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Expression of metalloproteinases and their inhibitors in degenerated and extruded intervertebral disks in chondrodystrophic dogs

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ABSTRACT. We analyzed the mRNA expression of matrix metalloproteinases (MMPs), metalloproteinases with thrombospondin motifs (ADAMTSs), and tissue inhibitors of metalloproteinases (TIMPs) in degenerated and herniated intervertebral disks (IVDs) in chondrodystrophic dogs. In degenerated IVDs, MMP3, 7, 13, and 14, ADAMTS4 and 5, and TIMP1–3 expression were significantly higher vs healthy controls ($P<0.05$). In herniated IVDs, MMP2, 3, 9, 13, and 14, ADAMTS4 and 5, and TIMP1 expression were significantly greater, and MMP7 expression was significantly lower vs degenerated IVDs ($P<0.05$). These results suggest that metalloproteinase may play a role in extracellular matrix degradation in IVD degeneration. Decreased MMP7 transcription may prevent proteoglycan degradation and may reduce macrophage infiltration, which might affect the resorption process of herniated IVDs.

KEY WORDS: degeneration, dog, intervertebral disk, metalloproteinase, resorption

Intervertebral disk (IVD) extrusion or Hansen type I herniation is a common disorder causing neurological deficits in dogs [5, 32]. IVD disease appears more commonly in the thoracolumbar region and in chondrodystrophic dogs [5, 32], which are characterized by genetically-related shortened limbs [6]. In addition to shortened limbs, chondroid metaplasia of the nucleus pulposus (NP) leads to premature IVD degeneration and correlates with a higher frequency of IVD extrusion [5, 32]. Extrusion of degenerated IVD material into the vertebral canal causes neuronal tissue compression and an inflammatory response in the epidural space; affected dogs present with signs of pain to paresis or paralysis [4, 17]. Treatments for thoracolumbar IVD extrusion in dogs include conservative treatments (rest and administration of anti-inflammatory or analgesic medications or both) and surgical decompression [4, 17]. Outcomes after conservative treatment are generally successful in 50% of affected dogs, with a treatment failure of approximately 15–20% and a recurrence rate of 30% [17, 22]. Additionally, affected dogs treated surgically experience successful recovery rates of 86–96% [9, 17], suggesting a substantial benefit of surgical vs conservative treatment [17]. In contrast, approximately 70–80% of humans with lumbar IVD herniation treated conservatively recover from hyperpathia within 6 weeks [36], and systematic reviews comparing surgical and conservative treatment show no marked difference during long-term follow-up, with approximately 10–20% recurrence rates for the symptoms [10, 16]. Thus, the natural course of IVD herniation is considered benign in humans, and relates to spontaneous resorption of the herniated IVD [1, 11, 12]. The major factors involved in spontaneous regression include neovascularization and infiltration of inflammatory cells, and phagocytosis by macrophages [1, 11, 12]. Recent studies indicated macrophage infiltration in 27–82% of herniated canine IVDs [8, 19, 24, 33]. Although multinucleate giant cells indicative of macrophage phagocytosis in a foreign body reaction were frequently observed in one study [33], these cells were absent or seen only in a few cases in other studies [8, 19, 24]. Thus, the potential for resorption of herniated IVD material in dogs remains unclear.

Matrix metalloproteinases (MMPs) and disintegrins, and metalloproteinases with thrombospondin motifs (ADAMTSs), are involved in the spontaneous regression of human herniated IVD material [12, 13]. Furthermore, macrophage infiltration facilitates spontaneous absorption through up-regulation of MMPs [11, 12]. Metalloproteinase families share a common catalytic core, and can degrade all of the components of the extracellular matrix (ECM) within the IVD [35]. The enzymatic activities of metalloproteinases are regulated by metalloproteinase tissue inhibitor genes (TIMPs) [25, 35]. Moreover, metalloproteinases are also believed to maintain ECM degradation in IVD degeneration in humans, which plays a key role in herniation [20, 31, 35].

Although the gross pathological process and ECM degradation are similar in humans and chondrodystrophic dogs [5, 18], limited

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reports describe the expression of MMPs in degenerated and herniated NP in chondrodystrophic dogs [15, 18]. Additionally, a previous study stated that the inflammatory processes occurring after IVD extrusion in dogs partially diverge from those in humans [24], and that inflammation can regulate the expression of metalloproteinases [25, 35]. Thus, we hypothesized that metalloproteinase expression for extruded IVDs may differ between chondrodystrophic dogs and humans, and that this difference may affects the potential for spontaneous resorption of extruded NP. The aim of the present study was to investigate the expression of the major metalloproteinases and their inhibitors in extruded NP in chondrodystrophic dogs.

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Kitasato University (Approval no. 18-102). Herniated disk material was collected from 20 consecutive chondrodystrophic dogs (all dogs were miniature dachshunds; 11 females (5 spayed) and 9 males (6 neutered)) with thoracolumbar IVD extrusion (Hansen type I) during decompressive surgery by hemilaminectomy at Kitasato University Veterinary Teaching Hospital (September 2015 to June 2017). The age, body weight, neurological grade (as previously described) [8], and the duration until surgery (time between the onset of clinical signs and surgery) of the dogs were summarized in Table 1. IVD extrusion was diagnosed according to the acute onset of clinical symptoms (<48 hr), neurological examination, computed tomography with or without myelography, and surgical findings. The collected herniated nucleus pulposus (HNP) material was snap frozen and stored at −80°C until RNA extraction. All dogs had good outcomes and recovered ambulation.

We also collected 12 IVDs from six clinically and neurologically normal beagles (median age: 14 months; range: 9–55 months; three males and three females) that were euthanized in unrelated animal experiments (Approval no. 17-126). After euthanasia, thoracolumbar spine magnetic resonance imaging (MRI) was performed using a 0.3 T open magnet (AIRIS-II; Hitachi, Tokyo, Japan) before NP isolation. T2-weighted sagittal images (4,000 msec repetition time, 125 msec echo time, and 4.0-mm-thick slices with a 1-mm gap) were obtained using the spin echo method. Using the MRI images, we examined the T13–L1 and L1–L2 IVDs using Onis 2.5 software (Digital Core Co., Ltd., Tokyo, Japan), and we classified the degeneration of each IVD according to the Pfirrmann grading system, as reported previously [2]. We classified six NP tissues as Pfirrmann grade 1, indicating healthy (nondegenerated), as control samples. We grouped five NPs with Pfirrmann grade 2, and one NP with Pfirrmann grade 3 together as degenerated NP. NPs graded as 1 or 2–3 were collected, snap frozen and stored at −80°C until RNA extraction.

Total RNA was extracted from NP tissue using ISOGEN II (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. For purification of total RNA, samples were treated with the RNeasy Mini Kit (QIAGEN, Tokyo, Japan). We quantified the RNA yield by measuring the optical density of a sample at 260 nm and 280 nm using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific Japan, Tokyo, Japan). Total RNA (1 µg) was reverse-transcribed using a Super Script VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Intron-spanning primers (Sigma-Aldrich Japan, Tokyo, Japan) for *canine* MMP2, 3, 7, 8, 9, 13, and 14; ADAMTS4 and 5; TIMP1–3; aggrecan (ACAN); collagen1α1 (Col1A1); and the housekeeping gene GAPDH were designed using Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi; Table 2). The uniqueness and specificity of each primer were verified using the Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/blast). Real-time polymerase chain reaction was performed using a StepOnePlus System (Thermo Fisher Scientific Japan) using the PowerUp SYBR Green Master Mix (Thermo Fisher Scientific Japan) according to the manufacturer's instructions. Quantification was initiated by incubation at 50°C for 2 min and 95°C for 2 min, followed by 40 cycles with the following profile: denaturation at 95°C for 15 sec and annealing at 60°C for 60 sec. The specificity and the size of the polymerase chain reaction products were tested by adding a melt curve at the end of the amplifications, and a single peak was observed. Each sample was run in triplicate for each gene, and the threshold cycle (C_T) value means were calculated and normalized to that of the housekeeping gene GAPDH. Fold differences were calculated using the $\Delta\Delta C_T$ method.

Statistical analyses were performed using Statcel 4 software (OMS Publishing Inc., Saitama, Japan). The results of the expression levels for each gene are presented as boxplots, and as median and interquartile range, unless otherwise stated. Statistical differences were determined using the Steel–Dwass test. Spearman's rank correlation was used to examine the relationship between gene expression (metalloproteinases and TIMPs) and the dogs' clinical characteristics (age, affected location, and duration until the surgery). Values of $P<0.05$ were considered statistically significant.

Col1A1 and ACAN mRNA levels in Pfirrmann grade 2–3 NP were significantly higher ($P<0.05$) than with grade 1. Col1A1 mRNA levels in the HNP were significantly elevated ($P<0.01$) compared with grade 1 and grade 2–3 NP. In contrast, ACAN mRNA in the HNP was significantly lower ($P<0.01$) compared with the grade 2–3 NP, but we found no significant difference compared with grade 1 NP (Fig. 1).

MMP3, 7, 13, and 14; ADAMTS4 and 5; and TIMP1-3 mRNA levels in the Pfirrmann grade 2–3 NP were significantly higher ($P<0.05$) than in the grade 1 NP. MMP, 2, 9, 13, and 14; ADAMTS4 and 5; and TIMP1 mRNA levels in the HNP were significantly greater ($P<0.05$) than in the grade 1 and 2–3 NP. MMP3 and 8; TIMP2 and 3 mRNA levels in the HNP were significantly higher ($P<0.01$) than in the grade 1 NP, while MMP7 mRNA expression in the HNP was significantly lower ($P<0.01$) than in the grade 2–3 NP (Fig. 2). MMP8 mRNA expression were positively correlated with the age of the dogs with IVD extrusion ($r_s=0.633$, $P<0.01$). TIMP1 mRNA expression in the HNP was negatively correlated with the age of the IVD patients ($r_s=-0.877$, $P<0.001$). Furthermore, MMP13 mRNA expression was positively correlated with the duration until surgery ($r_s=0.482$, $P<0.05$) in the HNP. No other significant differences were found.

IVD degeneration is typically described as chondroid degeneration secondary to genetic factors in chondrodystrophic dogs [6]. The NP compartment of the disk is first affected by degenerative changes. The NP functions as a compression–resistance tissue, and contains water, type II collagen, and proteoglycans (mainly aggrecan) [5, 7]. During IVD degeneration, ECM remodeling of the NP occurs as substitution of type II collagen with type I collagen, and decreased aggrecan leads to decreased water content [5].

Table 1. Clinical characteristics of the miniature dachshunds included in this study

Case	Gender[a]	Age (year)	Weight (kg)	Location[b]	Grade[c]	Duration[d] (day)
1	M	6	5.7	T12–T13	4	18
2	MC	12	7.2	L1–L2	3	33
3	F	4	5.9	T11–T12	5	17
4	F	10	5.8	L1–L2	4	23
5	FS	10	4.0	L3–L4	3	63
6	F	5	5.0	T11–T12	2	12
7	F	13	6.1	T13–L1	4	31
8	F	8	4.4	T13–L1	4	17
9	FS	10	3.6	T13–L1	4	25
10	MC	12	6.0	T12–13	4	7
11	M	6	5.5	L3–L4	4	30
12	M	11	5.6	L1–L2	4	63
13	MC	5	7.3	T13–L1	4	40
14	FS	12	4.7	L1–L2	3	27
15	M	6	10.5	L3–L4	5	48
16	FS	9	7.4	L2–L3	3	24
17	MC	13	10.0	T13–L1	4	16
18	MC	11	6.6	L2–L3	4	9
19	FS	3	5.6	T13–L1	4	17
20	F	7	5.1	T13–L1	3	33

[a] Gender: F=female intact, FS=female spayed, M=male intact, MC=male castrated. [b] The location of Intervertebral disc herniation. [c] Neurological grade of Intervertebral disc herniation [8]. [d] The time between the onset of clinical signs and surgery.

Table 2. Primer sequences for the real-time polymerase chain reaction testing

Gene	Ref.sequence	Sequence5′→3′
MMP2	XM_014109407	Forward: AGACGACATCAAGGGCATTC
		Reverse: TTGTTCCGTGGTGTCACTGT
MMP3	NM_001003301	Forward: ATGGAGATGCCCACTTTGAC
		Reverse: GGAGGAATCAGAGGGAGGTC
MMP7	NM_001242726	Forward: TGTGGTGTGCCTGATGTCG
		Reverse: CTCTGAAGCGTGGTAAGTCTGG
MMP8	XM_546547	Forward: GGACCAAGCACACCCACAAC
		Reverse: ATACCGTCAGGCAAGGATGG
MMP9	NM_001003219	Forward: TCCTGGTGTTCCTGGTGCTG
		Reverse: GGACTGCTTGTCGTTGCTCA
MMP13	XM_536598	Forward: TTCTGGCTCATGCTTTTCCT
		Reverse: GGTCCTTGGAGTGGTCAAGA
MMP14	XM_022421791	Forward: TGCCCAATGGAAAGACCTAC
		Reverse: CATCACTGCCCATGAATGAC
ADAMTS4	XM_545768	Forward: GCTGTTGTGGAGGATGATGG
		Reverse: CTTTGAGTTGTCGTGGAGCA
ADAMTS5	XM_846025	Forward: CTACTGCACAGGGAAGAG
		Reverse: GAACCCATTCCACAAATGTC
TIMP1	NM_001003182	Forward: CATCCTGCTGTTGCTGTGG
		Reverse: TCGGTCTGGTTGACTTCTGC
TIMP2	NM_001003082	Forward: ATCTACACGGCTCCTTCCTC
		Reverse: CTCTTCTTCTGGGTGCTGCT
TIMP3	NM_001284439	Forward: GCAAGGGGCTCAACTACAGG
		Reverse: TGGAGGTCAGCAAAGCAAGG
Col1A1	XM_001003090	Forward: CTGGCAAAGACGGACTCAAC
		Reverse: GCAGGAAGCTGAAGTCGAAAC
ACAN	NM_001113455	Forward: TCATTGCTACACCCGAACAG
		Reverse: CCTGGGAACTCATCCTTGTC
GAPDH	NM_001003142	Forward: GATGGGCGTGAACCATGAG
		Reverse: TCATGAGGCCCTCCACGAT

Phenotypic changes according to the Pfirrmann grade are reported to indicate the loss of water content and suggest decreased aggrecan in grade 2–3 NP [3, 27]. In contrast, in our study, ACAN mRNA was upregulated in Pfirrmann grade 2–3 NP compared with Pfirrmann grade 1 NP. This result suggests that upregulation is a compensatory reaction to reconstruct the ECM in the early stage of degeneration, as previously described [37]. Furthermore, the upregulation of Col1A1 mRNA seen in the grade 2–3 NP and HNP may be associated with the degeneration process and ECM remodeling, with increasing deposition of type I collagen [5, 7]. Therefore, we defined Pfirrmann grade 1 NP tissues as healthy and grade 2–3 NP as degenerated.

Although IVD degeneration is common in dogs, the expression profiles of metalloproteinases and their inhibitors are poorly understood. To our knowledge, ours is the first study to determine these expressions to evaluate whether metalloproteinase expression in HNP is a result of degeneration or herniation. MMPs can degrade all of the ECM within the IVD, and MMPs can be divided into subfamilies. Collagenases (MMP8, 13) cleave native interstitial collagen; stromelysins (MMP3) proteolyze proteoglycans, gelatins, and collagens; gelatinases (MMP2, 9) digest denatured collagens and gelatins; and matrilysin (MMP7) digests many ECM components, including aggrecan [25, 35]. Membrane type MMPs (MMP14) mainly activate other MMPs, and their enzymatic activities are regulated by TIMP1and 2 in a 1:1 ratio [25, 35]. ADAMTS4 and 5 show a particular affinity for aggrecan, and TIMP3 selectively inhibits the ADAMTS genes [25, 35]. In the present study, higher levels of mRNA were found in the degenerated vs healthy NP for many metalloproteinases, including MMP3, 7, 13, 14, and ADAMTS4, and 5. These results are consistent with findings in degenerated human NP [21, 28, 35], which suggests that upregulation of these metalloproteinases are also related to ECM degradation in the NP of chondrodystrophic dogs. Moreover, one study found that gene expression patterns were correlated with MMP activity, protein expression, and degeneration score in humans [2], which supports our results. In contrast, we found no significant difference in MMP2 and MMP9 expression, which is upregulated in human degenerated NP [28, 31, 35]. Among several mechanisms known to lead to MMP2 activation, the most common is activation by MMP14 and the tissue inhibitor, TIMP2 [30]. In the present study, MMP2 and 9 expression were unchanged, but conversely, MMP14 and TIMP2 expression was upregulated in degenerated NP. A recent study of canine IVD degeneration showed increasing gelatinase activity in the NP that correlated with increasing severity of IVD degeneration [4]. Thus, our findings may suggest that MMP2 activity may be initiated by MMP14 and TIMP2 upregulation in the early stage of NP degeneration in chondrodystrophic dogs. Furthermore, we found TIMP1 and 3 mRNA upregulation in degenerated NP; however, this finding is controversial because TIMP3 is not upregulated in degenerated human NP, and instead, ADAMTS levels are elevated, and an imbalance between MMPs/TIMP1 and 2

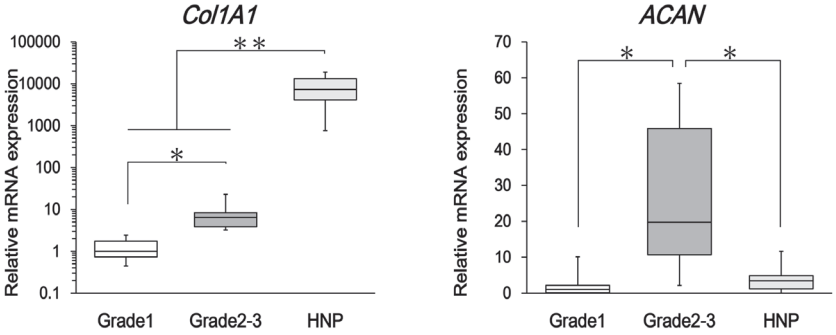

Fig. 1. Gene expression of collagen1α1 (Col1A1) and aggrecan (ACAN) in Pfirrmann grade 1 nucleus pulposus (Grade 1), Pfirrmann grade 2–3 nucleus pulposus (Grade 2–3), and herniated nucleus pulposus (HNP), shown as boxplots. **P*<0.05. ***P*<0.01.

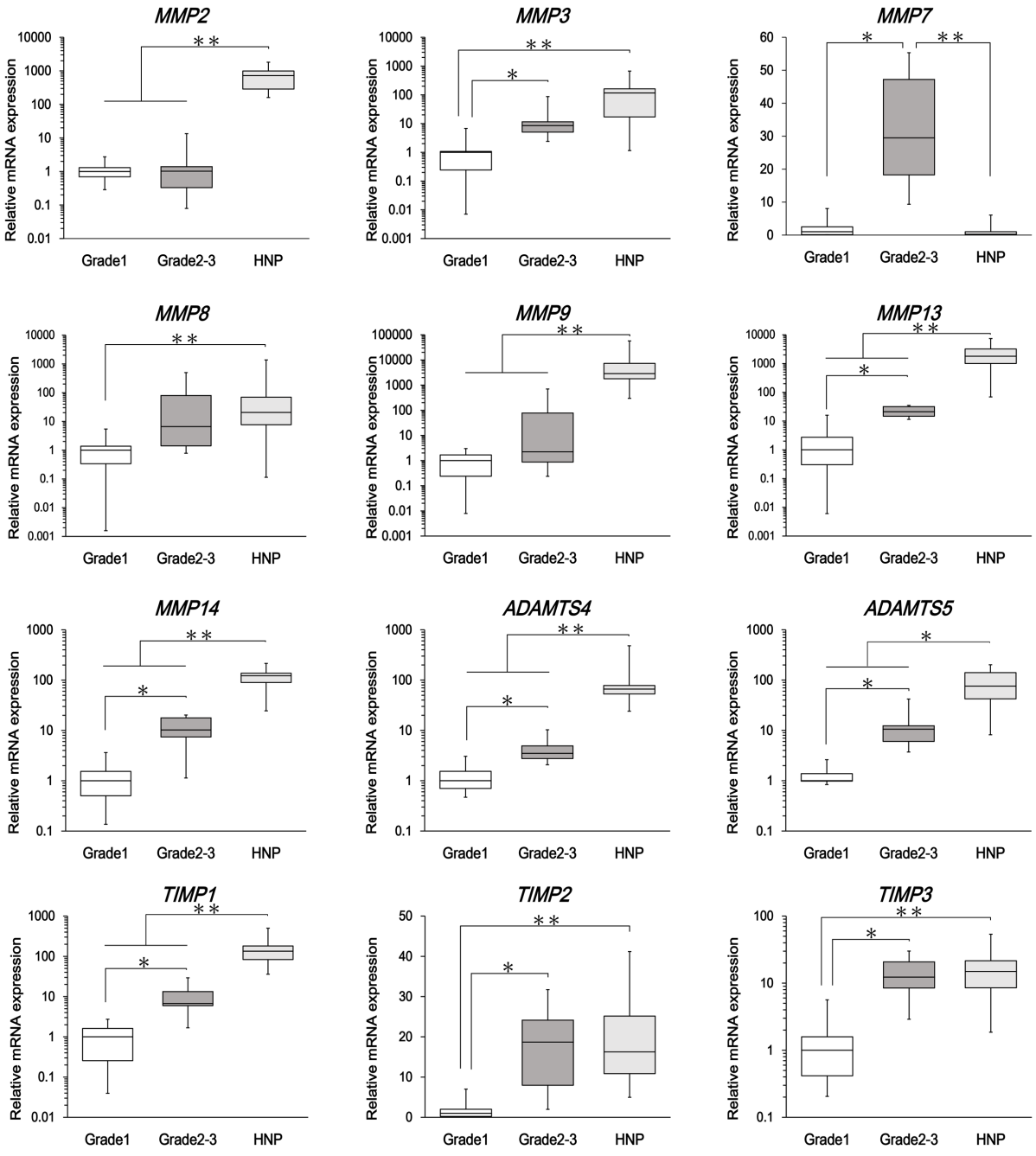

Fig. 2. Gene expression of matrix metalloproteinases (MMPs), metalloproteinases with thrombospondin motifs (ADAMTSs) and tissue inhibitors of metalloproteinases (TIMPs) in Pfirrmann grade 1 nucleus pulposus (Grade 1), Pfirrmann grade 2–3 nucleus pulposus (Grade 2–3), and herniated nucleus pulposus (HNP), shown as boxplots. **P*<0.05, ***P*<0.01.

and ADAMTSs/TIMP3 is considered to contribute to degradation [28, 35]. However, these imbalances were not found in an early-degeneration rat model [38], and the imbalances may occur in advanced stages of IVD degeneration in chondrodystrophic dogs.

In the HNP in our study, we found a strong and negative correlation between TIMP1 and age, and a positive correlation between MMP8 and age. If these changes resulted from the IVD degeneration progress, disease presentation within chondrodystrophic breeds may also be associated with age-related NP degradation. In contrast, we found high levels of mRNA for the MMPs except MMP7, ADAMTS4 and 5, and TIMP1–3 in the HNP. ADAMTS4 and ADAMTS5 were upregulated, while TIMP3 expression did not differ compared with degenerated NP, indicating an imbalance regarding TIMP3. Moreover, expression of MMP2, 3, 9, 13, and 14, and TIMP1 was also higher in HNP than in degenerated NP, while TIMP2 expression remained unchanged, also indicating an imbalance of TIMP2. Although in our study we did not evaluate the activity of these enzymes, similar findings were reported in several human reports [2, 11, 13, 20] and in chondrodystrophic dogs [15, 18]. Thus, our results suggest that the catabolic activity of metalloproteinases in HNP may be increased in degenerated NP in chondrodystrophic dogs. Additionally, the positive correlation between MMP13 expression and the duration until surgery suggest that catabolic potential may increase in a time-dependent manner following IVD extrusion.

Our finding that MMP7 expression in HNP was downregulated compared with degenerated NP, and remained unchanged compared with healthy NP is interesting. In humans, MMP3 and 7 are strongly expressed in surgically-removed herniated disks, and these MMPs are believed to play a crucial role in the natural resorption process of herniated IVDs [11, 12]. In human HNP, MMP7 expression occurred in the mononuclear cell infiltration in chondrocytes [11]. MMP7 secretion from macrophages enhanced proteoglycan degradation and macrophage infiltration in a cultured murine IVD model [11]. Moreover, recombinant human MMP7 decreased the wet weight of human herniated IVD, which was not observed with MMP3 *in vitro* [12]. With these considerations, our finding that lack of MMP7 upregulation in HNP in chondrodystrophic dogs may prevent proteoglycan degradation and further macrophage infiltration, which might affect the resorption process. The expression level of MMPs in macrophages differs between species [26]. To our knowledge, MMP7 expression in chondrodystrophic dog macrophages has not been reported; however, several reports describe macrophage infiltration in HNP [8, 19, 24, 33]. Additionally, a recent study described M2-polarized macrophages expression in canine HNP [34]. M2-polarized macrophages are anti-inflammatory [26], and this effect is associated with MMP7, 8, and 9 in humans [23, 26]. These findings support the lack of enhanced MMP7 expression with upregulated MMP8 and 9 in the HNP, in our study may derived from species difference of the macrophages. In wound healing, MMP7 knock-out mice have the most severe wound repair defects regarding MMPs [29], and decreased expression was observed in hypertrophic scar tissue [29]. Therefore, decreased MMP7 expression may lead to excessive granulation tissue in the HNP, which has been reported to remain in the human vertebral canal [14]. However, histological analysis and immunocytochemistry were lacking in our study because of sample size limitations, and further study is needed to evaluate the location of MMP7 expression and activity in HNP in both chondrodystrophic and non-chondrodystrophic dogs. Nevertheless, recombinant human MMP7 injection into protruding IVDs in beagles decreased the proteoglycan levels, and the protruded mass [12], which supports the MMP7 hypothesis.

In conclusion, to our knowledge, the present study is the first to reveal MMP7, ADAMTS, and TIMP transcript expression in the NP of chondrodystrophic dogs. Our results suggest that metalloproteinase transcription is enhanced in degenerated NP, except for MMP7 expression in extruded NP, in chondrodystrophic dogs. Although many of the metalloproteinase transcripts are upregulated, lack of enhanced MMP7 transcription seems to affect the resorption process of HNP. Further studies are needed to investigate the exact role of decreased MMP7 transcription in HNP in chondrodystrophic dogs.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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