

Transcript levels of major MMPs and ADAMTS-4 in relation to the clinicopathological profile of patients with lumbar disc herniation

Alexandros Tsarouhas · Giannoula Soufla ·
Pavlos Katonis · Dritan Pasku · Antonis Vakis ·
Demetrios A. Spandidos

Received: 25 April 2010 / Revised: 30 July 2010 / Accepted: 5 September 2010 / Published online: 22 September 2010
© Springer-Verlag 2010

Abstract The involvement of matrix metalloproteinases (MMPs) in both the pathogenesis of intervertebral disc (ID) herniation and the spontaneous regression of herniated ID fragments remains only partially elucidated. The purpose of the present study was to simultaneously examine the transcript levels of a large number of MMPs (-1, -3, -8, -9, -13 and -14) and ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs) and to investigate their correlation with the clinicopathologic profile of patients suffering from symptomatic lumbar ID herniation. mRNA expression levels were determined by means of the real-time polymerase chain reaction in 63 herniated and 10 control ID specimens. Our results showed multiple positive correlations among all MMPs and ADAMTS-4 mRNA in herniated samples, indicating their possible synergistic effect in ID herniation. MMP-9 and -13 mRNA levels were significantly elevated in patients with chronic pain, presumably as a consequence of neovascularization and chronic inflammation. Smoking habits were found to have

a negative dose-dependent effect on the transcript levels of MMP-3 and MMP-13 and a positive correlation with pain intensity, suggesting an unfavorable role for smoking in the regression process of herniated disc fragments. Our findings provide evidence of the molecular portrait of MMPs and ADAMTS-4 in lumbar ID herniation, as well as of its association with the clinicopathological profile of the patients included in this study, reinforcing the hypothesis of MMPs involvement in the natural history of ID herniation. However, further studies are necessary to elucidate the exact role of MMPs in the resorption process of herniated lumbar discs.

Keywords MMP · ADAMTS · Disc herniation · mRNA · Real-time PCR

Introduction

Scientific knowledge on the biology of intervertebral disc (ID) degeneration has improved considerably over the past decades. Genetic inheritance, aging, nutritional compromise and repetitive loading have been found to promote the disorganization of normal extracellular matrix turnover along with the degradation of its major structural proteins [1, 18]. Consequently, the structural integrity and hydration levels of the disc are compromised, leading to loss of the disc's functional properties.

Intervertebral disc herniation is the most prevalent clinical manifestation of this wear process. The type of herniation (protrusion, extrusion or sequestration) together with age and duration of symptoms have been correlated to different degrees of molecular changes within the disc cells and its extracellular matrix [16]. In addition, serial magnetic resonance imaging (MRI) studies have documented

A. Tsarouhas and G. Soufla have contributed equally to this study.

A. Tsarouhas · G. Soufla · D. Pasku · D. A. Spandidos (✉)
Laboratory of Virology, Medical School, University of Crete,
P.O. Box 1527, 710 03 Crete, Heraklion, Greece
e-mail: spandidos@spandidos.gr

G. Soufla
Department of Coagulation and Haemostasis,
Onassis Cardiac Surgery Center, Athens, Greece

P. Katonis · D. Pasku
Department of Orthopedics and Traumatology,
University Hospital of Heraklion, Crete, Greece

A. Vakis
Department of Neurosurgery, University Hospital of Heraklion,
Crete, Greece

the spontaneous resorption of non-operatively treated herniated discs [17, 31]. Contact between the herniated fragment of the ID and the epidural space was thought to trigger an autoimmune response with inflammatory cell infiltration and in situ neovascularization, leading to the progressive resorption of the herniated fragment and regression of symptoms. Mounting evidence has shown that growth factors, matrix metalloproteinases (MMPs) and inflammatory mediators, such as cytokines, play a key role in ID homeostasis and pathology [9, 15]. However, the role of such molecules in the pathogenesis of ID herniation as well as in the spontaneous regression of herniated disc fragments remains only partially elucidated.

Matrix metalloproteinases are a family of proteolytic enzymes that are known to be implicated in both the normal extracellular matrix turnover and degradation [28]. Currently, 24 members of the MMP family have been identified with a wide array of potential substrates. The ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) group of enzymes is a subfamily of metalloproteinases with the ability to degrade aggrecan acting at a different cleavage site than MMPs [25]. Among several members of the ADAMTS family, ADAMTS-4 plays a central role in cartilage degradation.

The involvement of several MMPs and ADAMTS in the pathogenesis and regression of ID herniation has been studied mainly by means of biochemistry and immunohistochemistry [26, 29]. However, few studies have investigated the mRNA transcription levels of the above enzymes, particularly in human ID herniation samples. A quantitative molecular analysis of the mRNA expression levels of the MMPs will provide insights into the molecular mechanism that regulates the expression of MMPs in disc herniation. In addition, the majority of the presented studies examined a limited number of MMPs in relatively small and heterogenic groups of ID specimens. Therefore, the present study aimed to concomitantly examine the transcript levels of a large number of MMPs (-1, -3, -8, -9, -13 and -14) and ADAMTS-4 in herniated and control disc specimens to elucidate their mRNA expression profile, as well as to investigate their correlation with the clinicopathological profile of patients suffering from symptomatic lumbar ID herniation.

Materials and methods

Tissue samples

Intervertebral disc tissue samples were collected from a total of 63 patients who underwent posterior open discectomy for lumbar ID herniation in our department during a recruitment period of 1 year (2008). The patients reported

symptoms of radiculopathy prior to surgery. Patients suffering from spinal stenosis, spondylolisthesis, scoliosis or systemic inflammatory disorders were excluded from the study. After excision, an experienced pathologist, in addition to the clinicians present, examined the tissue samples and identified the pulposus sections to limit the percentage of end-plates or annulus. Samples were then immediately refrigerated at -80°C until RNA extraction. Ten cadaveric ID tissue samples were also obtained within 12 h of the patient succumbing to the disease. The samples were used as controls of normal MMP mRNA expression. Donors were aged between 20 and 46 years and were known to have a negative history of low back pain, spinal trauma or systemic inflammatory disease. All the patients or relatives signed an informed consent form approved by the university ethics committee to participate in the study. The study design was in accordance with the Declaration of Helsinki guidelines.

Prior to surgery, an MRI examination was routinely performed to be used for ID herniation classification. To determine the type of herniation, recommendations of the combined task force from the North American Spine Society, the American Society of Spine Radiology and the American Society of Neuroradiology based on the imaging characterization of Milette were used [5]. Preoperative pain intensity was measured subjectively using the visual analog scale score (VAS). Patient employment was defined as heavy, provided it included manual labor with repetitive loading of the spine. A summary of patient and control group data are shown in Table 1a, b. There was no significant difference in terms of age, gender, body mass index, employment and smoking habits between the control and ID herniation groups (min $P > 0.24$).

RNA extraction, reverse transcription and real-time PCR

Total RNA was isolated from fresh tissue and homogenized with a power homogenizer using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration and purity were determined on a UV spectrophotometer (Hitachi Instruments Inc., USA) by absorbance measurements (260-nm absorbance and 260/280-nm absorbance ratio). RNA integrity was examined by 1% agarose gel electrophoresis and ethidium bromide staining.

Reverse transcription reactions for the preparation of first-strand cDNA from 2 μg of total RNA were performed using the AffinityScriptTM Multi Temperature cDNA synthesis kit (Stratagene, La Jolla, CA, USA). Random hexamers were used as amplification primers. Real-time PCR reactions were performed using the Mx3000P real-time PCR system (Stratagene, La Jolla, CA, USA) with SYBR[®] Green I Master Mix (Stratagene). Data were

Table 1 Patient clinical characteristics

Characteristic	No. of patients
A. ID herniation group	
Age (years)	
Mean (range)	47.3 (27–68)
20–39	27
40–59	23
>60	12
Sex	
Male/female	33/30
Body mass index (BMI)	
Mean (range)	27.2 (21.07–33.05)
Level of ID herniation	
L3–L4	11
L4–L5	24
L5–S1	28
Type of herniation	
Protrusion	23
Extrusion	33
Sequestration	7
Duration of symptoms	
<3 months	15
3–12 months	20
>12 months	28
Pain intensity (VAS)	
0–5	18
5–7	14
8–10	31
Employment	
Heavy/light	36/27
Smoking habits	
Smokers/non-smokers	26/37
<10 cigarettes per day	6
10–20 cigarettes per day	8
>20 cigarettes per day	12
B. Control group	
Age (years)	
Mean (range)	36.8 (20–46)
Sex	
Male/female	6/4
Body mass index (BMI)	
Mean (range)	27.0 (21.9–33.0)
Employment	
Heavy/light	7/3
Smoking habits	
Smokers/non-smokers	4/6

collected and analyzed using the Mx3000P real-time PCR software version 2.00, Build 215 Schema 60 (Stratagene). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and

beta-actin were used as internal controls and their geometric mean in each sample was employed to normalize the mRNA expression levels of the MMPs. The primer pair sequences used are shown in Table 2. PCR products were analyzed by electrophoresis on 2% agarose gels, stained with ethidium bromide and photographed on a UV light transilluminator. MMPs or ADAMTS-4 transcription levels were calculated using the formula: normalized sample or control = $(1 + E_{\text{MMP}})^{-\Delta\text{CtMMP}} / (1 + E_{\text{GAPDH}})^{-\Delta\text{CtGAPDH}}$. Procedures were repeated with a cDNA template synthesized three times from the same RNA. The mRNA levels of each sample for every gene tested represent the mean value of data acquired from three independent RT-PCR experiments. Reproducibility of the real-time PCR results for the same samples was 99%.

Statistical analysis

The one-sample Kolmogorov–Smirnov test was used to assess the normality of the distribution of the mRNA expression values for the genes studied. Accordingly, the mRNA expression of the MMPs/ADAMTS-4 in the control and herniated groups, as well as in groups of different clinicopathological and MRI features, were compared using non-parametric procedures (Kruskal–Wallis and Mann–Whitney tests). The Spearman rank correlation test (non-parametric) was employed to examine pairwise MMPs/ADAMTS-4 mRNA correlations in control and herniated discs. Probability values or differences less than 0.05 were considered to be significant. Statistical calculations were performed using SPSS software, version 11.

Results

MMPs and ADAMTS-4 mRNA levels in normal and herniated discs

Transcript levels of the MMPs/ADAMTS-4 examined in the present study did not differ significantly between normal and herniated IDs (Table 3). The mRNA of the MMPs/ADAMTS-4 examined was not detectable in all control or herniated ID samples. Specifically, no expression of MMP-1 mRNA was noted in either control or herniated samples. Expression of MMP-3, -13 and -14 mRNA ranged between 40 and 90% in the control and 63 and 66% in the herniated IDs. Finally, MMP-8 and ADAMTS-4 mRNA was expressed in 40% of the control group and only in 19 and 34% of the herniated discs, respectively (Table 4). Notably, 26% of the herniated ID specimens exhibited a complete absence of mRNA expression for the MMPs and ADAMTS-4 examined. However, all of these samples expressed both housekeeping genes, beta-actin and

Table 2 Primer sequences used for quantitative real-time RT-PCR

Gene	Primer pair sequence (5'–3')	Annealing temperature (°C)	Product size (bp)
MMP-1	GGCCACAAACCCCAAAAG ATCTCTGTCTGGCAAATTCGTAAGC	58	204
MMP-3	GATGCCCACTTTGATGATGATGAA AGTGTTGGCTGAGTGAAAGAGACC	57	94
MMP-8	TGGGGCTCGCTCACTCCTC ATCAAATGTCAAACCTGGGGTCAC	55	192
MMP-9	TGCCCGGACCAAGGATACAGTTT AGGCCGTGGCTCAGGTTTCAGG	60	198
MMP-13	CCCCAACCTAAACATCCAAAAAC TTAAAAACAGCTCCGCATCAACCT	58	152
MMP-14	GCCGGGGCATCCAGCAACTTTA TCCTCACCCGCCAGAACCAG	59	210
ADAMTS-4	TGTGCGGAGGGGACGGTTCTGGT ACATCTGTGGGGGAGGGCATCA	62	223
β -ACTIN	CGGCATCGTCACCAACTG GGCACACGCAGCTCATTG	60	70
GAPDH	GGAAGGTGAAGGTCGGAGTCA GTCATTGATGGCAACAATATCCACT	60	101

Table 3 Quantitative MMP mRNA expression in normal and herniated lumbar discs

	Normal discs (<i>N</i> = 10)	Herniated discs (<i>N</i> = 63)	<i>P</i> value*
MMP-3	3.9 ± 3.5	74.4 ± 63.5	NS
MMP-8	9.44 ± 9.43	3.35 ± 2.36	NS
MMP-9	1.68 ± 0.90	510 ± 315	NS
MMP-13	1.80 ± 1.50	13.7 ± 9.1	NS
MMP-14	0.66 ± 0.29	170 ± 118	NS

Data are presented as mean ± SEM (standard error of the mean)

* Mann–Whitney test

GAPDH, demonstrating that these results were indicative of the samples' molecular profile and not due to low cell numbers or bad RNA quality.

MMPs and ADAMTS-4 mRNA co-expression analysis pairwise

In the group of control discs, only a limited number of correlations were observed among the genes studied. Specifically, MMP-9 transcript levels were found to be positively correlated with those of MMP-13 and -14 ($P < 0.001$ and $P = 0.001$, respectively, Pearson correlation). MMP-13 and -14 mRNA were also found to be co-expressed in the normal discs ($P < 0.001$, Pearson correlation). However, in the group of herniated discs,

Table 4 Detected mRNA expression of MMPs and ADAMTS-4 in normal and herniated lumbar discs

Expressed gene	Normal discs % (<i>N</i>)	Herniated discs % (<i>N</i>)	<i>P</i> value*
MMP-1	–	–	–
MMP-3	90 (9/10)	63 (39/62)	NS
MMP-8	40 (4/10)	19 (12/62)	NS
MMP-9	80 (8/10)	65 (40/62)	NS
MMP-13	60 (6/10)	61 (38/62)	NS
MMP-14	80 (8/10)	66 (41/62)	NS
ADAMTS-4	40 (4/10)	34 (21/62)	NS

NS not significant

* χ^2 test. $P < 0.05$ is considered statistically significant

multiple positive correlations were observed among almost all of the MMP transcript levels studied (Spearman correlation; Table 5a, b).

The above-mentioned analysis was performed taking into consideration the mRNA expression status of the MMPs and ADAMTS-4 (mRNA expression or not) in the groups of control and herniated ID specimens. Our results showed that the MMP-8 expression status was significantly correlated to MMP-13 in the control group ($P = 0.035$, Pearson). Multiple positive correlations were found in the herniated group concerning the mRNA expression status of almost all of the MMPs and ADAMTS-4 (Table 6a, b).

Table 5 Spearman correlation tables demonstrating the co-expression profile of MMPs in the groups of A. normal and B. herniated lumbar discs

			MMP-3	MMP-8	MMP-9	MMP-13	MMP-14	
A. Control specimens								
Statistically significant correlations are highlighted in bold MMP-1 mRNA is not expressed in the control specimens CC correlation coefficient corresponding to Spearman correlation ρ values * Correlation is significant at the 0.05 level (two tailed) ** Correlation is significant at the 0.01 level (two tailed) ^a Spearman correlation ρ values ^b Pearson correlation values	MMP-3	CC	1.000					
		P						
	MMP-8	CC	-0.027 ^a	1.000				
		P	0.940					
	MMP-9	CC	-0.267 ^a	0.288 ^a	1.000			
		P	0.455	0.420				
	MMP-13	CC	-0.019 ^a	0.557 ^a	0.882 ^{b,**}	1.000		
		P	0.959	0.095	0.001			
	MMP-14	CC	-0.103 ^a	0.418 ^a	0.910 ^{b,**}	0.913 ^{b,**}	1.000	
		P	0.776	0.230	<0.001	<0.001		
	B. Herniated lumbar discs							
		MMP-3	CC	1.000				
			P					
		MMP-8	CC	0.380**	1.000			
		P	0.002					
	MMP-9	CC	0.741**	0.376**	1.000			
		P	<0.001	0.003				
	MMP-13	CC	0.672**	0.311*	0.784**	1.000		
		P	<0.001	0.014	<0.001			
	MMP-14	CC	0.604**	0.182	0.694**	0.723**	1.000	
		P	<0.001	0.158	<0.001	<0.001		

Correlation of transcript levels with clinicopathological features

Age and gender

Patients >60 years were found to have significantly higher MMP-8 transcript levels than those between 40 and 60 years old ($P = 0.005$, Mann–Whitney test). Gender did not significantly affect the transcript levels of any of the MMPs or ADAMTS-4 investigated.

Body mass index

Our data did not show any significant correlation between BMI and mRNA levels of the MMPs/ADAMTS-4 included in the present study.

Duration of symptoms

The duration of patient symptoms appeared to significantly affect the transcript levels of MMP-9 and -13 ($P = 0.017$ and $P = 0.037$, respectively; Kruskal–Wallis test). In detail, patients suffering from radicular pain for 3–12 months exhibited higher MMP-9 and -13 mRNA levels, compared to those in pain for <3 months ($P = 0.008$ and $P = 0.020$, respectively, Mann–Whitney

test; Fig. 1). MMP-9 transcript levels were also significantly elevated in patients with chronic pain (>12 months) compared to those in pain for <3 months ($P = 0.016$, Mann–Whitney test).

Pain intensity

High pain intensity (VAS between 8 and 10) significantly correlated with elevated MMP-9 mRNA levels, while a trend was observed in the same patient group for higher MMP-8 and -13 mRNA levels compared to discs excised from patients with low pain (VAS < 5) ($P = 0.024$, $P = 0.061$ and $P = 0.063$, respectively; Mann–Whitney test).

Level and type of ID herniation

The level or type of disc herniation (protrusion, extrusion or sequestration) as assessed by MRI did not significantly affect the transcript levels of the MMPs examined in the present study, with the exception of MMP-9. Specifically, sequestered discs exhibited significantly lower MMP-9 mRNA levels compared to protruding discs ($P = 0.035$; Mann–Whitney test). The ADAMTS-4 mRNA expression status was significantly associated with the ID herniation type ($P = 0.032$).

Table 6 Correlation tables demonstrating the pairwise co-expression profile of MMPs based on their expression status (expression or not) in the groups of C. normal and D. herniated lumbar discs

		MMP-3	MMP-8	MMP-9	MMP-13	MMP-14	ADAMTS-4
A. Control specimens							
MMP-3	CC	1.000					
	P						
MMP-8	CC	0.272 ^a	1.000				
	P	0.447					
MMP-9	CC	-0.167 ^a	0.408 ^a	1.000			
	P	0.645	0.242				
MMP-13	CC	-0.272 ^a	0.667 ^{b,*}	0.612 ^b	1.000		
	P	0.447	0.035	0.060			
MMP-14	CC	-0.167 ^a	0.408 ^a	0.375 ^a	0.612 ^a	1.000	
	P	0.645	0.242	0.286	0.060		
ADAMTS-4	CC	0.272 ^a	-0.250 ^b	-0.102 ^b	-0.167 ^b	-0.102 ^b	1.000
	P	0.447	0.486	0.779	0.645	0.779	0.300
B. Herniated lumbar discs							
MMP-3	CC	1.000					
	P						
MMP-8	CC	0.376**	1.000				
	P	0.003					
MMP-9	CC	0.756**	0.363**	1.000			
	P	0.000	0.004				
MMP-13	CC	0.654**	0.376**	0.687**	1.000		
	P	0.000	0.003	0.000			
MMP-14	CC	0.720**	0.720**	0.751**	0.791**	1.000	
	P	0.000	0.000	0.000	0.000		
ADAMTS-4	CC	0.290*	0.290*	0.409**	0.430**	0.317*	1.000
	P	0.022	0.022	0.001	0.000	0.012	

Statistically significant correlations are highlighted in bold

CC correlation coefficient corresponding to Spearman correlation *rho* values

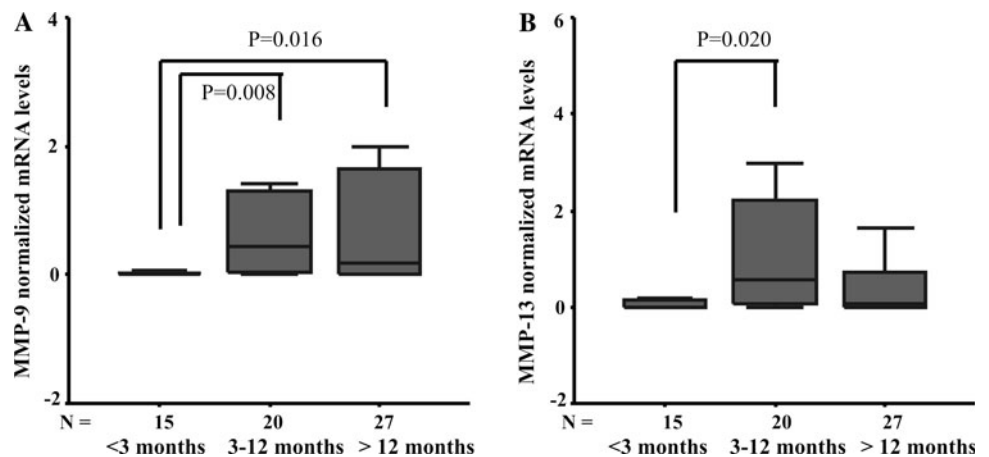
* Correlation is significant at the 0.05 level (two tailed)

** Correlation is significant at the 0.01 level (two tailed)

^a Spearman correlation *rho* values

^b Pearson correlation values

Fig. 1 Normalized transcript levels of **a** MMP-9 and **b** MMP-13 in herniated disc specimens with respect to the duration of patient symptoms ($P = 0.017$ and $P = 0.037$, respectively; Kruskal–Wallis test)



Employment

Patient employment, either light ($n = 27$) or heavy physical work ($n = 36$), did not correlate with MMPs/ADAMTS-4 transcript levels.

Smoking

Tobacco use was associated with lower MMP-3 mRNA levels in herniated discs ($P = 0.048$; Mann–Whitney test). The frequency of MMP-3 mRNA expression was significantly higher in non-smoking patients ($P = 0.048$; χ^2 test). Furthermore, the amount of cigarette smoking was found to affect MMP-3 and -13 transcript levels. Specifically, discs removed from patients smoking more than 20 cigarettes per day exhibited significantly lower MMP-3 and -13 mRNA levels than non-smoking patients ($P = 0.034$ and $P = 0.041$, respectively, Mann–Whitney test; Fig. 2).

Our data also revealed a significant association between smoking habits, duration of patient pain and pain intensity. Specifically, smoking was found to be negatively correlated to the duration of symptoms and positively correlated to the intensity of pain ($P = 0.001$ and $P = 0.01$,

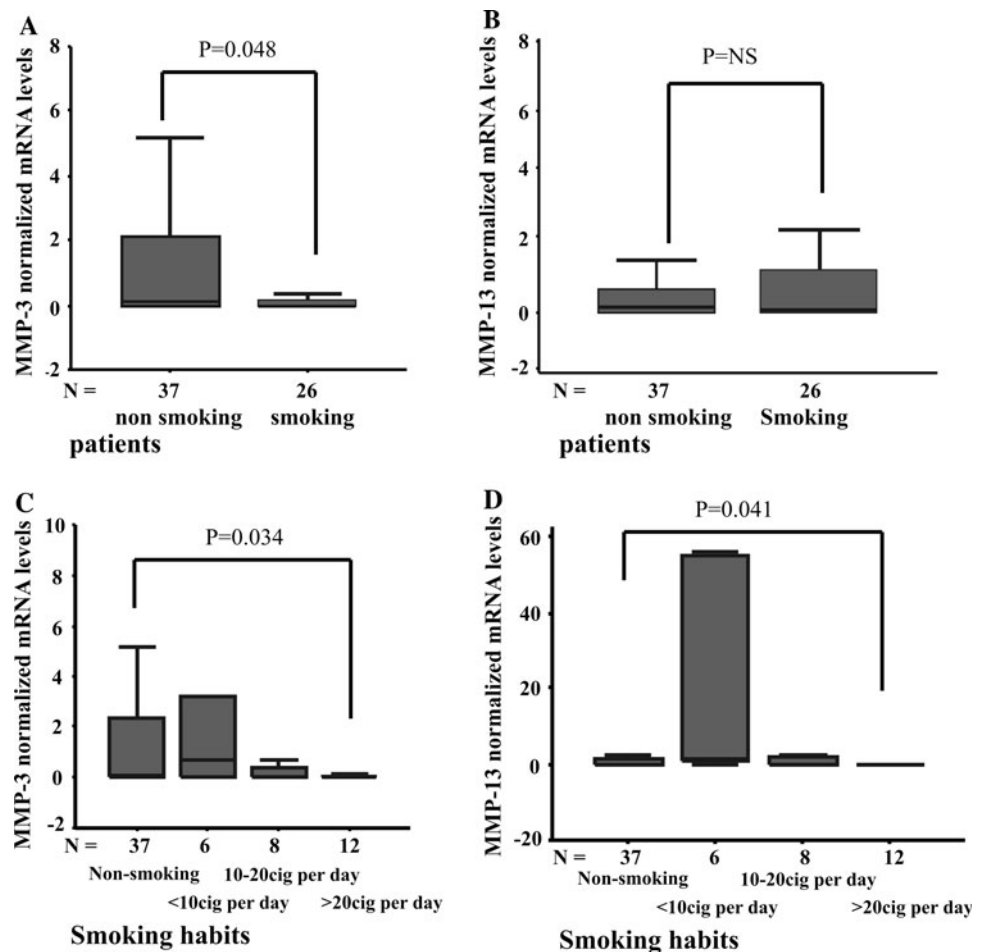
respectively; Spearman correlation). Furthermore, the duration of symptoms was negatively associated with pain intensity ($P = 0.01$; Spearman correlation).

Discussion

The present study investigated the mRNA expression profile of MMPs in a homogeneous, yet comparatively large, group of surgically resected lumbar-herniated IDs and in a control group of cadaveric specimens. Specifically, the transcript levels of several MMPs and ADAMTS-4 were examined by means of quantitative real-time PCR and correlated with the clinicopathological profile of the patients. Members of the major subgroups of metalloproteinases were included in the analysis, e.g., the *collagenases* (-1, -8, -13), the *gelatinases* (-9), the *stromelysins* (-3) and the *membrane-type* MMPs (-14) as well as ADAMTS-4, one of the most commonly studied members of the ADAMTS family of enzymes.

Numerous studies in literature have examined the protein expression levels of MMPs and ADAMTS in human herniated discs [10, 19, 20, 26, 29]. Using immunohistochemical

Fig. 2 Normalized transcript levels of **a** MMP-3 and **b** MMP-13 in smoking and non-smoking patients. Significantly lower MMP-3 (c) and MMP-13 (d) mRNA levels were observed in discs removed from patients smoking more than 20 cigarettes per day compared to non-smoking patients ($P = 0.034$ and $P = 0.041$, respectively; Mann–Whitney test)



techniques, Weiler et al. [29] found a significant correlation between MMP protein activity and histological signs of degeneration in human IDs. Similarly, Roberts et al. [26] found increased MMP activity in ID herniation compared to other disc disorders and control specimens. Nevertheless, limited evidence exists regarding the molecular profile of MMPs in ID herniation. In a recent study, Bachmeier et al. [3] examined the transcript levels of several MMPs in a group of lumbar-degenerated and -herniated discs. The authors found a substantial up-regulation of MMP-3 and -8 mRNA levels. However, a limited number of controls were used in the study (two samples), which impeded the use of standard statistical analysis. Our analysis did not show any significant differences between herniated and control discs in the mRNA expression levels of any of the MMPs examined. Given the increased MMP activity that has been observed in ID herniation compared to control specimens, [26] our results suggest that post-transcriptional modifications occur in these settings.

The findings of this study demonstrated multiple positive correlations between MMPs and ADAMTS-4 transcript levels in herniated discs. Fewer correlations, however, were observed in the control group. MMP production in ID tissue is mediated by cytokines, growth factors and inflammatory mediators. The role of interleukin-1 (IL-1) and tumor necrosis factor- α (TNF- α) has been documented in vitro [15]. Growth factors are known to affect the process of ID degeneration by inducing neo-vascularization and regulating MMP expression levels [23]. However, MMPs also play a regulatory role in the interactions between macrophages and chondrocytes. In recent studies, Haro et al. demonstrated a role for MMP-3 and -7 in the release of soluble bioactive factors, thus affecting macrophage infiltration in ID tissue [7, 8]. MMPs are also capable of activating other MMPs. Therefore, it appears that a cascade of events can be triggered by the initial activation of even only one enzyme. This process explains the transcriptional co-expression of several MMPs observed and indicates a possible synergistic effect of multiple MMPs in promoting the resorption process of herniated discs.

Several studies have documented the involvement of ADAMTS-4 in both articular cartilage and ID degradation [21, 22]. Recently, Pockert et al. [24] examined the expression of several members of the ADAMTS family, including ADAMTS-4, in ID samples of patients suffering from degenerative disc disease. Patients with radicular pain were excluded from their study. These authors found a significantly increased mRNA expression in degenerated compared with non-degenerated IDs, which, however, also expressed ADAMTS mRNA. In a study of herniated ID samples without any control group, Hatano et al. [11] demonstrated the expression of ADAMTS-4 mRNA and

protein, particularly in cases of transligamentous extrusion and sequestration. Our analysis showed that ADAMTS-4 mRNA was equally expressed in herniated and control ID samples. However, it was less frequently expressed in the two groups compared with MMPs, with the exception of MMP-8. Our findings are in accordance with Roberts et al. [26] who also found decreased aggrecanase activity, at the protein level, compared with MMPs in herniated discs. Imaging studies have suggested that aggrecan degradation occurs relatively early in the process of ID degeneration preceding the structural breakdown of the collagen network. The frequency of ADAMTS-4 mRNA expression in this study was higher in early compared to late stages of disc herniation, reinforcing the above hypothesis. We can only speculate that ADAMTS-4 plays a minor role in disc herniation. However, further in-depth studies are necessary to elucidate the exact role of ADAMTS-4 in the herniation process.

To the best of our knowledge, this is the first study in which the transcript MMP levels in human-herniated IDs are directly associated with smoking habits. Smoking was previously associated with the degree of disc degeneration and the occurrence of low back pain [6, 14]. It was suggested that cigarette smoking reduces blood circulation around the ID, thus limiting its nutrition and metabolite exchange [12]. Recently, Uei et al. [27] identified marked histological and molecular changes in the nucleus pulposus and annulus fibrosus of rat IDs after 7 weeks of passive cigarette smoking. A detrimental dose- and time-dependent effect of nicotine on cell proliferation and extracellular matrix synthesis has also been documented in vitro [2]. Our analysis showed that the amount of cigarette smoking significantly down-regulates the expression of MMP-3 and -13. Recent reports have shown MMP-3 to affect the resorption process of herniated ID tissue, directly through the proteolysis of extracellular matrix components and indirectly by inducing neoangiogenesis in the periphery of IDs and by promoting macrophage infiltration [7]. Similarly, MMP-13 is more potent than other MMPs in cleaving the helical region of collagen types I, III and particularly II which is found predominantly in the nucleus pulposus. Therefore, our data suggest a possible direct association of smoking with a reduced collagen degradation and resorption rate in herniated discs. Notably, the amount of cigarette smoking was significantly correlated with both the intensity and duration of patient symptoms (positive and negative correlations, respectively). Therefore, it appears that smoking is associated with intense, rather than chronic, pain. However, the latter patient characteristics may be under the influence of several diverse factors, such as individual, psychosocial and occupational. Psychosocial factors, in particular, have been found to independently affect the occurrence of low back pain, recovery rates, as

well as the transition from acute to chronic pain. Therefore, whether smoking alone has a clinically significant effect on the resorption rate of herniated disc fragments and, consequently, on pain regression itself should be further investigated.

Our analysis demonstrated that the duration of patient symptoms was associated with increased transcript levels of MMP-13 and -9 indicating that the MMP mRNA expression is associated with chronic pain. Moreover, MMP-9 and -13 were found to be elevated in patients with intense pain. The two enzymes are known to readily degrade collagen and denatured collagens, e.g., gelatins. The duration of disc herniation is believed to affect significantly the extent of neovascularization around herniated ID fragments. Newly formed blood vessels were found to play an important role as a passage for infiltrating macrophages, resulting in the secretion of proteolytic enzymes such as MMPs [16]. Of note, up-regulation of MMP-9 mRNA expression was established *in vitro* in the process of neurogenesis and vascularization during embryonic tissue development [4]. Furthermore, inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin-1 (IL-1), are produced by infiltrating mononuclear cells immediately after the onset of disc herniation [30] and, in turn, these cytokines induce the expression of MMPs. TNF- α in particular also contributes to the clinical manifestations of disc herniation by inducing endoneuronal edema, nerve fiber demyelination and eventually pain, in association with phospholipase A2 (PLA2) and nitric oxide (NO) [13]. Therefore, we assume that in the setting of chronic inflammation, the increased expression of inflammation-related molecules accounts for the up-regulated expression of MMP-9 and -13 in addition to the effect of extended neovascularization around herniated ID fragments.

Conclusions

Our study provides evidence on the molecular profile of MMPs and ADAMTS-4 in lumbar ID herniation. Transcript levels of the MMPs studied and ADAMTS-4 did not differ significantly between herniated and control ID samples as opposed to the increased MMP protein levels and activity described earlier, leading to the speculation that post-transcriptional modifications occur. Multiple positive correlations were identified in MMP transcript levels that imply a synergistic effect on the activity of these levels in ID herniation. Smoking habits were found to have a negative dose-dependent effect on the transcript levels of specific MMPs and a positive correlation with pain intensity, suggesting an unfavorable role for smoking in the regression process of herniated disc fragments. Pain intensity and duration of patient symptoms significantly

affected the expression levels of specific MMPs, possibly as a consequence of increased neovascularization and chronic inflammation.

References

- Adams MA, Roughley PJ (2006) What is intervertebral disc degeneration, and what causes it? *Spine* 31:2151–2161
- Akmal M, Kesani A, Anand B et al (2004) Effect of nicotine on spinal disc cells: a cellular mechanism for disc degeneration. *Spine (Phila Pa 1976)* 29:568–575
- Bachmeier BE, Nerlich A, Mittermaier N et al (2009) Matrix metalloproteinase expression levels suggest distinct enzyme roles during lumbar disc herniation and degeneration. *Eur Spine J* 18:1573–1586
- Canete Soler R, Gui YH, Linask KK et al (1995) MMP-9 (gelatinase B) mRNA is expressed during mouse neurogenesis and may be associated with vascularization. *Brain Res Dev Brain Res* 88:37–52
- Fardon DF, Milette PC (2001) Nomenclature and classification of lumbar disc pathology. Recommendations of the combined task forces of the North American spine society, American society of spine radiology, and American society of neuroradiology. *Spine (Phila Pa 1976)* 26:E93–E113
- Frymoyer JW (1992) Lumbar disk disease: epidemiology. *Instr Course Lect* 41:217–223
- Haro H, Crawford HC, Fingleton B et al (2000) Matrix metalloproteinase-3-dependent generation of a macrophage chemoattractant in a model of herniated disc resorption. *J Clin Invest* 105:133–141
- Haro H, Crawford HC, Fingleton B et al (2000) Matrix metalloproteinase-7-dependent release of tumor necrosis factor- α in a model of herniated disc resorption. *J Clin Invest* 105:143–150
- Haro H, Komori H, Kato T et al (2005) Experimental studies on the effects of recombinant human matrix metalloproteinases on herniated disc tissues—how to facilitate the natural resorption process of herniated discs. *J Orthop Res* 23:412–419
- Haro H, Shinomiya K, Murakami S et al (1999) Up-regulated expression of matrilysin and neutrophil collagenase in human herniated discs. *J Spinal Disord* 12:245–249
- Hatano E, Fujita T, Ueda Y et al (2006) Expression of ADAMTS-4 (aggrecanase-1) and possible involvement in regression of lumbar disc herniation. *Spine* 31:1426–1432
- Holm S, Nachemson A (1988) Nutrition of the intervertebral disc: acute effects of cigarette smoking. An experimental animal study. *Ups J Med Sci* 93:91–99
- Igarashi T, Kikuchi S, Shubayev V et al (2000) 2000 Volvo award winner in basic science studies: exogenous tumor necrosis factor- α mimics nucleus pulposus-induced neuropathology. Molecular, histologic, and behavioral comparisons in rats. *Spine (Phila Pa 1976)* 25:2975–2980
- Kaila-Kangas L, Leino-Arjas P, Riihimaki H et al (2003) Smoking and overweight as predictors of hospitalization for back disorders. *Spine (Phila Pa 1976)* 28:1860–1868
- Kato T, Haro H, Komori H et al (2004) Sequential dynamics of inflammatory cytokine, angiogenesis inducing factor and matrix degrading enzymes during spontaneous resorption of the herniated disc. *J Orthop Res* 22:895–900
- Koike Y, Uzuki M, Kokubun S et al (2003) Angiogenesis and inflammatory cell infiltration in lumbar disc herniation. *Spine* 28:1928–1933

17. Komori H, Shinomiya K, Nakai O et al (1996) The natural history of herniated nucleus pulposus with radiculopathy. *Spine (Phila Pa 1976)* 21:225–229
18. Korecki CL, Kuo CK, Tuan RS et al (2009) Intervertebral disc cell response to dynamic compression is age and frequency dependent. *J Orthop Res* 27:800–806
19. Le Maitre CL, Freemont AJ, Hoyland JA (2004) Localization of degradative enzymes and their inhibitors in the degenerate human intervertebral disc. *J Pathol* 204:47–54
20. Liu J, Roughley PJ, Mort JS (1991) Identification of human intervertebral disc stromelysin and its involvement in matrix degradation. *J Orthop Res* 9:568–575
21. Majumdar MK, Askew R, Schelling S et al (2007) Double-knockout of ADAMTS-4 and ADAMTS-5 in mice results in physiologically normal animals and prevents the progression of osteoarthritis. *Arthritis Rheum* 56:3670–3674
22. Patel KP, Sandy JD, Akeda K et al (2007) Aggrecanases and aggrecanase-generated fragments in the human intervertebral disc at early and advanced stages of disc degeneration. *Spine (Phila Pa 1976)* 32:2596–2603
23. Pattison ST, Melrose J, Ghosh P et al (2001) Regulation of gelatinase-A (MMP-2) production by ovine intervertebral disc nucleus pulposus cells grown in alginate bead culture by transforming growth factor-beta(1) and insulin like growth factor-I. *Cell Biol Int* 25:679–689
24. Pockert AJ, Richardson SM, Le Maitre CL et al (2009) Modified expression of the ADAMTS enzymes and tissue inhibitor of metalloproteinases 3 during human intervertebral disc degeneration. *Arthritis Rheum* 60:482–491
25. Porter S, Clark IM, Kevorkian L et al (2005) The ADAMTS metalloproteinases. *Biochem J* 386:15–27
26. Roberts S, Caterson B, Menage J et al (2000) Matrix metalloproteinases and aggrecanase: their role in disorders of the human intervertebral disc. *Spine* 25:3005–3013
27. Uei H, Matsuzaki H, Oda H et al (2006) Gene expression changes in an early stage of intervertebral disc degeneration induced by passive cigarette smoking. *Spine (Phila Pa 1976)* 31:510–514
28. Visse R, Nagase H (2003) Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 92:827–839
29. Weiler C, Nerlich AG, Zipperer J et al (2002) 2002 SSE award competition in basic science: expression of major matrix metalloproteinases is associated with intervertebral disc degradation and resorption. *Eur Spine J* 11:308–320
30. Yoshida M, Nakamura T, Sei A et al (2005) Intervertebral disc cells produce tumor necrosis factor alpha, interleukin-1beta, and monocyte chemoattractant protein-1 immediately after herniation: an experimental study using a new hernia model. *Spine (Phila Pa 1976)* 30:55–61
31. Yukawa Y, Kato F, Matsubara Y et al (1996) Serial magnetic resonance imaging follow-up study of lumbar disc herniation conservatively treated for average 30 months: relation between reduction of herniation and degeneration of disc. *J Spinal Disord* 9:251–256