractions during flexion and extension (Nihei et al. 2003). This elastic structure is replaced with collagen in LF hypertrophy and causes LSS. For this study, the researchers investigated the association of MMP-3 and MMP-13 gene polymorphisms and LF hypertrophy and the correlation of expression of these genes and LF hypertrophy.

There exists no genetic study conducted previously on effects of both MMP-3 and MMP-13 gene polymorphisms on LF hypertrophy in the literature. However, there are some studies that researched effects of MMP-3 and MMP-13 genes on the etiology of intervertebral disc degeneration. Takahashi et al (2001) found a relation between MMP-3 5A allele and disc modifications of elder patients in their paper on MMP-3 gene polymorphism and intervertebral disc hernia. A paper conducted on the Chinese population showed that MMP-3 5A allele increases the predisposition to lumbar disc degeneration due to occupational factors (Yuan et al. 2010). It was reported in the paper investigating the association between rheumatoid arthritis and MMP-3 and MMP-13 gene polymorphisms that both polymorphisms might be useful in determining the functional status of rheumatoid arthritis (Ye et al. 2007). Rajasekaran et al. (2013) reported a strong relation between end plate damage and MMP-13 gene polymorphism in degenerative disc disease. No relationship was detected between MMP-3, MMP-13 gene polymorphisms and LF hypertrophy in the present study. The researchers believe that this outcome may be associated with a small sample count. Thus, the current study of these polymorphisms should be repeated on different populations and in larger patient series.

The LF tissue in the lumbar area consists of elastic fibrils comprising of elastin and fibrillin components (Nihei et al. 2003). MMP-13 degrades fibrillin and acts in remodeling of the connective tissue (Oh and Ha 2009). Hypertrophy, fibrosis and loss of elastin were detected in the studies carried out on LF tissue of the patients with spinal stenosis. Sairyo et al. (2007) reported a positive association between LF thickness and elasticity loss and fibrosis in their paper conducted on LF samples of the patients with spinal stenosis and discogenic pain. In the present study, regularly located and rich elastic fibrils were observed in the low grade patients whereas elastic fibrils disorganized and disappeared in the high grade patients. MMP-13 degrades collagen and elastic fibrils and provides remodeling of the extracellular matrix (Visse and Nagase 2003; Oh and Ha 2009). However, degradation in elastic fibrils as well as proliferation in the collagen fibers was detected in the present study. This outcome may seem conflicting when it was considered that MMP-13 degrades collagen. However, in the detailed examination of collagen fibers of LF, small and large cystic degenerations were detected. In this case, it may be claimed that collagen proliferated in the hypertrophic LF tissue is in abnormal collagen fiber formation due to collagen-degradation characteristics of MMP-13 (Park et al. 2009).

MMP-3 is an enzyme acting in degradation and modification of the extracellular matrix. It is reported that MMP-3 released by LF fibroblasts in the patients with LSS may cause fibrosis and elastin degradation rather than collagen degradation (Park et al. 2009). Weber et al. (2016) did not determine significantly elevated serum MMP-3 level in patients with low back pain including spinal stenosis. No significant relationship was reported between MMP-3 and LF hypertrophy and more MMP-13 expression than MMP-3 in the paper of Park et al. (2009). It was observed in the present study that immunopositive cell density of MMP-3 is higher in quantity than MMP-13 in the LF samples obtained from the patients with LSS. Above all, excessive MMP-3 immunopositive fibroblast and chondroblast cell density was detected in some LF areas where elastic fibers were limited or absent. Increased MMP-3 activity is considered to cause a fibro-cartilaginous tissue and loss of elasticity by degrading the extracellular matrix components, especially in grade 3 and grade 4 patients.

CONCLUSION

The researchers could not detect any association between LF hypertrophy and the poly-

morphisms of MMP-3 and MMP-13 genes. On the other hand, the researchers detected immunopositive cells of both MMP-3 and MMP-13 in the fibroblasts of LF tissue. Both MMP-3 and MMP-13 were expressed (MMP-3 in higher quantities) in high grade hypertrophied LF tissue in particular.

RECOMMENDATIONS

This study needs to be repeated using normal (thin) LF tissues. The expressions of MMP-3 and MMP-13 should be compared in thin and thick LF tissues.

LIMITATION OF THE STUDY

The ethical committee did not permit the collection of normal LF. Thus, the researchers could only obtain LF tissues from the patients with LSS, not from healthy individuals. The researchers decided that patients were divided into two groups according to LF thickness measurements and these groups were compared with each other. Although these groups are not definitely ideal, the researchers believed that this arrangement was convenient to investigate the hypothesis of the present study.

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