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Gene Expression of Matrix Metalloproteinases and their Inhibitors (TIMPs) in Meningiomas of Dogs

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Background: Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are considered to be key mediators of tumor invasion and metastasis. MMP-2 and MMP-9 are expressed in meningiomas of dogs, but TIMP expression, and variations of specific MMP/TIMP ratios still are unknown in this tumor.

Hypothesis/Objectives: Expression of MMP/TIMP might increase progressively from grade I to grade III meningioma. Therefore, genetic expression of MMP-2 and MMP-9, and specific TIMP-2 and TIMP-1, respectively, has been investigated in meningiomas of different grades.

Animals: Selected formalin-fixed paraffin-embedded tissue from 43 meningiomas of dogs was evaluated.

Methods: Genetic material was obtained from pathologic samples and used for quantitative reverse transcriptase real-time polymerase chain reaction (RT-qPCR).

Results: MMP-9 was not expressed in all of the tumors, but MMP-2 was significantly more expressed in papillary meningioma. Likewise, the MMP-2/TIMP-2 ratio was numerically higher in papillary meningiomas compared to all grades (>3.5 times) showing a strong bias in favor of metalloproteinase. In the papillary meningioma, TIMP-1 gene expression was significantly higher than in grades I and III.

Conclusions and Clinical Importance: MMP-2/TIMP-2 imbalance might contribute to the aggressive biologic behavior of papillary meningiomas in dogs. TIMP-1 expression may play a role independent of MMP-9 expression in neoplastic progression. These results further support that therapeutic and prognostic evaluations of dogs with meningioma need to be addressed according to different histologic patterns as is performed in humans.

Key words: Dog; Meningioma; Metalloproteinases; Tissue Inhibitors of metalloproteinases.

Meningioma is an extra-axial central nervous system tumor, which arises from the cap cells covering the arachnoid granulations.[1] In the dog, meningioma is the most common brain tumor[2,3] and it shares several similarities with meningioma in humans in neuroimaging,[4,5] gross, and histologic patterns as well as in the expression of growth factors and hormone receptors.[6–8] Except for the anaplastic histotype, this neoplasm generally is associated with benign biologic behavior.[9,10] Thus, surgery still represents the treatment of choice and the postexcision prognosis is quite satisfactory.[11,12] In humans, it has long been recognized that the presence of brain invasion in World Health Organization (WHO) grade I meningioma confers recurrence and mortality rates similar to those of WHO grade II meningioma.[13,14] Data about postsurgery recurrence of meningioma are inconsistent in dogs. In a recent study, 87.5% of dogs (7/8 animals) affected by papillary meningioma that underwent surgery alone experienced tumor recurrence from 2 to 17 months after surgery.[15]

Abbreviations:

MMPs	matrix metalloproteinases
TIMPs	tissue inhibitors of metalloproteinases

Degradation of extracellular matrix (ECM) and subsequent infiltration of adjacent tissue currently is recognized as the triggering event of tumor progression and recurrence.[14,16] In the complex process of ECM degradation, a key role is played by matrix metalloproteinases (MMPs), a family of endopeptidases[17] primarily involved in tissue remodeling by selective proteolytic activity.[18] This action is countered by another class of molecules that function as tissue inhibitors of matrix metalloproteinases (TIMPs) and hence as important regulators of ECM turnover and remodeling.[19,20] In recent years, several studies have identified a positive correlation between increased MMP concentration expressed by tumor stroma and tumor cell invasion or metastasis,[18,21,22] suggesting a modified MMPs/TIMPs imbalance in malignant tissues.[18] Therefore, changes in the expression of molecules related to invasion, such as MMPs and TIMPs, have been proposed as prognostic factors in cancer development and progression and as potential novel therapeutic targets.[23]

The expression of MMP-2 and MMP-9, known as type-IV collagenases,[17] has been investigated in both humans[14,20,24–27] and dogs with meningiomas,[28,29] generally with phenotypic methods such as immunolabeling. These studies have provided contradictory results about the potential role of these molecules in meningioma recurrence and invasion. On the contrary, a more specific relationship between MMP and TIMP expression has yet to be adequately investigated. Although radiotherapy and chemotherapy are considered

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therapeutic options for adjacent tissue invasion from neoplastic meningeal cells,[30] meningiomas are not always responsive to radiotherapy and not infrequently develop pharmacologic resistance against chemotherapy drugs.[17,31,32]

Based on these considerations, the aim of our study was to investigate gene expression of MMP-2 and MMP-9 and related specific inhibitors, TIMP-2 and TIMP-1, respectively, in meningiomas of dogs of different grades. Moreover, the relationship between MMP and TIMP expressions also was investigated to identify clinically relevant imbalance of the MMP/TIMP ratio associated with the progression of the tumor.

Materials and Methods

Selection of Cases

Forty-three tumors from dogs histologically classified as meningiomas were selected. They consisted of formalin-fixed paraffin-embedded (FFPE) blocks stored in the archive of this Neuropathology Laboratory as necropsy samples (12 cases) and biopsy samples (31 cases). In 33 cases, they developed in the brain, and in the remaining 10 cases, they developed in the spinal cord. Four FFPE normal adjacent tissues (NATs) also were collected as controls for a comparative evaluation of results.

Histopathologic Study

Five-micrometer of FFPE sections were stained with hematoxylin and eosin (H&E) for routine histologic diagnosis and graded according to the criteria of the human international WHO histologic classification of central nervous system (CNS) tumors[9] as follows:

- Grade I (benign meningioma): <4 mitoses/10 high power field (HPF) for variants lacking criteria of atypical histotype
- Grade II (atypical meningioma): ≥ 4 mitoses/10 HPF or brain invasion or ≥ 3 of the following criteria: increased cellularity, patternless sheets, high N/C ratio, macronucleoli, spontaneous necrosis
- Grade III (anaplastic meningioma): >20 mitoses/10 HPF or morphologic findings of frank anaplasia.

The papillary histotype, known to be 1 of the most aggressive meningiomas (grade III) in humans and suspected to have a similar aggressive behavior in the dog, despite its benign histologic appearance, has been considered as a separate group in our study.[15]

Real-Time PCR Analysis

RNA was extracted from all FFPE samples with RecoverAll Total Nucleic Acid Isolation Kit[a]. Twenty nanograms of total RNA was reverse-transcribed in 20 μL of iSCRIPT cDNA[b] with random hexamers according to the manufacturer's guidelines. Stored FFPE mammary gland tumors and artificial plasmids were used as positive controls. RNA was submitted for qualitative and quantitative assessments using the Bioanalyzer, Nanodrop, and Qubit RNA HS Assay Kit[c]. This test expresses the mean RNA integrity number (RIN) according to the whole electrophoretic profile of the RNA sample, including the presence or the absence of degradation products.

No-RT controls were included to check genomic DNA contamination. Taqman gene expression assays for MMP-9, MMP-2, TIMP-1, and TIMP-2 were used[d] (Table 1). The optimized PCR assay of 20 μL PCR reaction volume contained 10 μL of iTaq Universal Probes Supermix[b], 1 μL of TaqMan Gene Expression Assay[d], and water to 20 μL. All reagents were mixed and distributed into a 96-well PCR plate before adding 4 μL of cDNA (1–100 ng). Sample amplification fidelity was verified by agarose gel electrophoresis. The PCR was performed on iCycler iQ[b] with an initial incubation at 95°C for 10 minutes, followed by 45 cycles at 95°C for 15 seconds and 60°C for 60 seconds, during which fluorescence data were collected. The Ct was automatically computed for each trace. Subsequently, PCR products were purified and sequenced by QIAquick PCR Purification Kit according to the manufacturer's protocol. Each sample was run in triplicate, and actin beta (ACTB) was chosen as a housekeeping gene. The expression level was determined by the $2^{-\Delta\Delta Ct}$ method[33] (ΔCt = [Ct target gene – Ct Housekeeping gene]; $2\Delta\Delta Ct$ = [ΔCt– Ct NAT]), and the results were normalized.

Table 1. List of TaqMan Gene Expression Assays used in the gene expression study. TaqMan Gene Expression consists of a pair of unlabeled PCR primers and a TaqMan probe with a FAM dye label on the 5′ end, and minor groove binder (MGB) nonfluorescent quencher (NFQ) on the 3′ end. TaqMan Gene Expression Assays are listed by the assay numbers.

Gene	Name	GenBank	Connected Exons	Amplicon
MMP-9	TaqMan Cf02621847_g1	AF169244.2	2–3	81 bp
MMP-2	TaqMan Cf02623422_m1	AF177217.1	2–3	74 bp
TIMP-1	TaqMan Cf02621937_g1	AF077817.1	3–4	63 bp
TIMP-2	TaqMan Cf02623335_m1	AF112115.1	1–2	81 bp

The PCR amplification efficiency was determined using the slope of the standard curve (efficiency = $10^{(-1/\text{slope})}-1$). The slope of these graphs was utilized to determine the amplification efficiency. The PCR conditions were optimized to generate >95% PCR efficiency. Only reactions from 95 to 100% efficiency were included in the subsequent analysis.

The mRNA expression levels referred to MMP-2, MMP-9, TIMP-2, and TIMP-1 were studied for each grade of tumor and for the papillary histotype. Specifically, MMP-2/TIMP-2 and MMP-9/TIMP-1 ratios also were evaluated and interpreted for each grade of tumor and for the papillary histotype.

Statistical Analysis

The expression levels of MMP-2, TIMP-1, and TIMP-2 in different grades of tumor and papillary meningioma were analyzed by one-way ANOVA followed by a Tukey's multiple comparison test performed by GraphPad Prism version 7.00 for Windows.[e] A P value <0.05 was considered statistically significant.

Results

Pathologic Findings

Sixty percent of affected dogs were male. Most were of mixed breed (33%), followed by Boxer dogs (18%) and German Shepherd dogs (16%). The mean age of the dogs was 9.9 years. Based on the WHO classification system of CNS tumors in humans, we identified

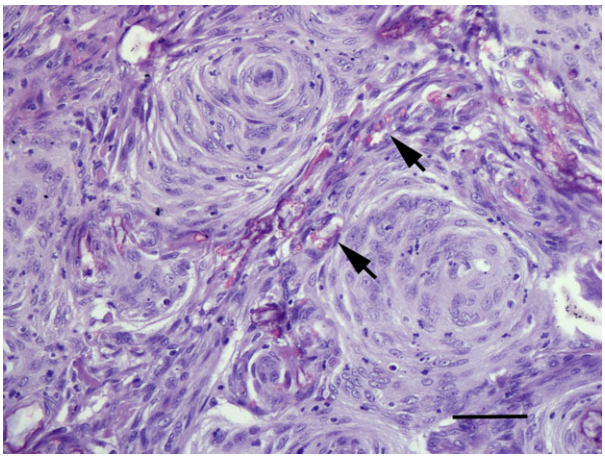

Fig 1. Brain grade I meningioma. Transitional histotype characterized by the prevalence of whirls and diffuse calcifications (arrows) (H&E; Bar = 90 μm).

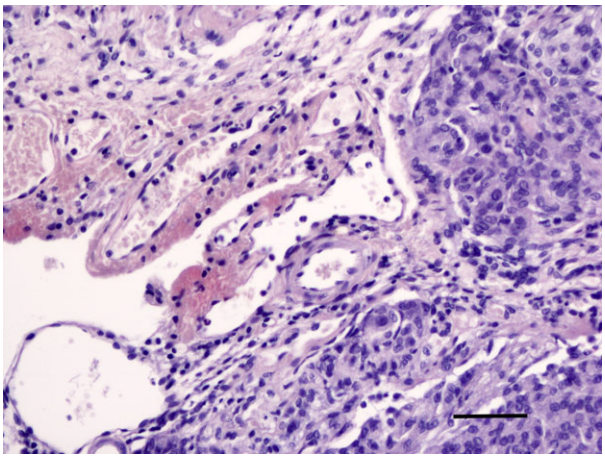

Fig 2. Brain grade II meningioma. Meningothelial histotype showing an aggressive behavior to the cerebral cortex (H&E; Bar = 90 μm).

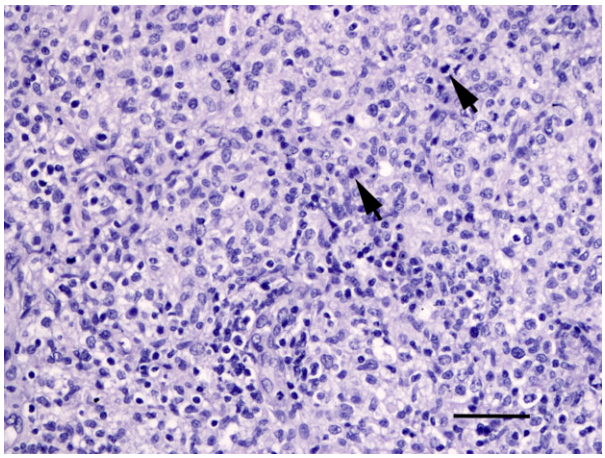

Fig 3. Brain grade III meningioma. The tumor shows a solid pattern associated with high mitotic index (arrows) (H&E; Bar = 130 μm).

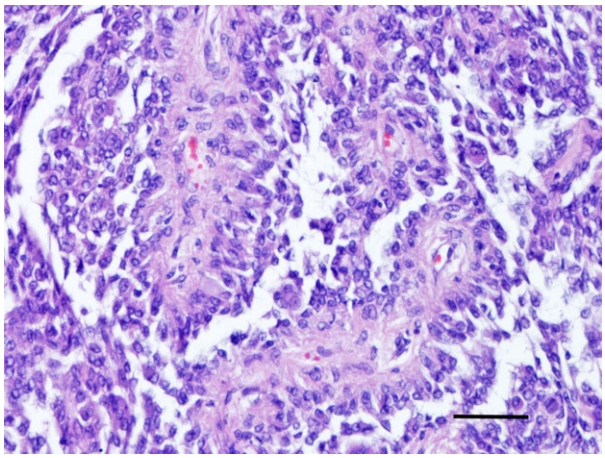

Fig 4. Brain papillary meningioma. The tumor is characterized by a perivascular pseudopapillary pattern (H&E; Bar = 100 μm).

13/43 grade I meningiomas (30.2%; Fig 1), 14/43 grade II meningiomas (32.5%; Fig 2), and 6/43 grade III meningiomas (13.9%; Fig 3). They consisted of 23 brain tumors and 10 spinal cord tumors. The remaining 10 meningiomas, consisting of brain tumors, were diagnosed as the papillary histotype (23.2%; Fig 4). Except for 7 cases submitted for histologic examination in the absence of adjacent nervous tissue, for which we were unable to evaluate infiltration, infiltration was present in 11/28 (39.2%) brain tumors and in 5/8 (62.5%) spinal cord tumors. As for the papillary histotype, infiltration was confirmed in 5 of 9 evaluable cases (55.5%).

Qualitative and Quantitative Assessment of RNA

Mean RNA integrity number (RIN) assessed with an Agilent 2100 Bioanalyzer was 8.7 (range, 8.3–9.0). Total recovery of RNA was not significantly different among samples and the RNA yield varied from 3.5 to 8 ng/μL. Minimal variations in total RNA content were detected during reverse transcription using fixed RNA input (20 ng/total RNA).

mRNA Quantification by Real-Time PCR Analysis

The mRNA expression of MMP-9 was not valuable in normal adjacent tissue (NAT) or in the meningiomas submitted to the study. Storage FFPE mammary gland tumors and artificial plasmids gave the expected MMP-9 real-time PCR product lengths.

As for MMP-2, mRNA expression levels did not change significantly in different grades (Fig 5, Table 2). On the contrary, MMP-2 mRNA was significantly more expressed in the papillary meningioma compared to grade I ($P < 0.0001$), grade II ($P < 0.0001$), or grade III ($P < 0.0001$; Fig 5; Table 2). Expression of TIMP-2 significantly increased from grade I to grade II ($P = 0.0031$). Additional significant variations were not

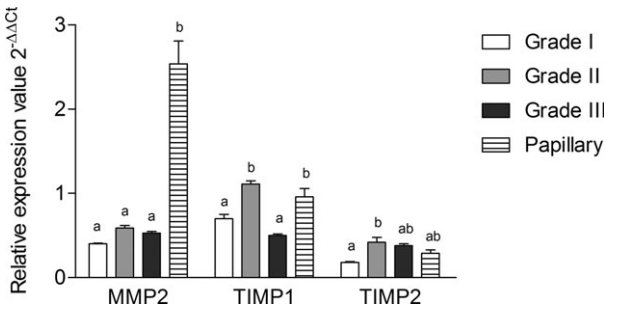

Fig 5. Gene expression levels referred to MMP-2, TIMP-1, TIMP-2 in grade I, grade II, grade III, and papillary meningiomas are indicated (y) as mean ± SD of $2^{-\Delta\Delta Ct}$ [normalization of mRNA levels to reference gene and NAT]. [a,b,c]Different letters indicate statistically significant differences ($P < 0.05$). Equivalent letters indicate not statistically significant differences.

observed (Fig 5). As for TIMP-1 expression, mRNA expression significantly increased from grade I to grade II ($P < 0.0001$) whereas it significantly decreased from grade II to grade III ($P < 0.0001$). Significant variations were not observed from grade I to grade III (Fig 5). In the papillary meningiomas, TIMP-1 expression was somewhat similar to that observed in grade II ($P = 0.2625$), whereas it was higher than grade I ($P = 0.0209$) and grade III ($P = 0.0003$; Fig 5) expressions. The MMP-2/TIMP-2 mRNA ratio was 2.22 in grade I tumors, 1.40 in grade II tumors, 1.39 in grade III tumors, and 8.76 in the papillary meningiomas, respectively (Table 2). The MMP-2/TIMP-2 ratio was numerically higher in the papillary meningioma compared to all other grades (>3.5 times).

In normal adjacent tissue (NAT), MMP-2, TIMP-2, and TIMP-1 mRNA expressions were not significantly different.

Discussion

In the last 20 years, involvement of MMPs in the progression of several tumors in humans[34–37] and dogs[38–40] has been demonstrated. However, results for meningioma still are incomplete or contradictory. To date, in meningiomas of dogs, MMPs have been investigated with phenotyping studies (e.g., immunohistochemistry) instead of genotyping studies,[28,29] and specific investigations of MMP/TIMP ratios have not been reported. The MMP-2/TIMP-2 expression ratio has been considered a critical factor in the invasion and metastasis of mouse renal cell carcinoma,[41] and MMP-2 and TIMP-2 polymorphisms have been considered genetic mechanisms of meningioma pathogenesis in humans.[42]

We investigated mRNA expression of MMP-2 and MMP-9 and correlated TIMP-2 and TIMP-1, respectively, in different grades of meningiomas in dogs. Special consideration has been given to the papillary meningioma for which an aggressive behavior is known in humans[9] and is suspected in the dog,[15] despite its benign histologic appearance.

Matrix metalloproteinase-9 was not identified in any of the selected cases. The assessment of RNA purity indexes excluded the possibility of RNA fragmentation resulting in an incorrect evaluation in real-time PCR. However, this finding needs to be interpreted with caution because MMP-9 phenotyping expression previously has been reported in meningiomas of dogs.[28,29] As reported in humans,[43] this result might be justified considering the potential for genetic single nucleotide polymorphisms of the MMP-9 gene, making it not traceable. Moreover, the observation that in NAT MMP-9 mRNA expression was much lower as compared to the housekeeping gene supports down regulation of the MMP-9 gene in the central nervous tissue of dogs as occurs in humans.[44] It might also be that in our study, rare transcripts were affected more than abundant transcripts[45] or that paraffin embedding had negative effects on evaluation of minimally expressed mRNA. In the absence of MMP-9 expression, consistent TIMP-1 mRNA expression is of doubtful relevance. It is feasible that a generalized increase in expression of TIMP-1 in all of the tumors resulted in MMP-9 expression that could not be evaluated. On the other hand, additional investigations to determine whether canine meningioma TIMP-1 also might play an independent role in increasing the proliferative activity of neoplastic cells or have an antiapoptotic effect[46] are warranted. The consistent increase in TIMP-1 mRNA expression from grade I to grade II meningiomas, but not from grade II to grade III tumors, seems to support different pathways in the neoplastic progression.

Table 2. Gene expression of MMP-2, TIMP-1, and TIMP-2, and data analysis referred to different grades of tumor. Real-time data are expressed as a mean ± SD of $2^{-\Delta\Delta Ct}$.

	Gene Expression				MMP-2, TIMP-1, and TIMP-2 Data Analysis			
	Grade I	Grade II	Grade III	Papillary Meningioma	RSD	*P*	F	df
MMP-2	0.40[a]	0.59[a]	0.53[a]	2.54[b]	3.39	<0.0001	98.59	3–41
TIMP-1	0.70[a]	1.11[b]	0.50[a]	0.96[b]	1.37	<0.0001	23.31	3–41
TIMP-2	0.18[a]	0.42[b]	0.38[ab]	0.29[ab]	1.27	<0.005	4.96	3–40
MMP-2/TIMP-2	2.22	1.40	1.39	8.76				

[a,b,c]Values within a row differ significantly at $P < 0.05$. Different letters indicate statistically significant differences, whereas equivalent letters indicate not statistically significant differences. RSD, residual standard deviation of variance analysis; df, denominator degree of freedom; F, Fisher's test; *P*, *P*-value.

Contrarily to MMP-9, MMP-2 mRNA was expressed in all of the selected tumors. However, statistically significant differences among the tumor grades were not observed. This result seems to suggest that MMP-2 expression from neoplastic meningeal cells is not strictly correlated with histologic malignancy. Even though a number of studies in veterinary and human medicine support a role for MMP-2 in neoplastic progression,[34,39,47–49] these results are consistent with previous data about meningiomas in dogs.[29] As for human meningiomas, the literature is contradictory. In the last few years, the role played by MMP-2 and MMP-9 in neoplastic progression of meningioma in humans has been considered marginal[50] to basic,[51] and there is more support for the ratio of metalloproteinases to their inhibitors to be more specifically involved in extracellular matrix enzymatic degradation. Most recently, a marked imbalance between MMP-2 and TIMP-2 has been more closely associated with meningioma progression.[42] In our study, descriptive statistics did not show significant differences of MMP-2/TIMP-2 ratio associated with the morphologic progression of tumors, perhaps suggesting alternative molecular pathways involved in MMP-2 expression or biologic variation between meningiomas in dogs and humans. On the contrary, in papillary meningioma, we found the MMP-2/TIMP-2 ratio numerically higher than in other grades. In humans, despite its moderate morphologic evidence of malignancy and given its aggressive clinical behavior, the papillary meningioma is graded as WHO grade III. Based on follow-up data (postsurgery survival time and survival time in the absence of surgery), a more aggressive biologic behavior of papillary meningioma recently has been suspected in dogs,[15] supporting that the papillary meningioma should be studied as a histotype separate from grades I, II, and III. The excessive expression of MMP-2 compared to TIMP-2 in this histologic variant of meningioma seems to confirm its more aggressive biologic behavior also associated with high recurrence rate in the dog.[15]

In our study, grade I and grade II meningiomas accounted for 30% and 32%, respectively. Even though this result is different from that reported in the literature (from 47[52] to 57%[3] for grade I, from 46[52] to 43%[3] for grade II), it seems to be consistent with the fact that in our study the papillary meningioma was excluded from histologic grading. On the contrary, compared to previous data in dogs,[3,52] a consistent increase in grade III meningioma was recorded.

Our study represents a first effort to clarify the genetic expression profile of meningiomas in dogs and to identify MMP/TIMP ratios as biomolecular markers that reflect the neoplastic progression of this neoplasm. In this context, our results further support the malignant behavior of the papillary meningioma and strongly suggest that specific therapeutic and prognostic evaluation of meningiomas in dogs needs to be applied to different histologic patterns as in humans.

Footnotes

[a] Ambion, Austin, Tx
[b] Bio-Rad, Berkeley, CA
[c] Life Technologies
[d] Applied Biosystems, Carlsbad, CA
[e] GraphPad Software, La Jolla, CA

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Conflict of Interest Declaration: Authors declare no conflict of interest.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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