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Expression of Matrix Metalloproteinases, Tissue Inhibitors of Metalloproteinases and Vascular Endothelial Growth Factor in Canine Mast Cell Tumours

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Summary

Degradation of the extracellular matrix and angiogenesis are associated with tumour invasion and metastasis in human and canine neoplasia. Matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs) and vascular endothelial growth factor-A (VEGF-A) are key mediators of these respective processes. Mast cell tumour (MCT) is the most common malignant cutaneous tumour in dogs. MCTs are always consid-ered potentially malignant, but their true metastatic potential is unknown. In the present study, samples from seven grade 1, 22 grade 2 and six grade 3 MCTs were subjected to quantitative real-time polymerase chain reaction and immunohistochemistry (IHC) to evaluate MMP-2, MMP-9, membrane-type 1 MMP (MT1-MMP), TIMP-2 and VEGF-A mRNA and protein expression. Gelatin zymography (GZ) was also performed to evaluate MMP-2 and MMP-9 activity. MMP-9 and VEGF-A mRNA increased with histological grade, while TIMP-2 decreased with increasing grade. Gene expression data obtained for MMP-9, VEGF-A and TIMP-2 were confirm ed by IHC for evaluation of the respective proteins. In contrast, MMP-2 and MT1-MMP had variable, but similar, expression for both mRNA and protein. Despite the high variability observed, there was correlation between MMP-2 and MT1-MMP mRNA expression ($r = +0.91$, $P < 0.0001$). The MMP-2:TIMP-2 and MMP-9:TIMP-1 mRNA ratios showed an imbalance between MMPs and their specific inhibitors in MCTs, which increased with the histological grade. Finally, the activities of both latent and active forms of MMP-2 and MMP-9 were evaluated by GZ and there were significant increases in their activities with increasing histological grade and

immunohistochemical expression. This study demonstrates that MMP-9, TIMP-2 and VEGF-A expression is related to histological grade and suggests that these markers are possible indicators of malignancy and targets for therapeutic strategies.

Introduction

The degradation of the extracellular matrix (ECM) and vascular angiogenesis are key factors for the growth and progression of tumours (Liotta *et al.,* 1991; Kim *et al.,* 1993; MacDougall and Matrisian, 1995; Stetler-Stevenson, 1996; Kawai *et al.,* 2006; Nakaichi *et al.,* 2007).The invasion of a tumour relies on extracellular proteolysis, which is largely mediated by matrix metalloproteinases (MMPs) produced by neoplastic cells. MMPs are a family of zinc-containing, calcium-dependent endopeptidases secreted by various cell types and implicated in a wide range of physiological events (e.g. trophoblast inva-sion, development, endometrial remodelling, ovula-tion, angiogenesis, wound healing, bone resorption and cell migration) as well as in pathological processes (Loukopoulos *et al.,* 2003; Nagel *et al.,* 2004). In veterinary medicine, the role of MMPs has been evaluated in canine meningitis-arteritis (Schwartz *et al.,* 2010), chronic valvular disease (Aupperle *et al.,* 2009), arthritis (Muir *et al.,* 2007) and tumours (Loukopoulos *et al.,* 2003; Takagi *et al.,* 2005) including oronasal tumours (Nakaichi *et al.,* 2007), mammary tumours (Hirayama *et al.,* 2002; Papparella *et al.,* 2002; Kawai *et al.,* 2006; Aresu *et al.,* 2011) and mast cell tumours (MCTs; Leibman *et al.,* 2000). In these tumours, invasion and metastasis have been associated with MMP-mediated breakdown of the ECM.

Within the MMP family, the group of gelatinases or type-IV collagenases, including MMP-2 (gelatinase-A), MMP-9 (gelatinase-B) and membrane-type 1 MMP (MT1-MMP), are studied most often. Like all MMPs, MMP-2 and MMP-9 are produced as zymo-gens, which are activated by protein cleavage to be-come proteolytic enzymes (Loukopoulos *et al.,* 2003). MT1-MMP was the first MT-MMP identified, as a major physiological activator of pro-MMP-2 (Sato *et al.,* 1994). MMP proteolytic activity is controlled at both the mRNA and protein levels by specific en-dogenous inhibitors, the tissue inhibitors of metallo-proteinases (TIMPs) (Gomez *et al.,* 1997). TIMP-2 is the specific inhibitor of MMP-2, but there is evidence that the process of pro-MMP-2 activation requires the formation of a ternary complex consisting of the C-terminal domain of pro-MMP-2, TIMP-2 and MT1-MMP (Papparella *et al.,* 2002). The balance between MMPs and TIMPs appears to be important for ECM regulation and homeostasis (Aupperle *et al.* 2009).

The infiltrative growth of tumours also depends on angiogenesis. Tumours may produce blood vessel growth-stimulating factors such as vascular endothelial growth factor (VEGF, also called VEGF-A), which acts as a potential autocrine growth factor for neoplas-tic cells. VEGF is a homodimeric heparin-binding protein with several different isoforms generated by alternative splicing (Ladomery *et*

al., 2007). In physiological conditions, VEGF is essential for the formation of blood vessels in embryos, induces vascular perme-ability and stimulates endothelial cell proliferation (Wolfesberger *et al.*, 2007). It also stimulates abnormal angiogenesis in solid tumour growth and metastasis (Ribatti *et al.*, 1999; Takanami *et al.* 2000; Elpek *et al.*, 2001; Ranieri *et al.*, 2002, 2003; Iamaroon *et al.* 2003; Kondo *et al.*, 2006; Tuna *et al.*, 2006). VEGF expression has been evaluated in canine mammary carcinoma (Restucci *et al.*, 2002; Qiu *et al.*, 2008), seminoma (Restucci *et al.*, 2003), intracranial meningi-oma (Matiasek *et al.*, 2009) and lymphoma (Gentilini *et al.*, 2005; Wolfesberger *et al.*, 2007, 2008). Crosstalk between VEGF and MMPs has recently been described: VEGF signalling via VEGFR-2 induces the expression of MMP-2, MMP-9 and MT1-MMP, which degrade the matrix to allow for endothelial sprouting (Funahashi *et al.*, 2011).

MCTs are the most common cutaneous tumour of the dog (Thamm and Vail, 2007). MCTs are always considered potentially malignant, but their true met-astatic potential is not entirely known (Welle *et al.* 2008). Currently, the prognostic significance of MCT is assigned through histological grading, but numerous studies have shown significant differences between well- and poorly-differentiated MCTs in terms of survival times and disease-free intervals. In particular, the prognosis for intermediate MCT (grade 2) is difficult to predict: the tumour might be-have in a benign fashion or recur and metastasize. Well-differentiated tumours have a metastatic rate of <10%, intermediate tumours are considered low to moderate in metastatic potential and undifferenti-ated tumours have a much higher metastatic rate (55e96%; Welle *et al.*, 2008). Uncontrolled cellular proliferation plays a significant role in the progression of canine MCTs (Sakai *et al.*, 2002; Scase *et al.*, 2006; Webster *et al.*, 2007; Gil da Costa *et al.*, 2007). Despite these issues, information about ECM degradation and vascular angiogenesis in canine cutaneous MCT is sparse. MMPs have been evaluated only at the catalytic activity level through gelatin zymography (GZ) in samples from grade 2 and 3 tumours (Leibman *et al.*, 2000) and VEGF has been detected at the protein level by immunohistochemis-try (IHC) (Mederle *et al.*, 2010) and by enzyme-linked immunosorbent assay (ELISA) analysis (Patruno *et al.*, 2009). Thus, in order to better under-stand the role of ECM degradation and angiogenesis in canine MCTs of different histological grades, the aim of the present study was to evaluate the mRNA and protein expression of MMP-2, MMP-9, MT1-MMP, TIMP-2 and VEGF-A in MCTs subdivided by histological grade (Patnaik *et al.*, 1984). The catalytic activities of both latent and active forms of MMP-2 and MMP-9 were also evaluated.

Materials and Methods

Case Selection and Tissue Sampling

Fresh tissue samples were obtained from 35 dogs that underwent surgery for MCT. The consent of the owners to use the tissues for research purposes was obtained. Only samples of suitable size and

quality were included in the study. After excision of the entire tu-mour, multiple samples (50e100 mg) were collected from the central core of the mass. For total RNA iso-lation, aliquots were immersed in RNAlater solution (Applied Biosystems, Foster City, California, USA) and stored at -20°G until used. For GZ, aliquots were frozen at -20°G until used. The remaining tissue was formalin-fixed and paraffin wax-embedded for microscopical examination and IHC.

Gene Expression

Total RNA was isolated using TRIzol reagent (In-vitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. The samples were purified with classical phenolechloroform extraction. The total RNA concentration and quality were evaluated with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) and by denaturing gel electrophoresis.

First-strand cDNA was synthesized from 2 mg of total RNA in a final volume of 20 ml using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol and stored at -20°G until used. The cDNA was used as a template for the quantification of MMP-2, MMP-9, MT1-MMP, TIMP-2 and VEGF-A by quantitative real-time reverse transcriptase polymerase chain reaction (qPCR) in a Light Cycler 480 Instrument (Roche Diagnostics, Basel, Switzerland) using standard PCR conditions. Canine VEGF-A (GenBank reference sequences: NM_001003175.2, NM_001110502.1 and NM_001110501.1) was ampli-fied with 5^0-CGT GCC CAC TGA GGA GTT-3^0 (for-ward primer, 300 nM final concentration) and 5^0-GCC TTG ATG AGG TTT GAT CC-3^0 (reverse primer, 300 nM) and human Universal Probe Library (UPL) probe number 9 (100 nM), while ca-nine MMP-2, MMP-9, MT1-MMP, TIMP-2 and the reference genes transmembrane Bcl-2-associated X protein inhibitor motif containing 4 (CGI-119) and golgin A1 (GOLGA-1) were amplified with the primer pairs and UPL probes reported in Aresu *et al.* (2011). The qPCR assay was performed as described by Aresu *et al.* (2011). Calibration curves revealed PCR efficiencies close to 2.0 and error values < 0.2. Reference gene amplification revealed the absence of significant differences in expression profile between groups. Crossing point (CP) values were obtained using the Light Cycler 480 software release 1.5.0 (Roche Diagnostics) and the second derivative maximum method (Rasmussen, 2001). The normalization of qPCR target genes in each sample was performed us ing firstly the arithmetic mean of reference gene C_P values ($\grave{A}Gp_{sample} = C_{P\ target\ gene} — mean\ C_{P\ reference\ genes}$) and secondly using the ÀGp of a calibrator sample ($\grave{A}\grave{A}C_{P\ sample} = AG_{P\ sample} - AG_{P\ calibrator}$). Finally, relative quantification values (RQ) were calculated using the following formula: $RQ_{sample} = 2\ ''^{AACP\ sample}$ (Livak and Schmittgen, 2001).

Immunohistochemistry

Serial sections (4 mm) on charged slides were dewaxed for IHC. Primary antibody incubation was performed by an automated system (Ventana Medical Systems, Tucson, Arizona, USA). The details of IHC for MMP-2, MMP-9, MT1-MMP and TIMP-2 have been described previously (Aresu *et al.*, 2011). A polyclonal rabbit antiserum specific for VEGF (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA; diluted 1 in 200) was also used. Subsequent incubation with biotinylated anti-mouse IgG secondary antibody, the addition of $3,3^0$-diaminobenzidine substrate and the haematoxylin counterstain were performed using the automated system. Negative control slides were incubated with isotype-matched immunoglobulin.

Ten fields on each slide in which labelling was apparent were evaluated at x400 magnification. Labelling was considered negative or positive according to whether there was cytoplasmic expression of the target molecule. The intensity and the percentage of labelled tumour cells were recorded. All evaluations were performed in blinded fashion by three independent pathologists (LA, SB and AA) .Discordant samples were re-evaluated by the investigators and a consensus evaluation was used for definitive analysis. An intensity score of 0 was given if no labelling was detected, 1 if there was weak to moderate labelling, 2 if moderate to strong labelling was present and 3 if strong labelling was detected. A total score for each field examined was obtained by multiplying the intensity score by the percentage of labelled cells. A final ratio (mean i standard deviation) was obtained after averaging the 10 selected fields. An image analysis system that consisted of an Olympus BX51 microscope and software analysis (analySIS, Soft im-aging system, Muenster, Germany) was used.

Gelatin Zymography

The gelatinase activities of both latent (proenzymes or zymogens) and mature MMP-2 and MMP-9 were evaluated by GZ (Aresu *et al.*, 2011). Culture medium conditioned by A2058 melanoma cells was used as a control to identify the pro-MMP-9 gelatinolytic band, while conditioned media from HT1080 fibrosarcoma cells was used for the active forms of MMP-2 and MMP-9 and small amounts of pro-MMP-2 (Davies *et al.*, 1993). The activities of MMP-9 and MMP-2 in 20 ml of conditioned media from the two respective cell lines were defined as 100 arbitrary units (AU). The bands were quantified using an image analysis system that consisted of a Gel-Doc 2000 and Quantity One software (Bio-Rad, Hercules, California, USA).

Statistical Analysis

Statistical analysis of the gene expression and GZ data was performed using the non-parametric KruskaleWallis test followed by Dunn's post test. The correlation between target gene mRNA data and between mRNA and protein activity results was performed using a Spearman non-parametric test. For all of these analyses, GraphPad InStat 2.01 software (San Diego, California, USA) was used and a *P*

value < 0.05 was considered significant.

To compare the IHC scores, differences in percent-ages were calculated with the Chi-square test. The SPSS 17.00 (SPSS Inc., Chicago, Illinois, USA) pro-gramme was used.

Results

The study included samples from 35 MCTs from 35 dogs with a median age of 7.9 ±2.7 years. Of the 35 tumours, seven were histological grade 1, 22 were grade 2 and six were grade 3. Male (14; three neu-tered) and female (17; two neutered) dogs were included in the study and in four cases the gender was not recorded. The following breeds were represented: crossbred (10), boxer (6), Labrador retriever (5), dogo Argentino (2), golden retriever (2), American Staffordshire bull terrier (1), dachshund (1), beagle (1), Boston terrier (1), cocker spaniel (1), Dalmatian (1), dogue de Bordeaux (1), rottweiler (1), English setter (1) and Siberian husky (1).

Gene Expression

All of the target genes were expressed in each MCT sample (Table 1). Between tumour grades, significant differences were found only for TIMP-2 where less TIMP-2 mRNA was found in grade 3 compared with grade 2 tumours (P<0.05). In contrast, there was a progressive, but not significant, increase in MMP-9 and VEGF-A expression with increasing histological grade. MMP-2 and its specific activator (MT1-MMP) showed the same gene expression pro-file ($r = +0.91$, $P < 0.0001$; Fig. 1). The MMP-2:TIMP-2 and MMP-9:TIMP-1 mRNA ratios were also determined. Progressive, but not significant, increases in both ratios were observed with increasing tumour grade (Fig. 2).

Table 1

MMP-2, MMP-9, MT1-MMP, TIMP-2 and VEGF-A mRNA expression

	mRNA expression (AU)		
	Grade 1	*Grade 2*	*Grade*
MMP-2	2.54 ±0.68	7.45 ±3.50	2.03 ± 1.61
MMP-9	0.62 ±0.20	1.27 ±0.39	5.66 ±3.67
MT1-MMP	**1.19** ±0.22	3.43 ± 1.42	1.40 ±0.66
TIMP-2	1.14±0.29	1.69±0.25[c]	0.54±0.21
VEGF-A	0.86 ±0.37	1.56 ±0.42	1.92 ±0.75

Data are expressed as the mean ± standard error.

[a, b, c]Significant differences between grade 1 and 2, grade 1 and 3 and grade 2 and 3, respectively (KruskaleWallis test followed by Dunn's post test; c: P<0.05).

Immunohistochemistry

MMP-2 and MMP-9 were present in all of the tumours examined (Table 2). Both molecules were lo-calized to the cytoplasm of mast cells, while eosino-phils appeared negative. MMP-9 expression was diffuse, while MMP-2 expression was multifocal (Fig. 3). Significant differences in the IHC scores were observed for MMP-9 between the three grades of MCT ($P<0.05$). The most intense expression was in grade 3 MCTs (Fig. 4 and Fig. 1 supplementary material). The same behaviour was also observed for MMP-2, but this did not reach statistical significance (Fig. 3 and Fig. 1 supplementary material). MT1-MMP protein was observed in all tumours with cytoplasmic immunolabelling of few scat-tered neoplastic cells (data not shown). MT1-MMP expression increased with tumour grade without reaching statistical significance. In contrast, TIMP-2 expression was stronger in grade 1 than in grade 3 MCTs ($P<0.01$) (Fig. 5 and Fig. 1 supplementary material). Finally, VEGF-A expression was stronger in grade 3 than in grade 1 MCTs ($P < 0.001$; Fig. 6 and Fig.1 supplementary material). Labelling was mainly of the cytoplasm of neoplastic cells, in particular those cells adjacent to blood vessels.

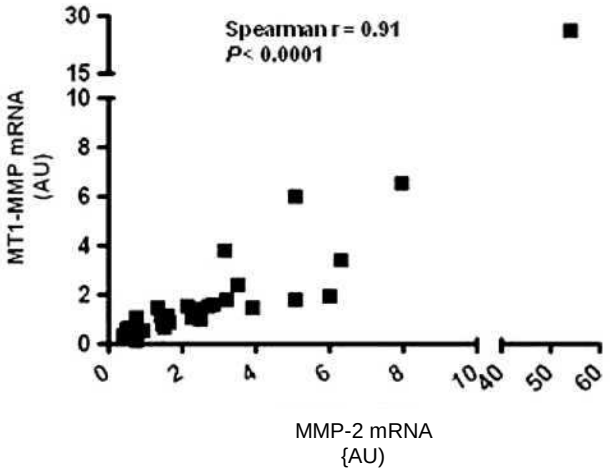

Fig. 1. Spearman correlation analysis between MMP-2 and MT1 -MMP mRNA expression.

Table 2

MMP-2, MMP-9, MT1-MMP, TIMP-2 and VEGF-A IHC

	IHC score			*Significance*
	Grade 1	*Grade 2*	*Grade 3*	
MMP-2	34.1 ± 29.2	78.3 ± 39.1	159.0 ± 21.2	ns
MMP-9	91.9 ± 10.3	187.2 ± 54.3	279.3 ± 23.2	$P < 0.05$
MT1-MMP	45.3 ± 23.0	103.4 ± 25.2	183.3 ± 31.4	ns
TIMP-2	182.4 ± 12.2	143.2 ± 17.8	97.3 ± 10.3	$P < 0.01$
VEGF-A	106.6 ± 9.6	201.3 ± 15.5	291.3 ± 4.7	$P < 0.001$

ns, not significant.

Gelatin Zymography

Active and inactive forms of MMP-9 and MMP-2 were measured by GZ (Fig. 7). The activities of MMP-9 and MMP-2 were calculated in AU per 10 ng of protein. The pro-MMP-9 band was present in all samples examined by GZ. The activity range in grade 1 tumours was 2.3e60.2 AU, in grade 2 tumours 71.0e134.3 AU and in grade 3 MCTs 151.2e218.9 AU. Thirty-four tumours exhibited bands for the active form of MMP-9. The activity ranged from 0 to 40.3 AU for grade 1, 54.3e192.6 AU for grade 2 and 160.3e248.0 AU for grade 3 tumours. Pro- and active-MMP-9 activities progres-sively increased from grade 1 to 3 MCTs. Both were higher in grade 3 than in grade *2 (*$P < 0.05$), in grade3 than in grade 1 (*P* < 0.001) and in grade 2 than in grade 1 tumours *(P<0.01;* Fig. 7B). Furthermore, the activity of the MMP-9 latent form was significan tly higher than that of the active form (*P* < 0.05) in grade 2 and 3 MCTs. Densitometric values of the active form of MMP-9 were also correlated to MMP-9 mRNA expression (*r* = 0.37, *P* < 0.05).

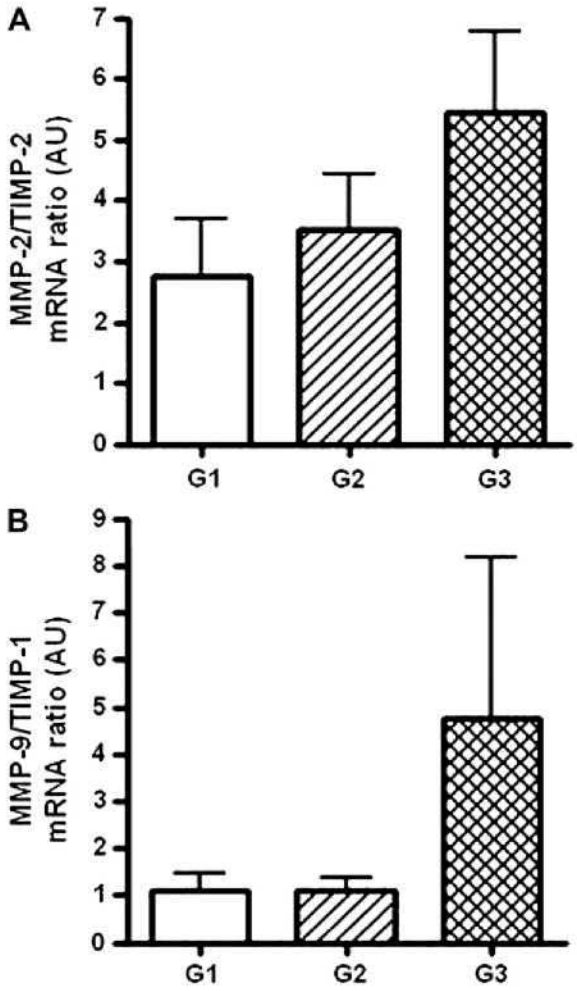

Fig. 2. (A) MMP-2:TIMP-2 and (B) MMP-9:TIMP-1 mRNA ratios in grade (G) 1, 2 and 3 MCTs. Data (mean ± standard error) are expressed in AU. Statistical analysis: Kruskale Wallis test followed by Dunn's post test.

Bands for the latent and active forms of MMP-2 were found in 97% and 94% of MCTs, respectively. A progressive increase in MMP-2 gelatinolytic activity with histological grade was observed. The activity of pro-MMP-2 was 0e31.4 AU, 28.7e164.0 AU and 65.3e149.7 AU in grade 1, 2 and 3 tumours, respectively. The activity of active-MMP-2 was 0e23.9 AU, 13.2e169.7AU and 70.8e161.2 AU in grade 1, 2 and 3 MCTs, respectively. Significant differences were obtained for pro-MMP-2 in grade 1 versus grade 2 and in grade 1 versus grade 3 ($P < 0.001$) and for active-MMP-2 in grade 1 versus grade 2 ($P<0.01$) and in grade 1 versus grade 3 ($P < 0.001$) (Fig. 7C). Pro-MMP-2 activity was significantly higher than active-MMP-2 only in grade 1 samples ($P < 0.05$).

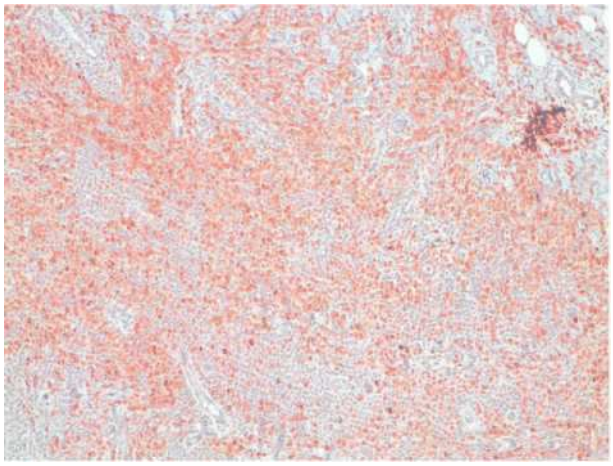

Fig. 3. Grade 3 MCT showing intense expression (multifocal to coalescing distribution) of MMP-2. IHC.x200.

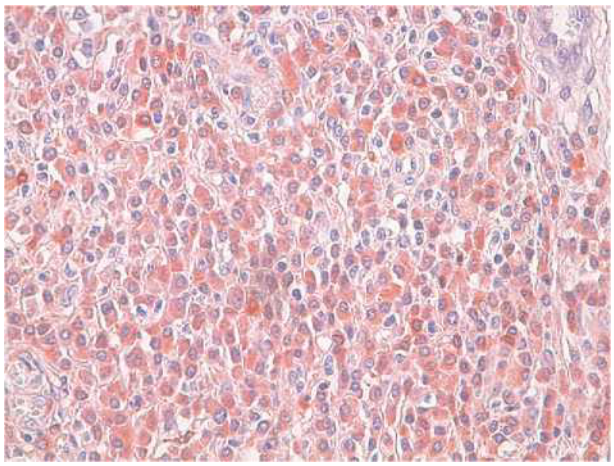

Fig. 4. Grade 3 MCT showing intense and diffuse expression of MMP-9. IHC. x400.

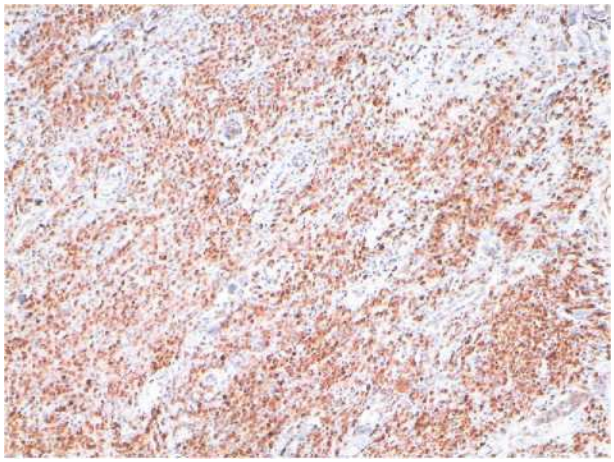

Fig. 5. Grade 1 MCT showing intense and diffuse expression of TIMP-2. IHC. x200.

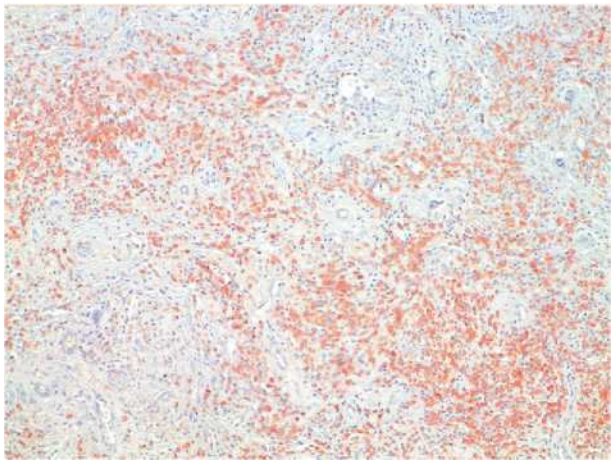

Fig. 6. Grade 3 MCT showing expression of VEGF antigen by tumourcells. IHC. x200.

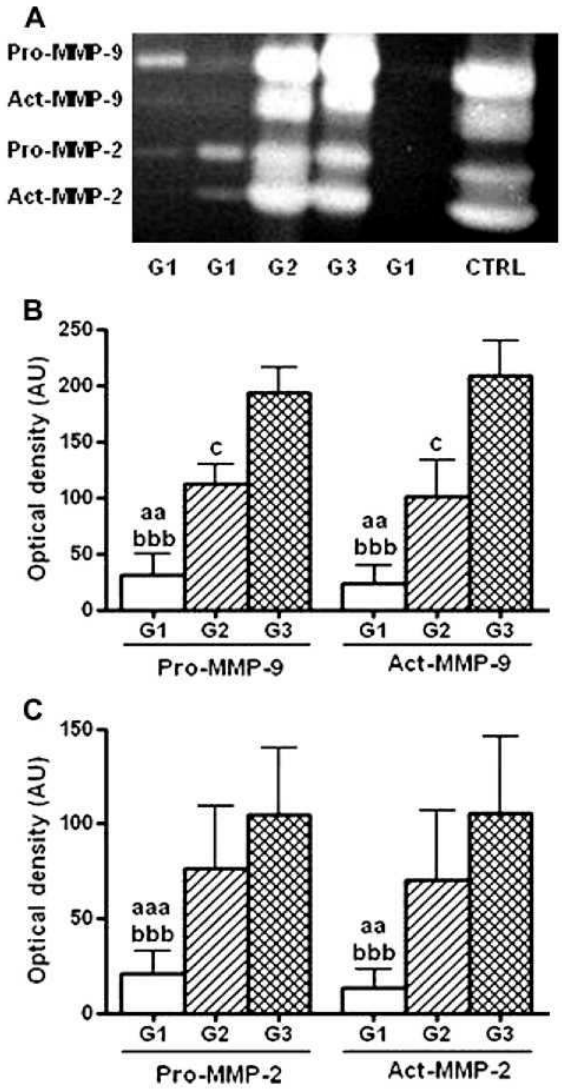

Fig. 7. (A) Zymographic assay of gelatinase activity in grade 1, 2 and 3 MCTs and in HT1080 fibrosarcoma cells (CTRL). Bands corresponding to both latent (pro-) and active (act-) forms of MMP-9 and MMP-2 were observed in MCTs. HT1080 fibrosarcoma cells were used as the control for pro-MMP-9, pro-MMP-2 and act-MMP-2. (B and C) Densitometric analysis of the bands corresponding to latent and active forms of MMP-9 (B) and MMP-2 (C) in grade 1, 2 and 3 MCTs. Integrated density values for each group (mean ± standard deviation) are expressed as AU as a per-centage of the density recorded in CTRL (100 AU). [a, b, c]Significant differences between grade 1 and 2, grade 1 and 3 and grade 2 and 3, respectively (KruskaleWallis test followed by Dunn's post test; c: $P<0.05$; aa: $P<0.01$;aaa, bbb: $P< 0.001$).

Discussion

Dogs are at risk for cutaneous MCT, which accounts for up to 21% of all skin tumours (Thamm and Vail, 2007). The diagnosis of MCT by cytology or his-topathology is straightforward in the majority of cases, but forming an accurate prognosis is more challenging (Welle *et al.*, 2008). The biological behaviour of MCT is highly variable, in particular for intermediate-grade tumours. Some

intermediate-grade tumours have a benign behaviour, while others exhibit aggressive growth and a high frequency of local and distant recurrence (Welle *et al.,* 2008).

Degradation of the ECM and basement membrane components and angiogenesis act in a coordinated manner in the growth and progression of several hu-man and animal tumours (Loukopoulos *et al.,* 2003; Patruno *et al.,* 2009). These complex processes are mainly regulated by MMPs and their specific inhibitors (ECM and basement membrane degradation) and VEGF (angiogenesis). Few data are available on the roles of MMPs and VEGF-A in canine MCTs (Leibman *et al.,* 2000; Loukopoulos *et al.,* 2003; Patruno *et al.,* 2009; Mederle *et al.* 2010). To the best of our knowledge, this is the first study describing the expression of MMP-2, MMP-9, MT1-MMP, TIMP-2 and VEGF-A in canine MCTs using an integrated approach from mRNA to protein, by means of both qPCR and IHC. GZ was also performed to evaluate MMP-2 and MMP-9 gelatinolytic activity. MMP-2 and MMP-9 have been identified by GZ in 24 cases of canine MCT of histological grades 2 and 3 (Leibman *et al.,* 2000) and in two cases of oral multifocal MCT by GZ and IHC (Loukopoulos *et al.* 2003). In those studies, normal tissue adjacent to the tumour was also considered. In the present study the activity of both latent and active forms of MMP-9 and MMP-2 was evaluated in grade 1, 2 and 3 MCTs and these data were compared with IHC and qPCR results. Both latent and active forms of MMP-9 and MMP-2 were found in almost all tumours and proen-zyme activity was generally higher than that of the active form for both gelatinases. GZ showed a signifi-cant increase of the pro- and active forms of MMP-9 and MMP-2, correlated with histological grade, as previously observed by Leibman *et al.* (2000) in canine MCT and by Loukopoulos *et al.* (2003) in other tumour types. In general, the production of gelatinases is correlated with its biological behaviour: tumours produce more MMP-9 and MMP-2 than non-neoplastic tissue and malignant tumours produce significantly more MMP than their benign counter-parts (Loukopoulos *et al.,* 2003). The grade-dependent expression observed through zymography was confirmed at the mRNA and protein levels. Increased MMP-9 mRNA expression was correlated with IHC score and with act-MMP-9 optical density by GZ. In contrast, MMP-2 expression was more variable: despite a grade-dependent increase in MMP-2 activity, the immunohistochemical expression was of multifocal distribution and there were no significant differences between grades in terms of percentage cells labelled or intensity of labelling. Moreover, there was high variability at the level of gene expression for MMP-2.

The results obtained here for MMP-2 are in contrast with previously published data. The invasion and malignant potential of many canine solid tumours (e.g. lymphoma, mammary tumours and oro-nasal tumours) have been associated with increased expression of MMP-2 (Hirayama *et al.,* 2002; Papparella *et al.,* 2002; Loukopoulos *et al.,* 2003; Gentilini *et al.,* 2005; Nakaichi *et al.,* 2007; Aresu *et al.,* 2011), while a minor role for MMP-9 in these tumours has been hypothesized because of its wide expression in normal cells, including macrophages and vascular smooth muscle cells (Nakaichi

et al., 2007) or for the fact that the active form of MMP-9 was identified in only three cases among 51 tumours (Loukopoulos *et al.*, 2003). In contrast, the results of the present study might suggest that MMP-9, rather than MMP-2, represents the key factor in ECM degradation by mast cells in canine MCTs and conse-quently in tumour aggressiveness and malignancy.

MT1-MMP is one of the main activators ofMMP-2. Mature active MT1-MMP is expressed on the cell surface, where it binds and activates pro-MMP-2 (Sato *et al.*, 1994). Overexpression of MT1-MMP in tumour cells enhances human tumour growth and metastasis (Soulié *et al.*, 2005). In veterinary oncol-ogy, MT1-MMP has been detected by IHC in canine mammary tumours, where its expression was observed in the cytoplasm of tumour and stromal cells (Papparella *et al.*, 2002; Aresu *et al.*, 2011). Both studies revealed increased expression of MT1-MMP in association with MMP-2 in canine mammary car-cinomas and emphasised the contribution of stromal cells to the development of a pro-invasive micro-envi-ronment (Papparella *et al.*, 2002; Aresu *et al.*, 2011). In the present study, MT1-MMP mirrored MMP-2 behaviour at both the protein and mRNA levels. MMP-2 and MT1-MMP were both detected by IHC predominantly in the cytoplasm of tumour cells, and they showed multifocal distribution that increased in terms of percentage of labelled cells and intensity score with histological grade. At the gene expression level, MT1-MMP showed the same pat-tern of expression of MMP-2 and was positively correlated with MMP-2 expression. Thus, these results confirm the relationship between these two proteins in ECM degradation (Will *et al.*, 1996).

The gelatinolytic function of MMPs is also controlled by TIMPs. In particular, TIMP-2 has dual functions of inhibition/activation of MMP-2 (Lambert *et al.*, 2004). No reports are available of the role of TIMP-2 in canine MCT; this has been in-vestigated only in canine mammary tumours (Kawai *et al.*, 2006; Aresu *et al.*, 2011). In the present study IHC and qPCR showed the lowest level of TIMP-2 expression in undifferentiated MCTs, which appears to be consistent with the primary MMP inhibitory role played by TIMPs (Lambert *et al.*, 2004).

Imbalances in the activities of MMPs and TIMPs are involved in tumour progression (Liotta *et al.*, 1991). MMP-2 mRNA increases with respect to TIMP-2 in more aggressive tumours (Onisto *et al.*, 1995; Caenazzo *et al.*, 1998; Nagel *et al.*, 2004; Nakaichi *et al.*, 2007); thus, the ratio of enzyme:inhibitor mRNA has been proposed as an early indicator of aggressiveness (Caenazzo *et al.*, 1998; Nakaichi *et al.*, 2007). In line with previous literature, a progressive increase in the MMP-2:TIMP-2 mRNA ratio according to tumour grade and malignancy was obtained here.

To exert MMP-inhibiting or MMP-activating functions, TIMP-2 binds preferably to MMP-2, while TIMP-1 binds to MMP-9 (Stetler-Stevenson *et* al., 1989; DeClerck *et al.*, 1991). Based on this evidence, the mRNA expression profile of TIMP-1 was exam-ined here, but no statistically significant differences were obtained (data not shown). Nevertheless, the disturbed balance between MMPs and TIMPs in MCTs, as evidenced by the MMP-2:TIMP-2 mRNA ratio, was also confirmed by the

MMP-9:TIMP-1 mRNA ratio, where in more aggressive MCTs (grade 3) an imbalance of about fourfold ver-sus grade 1 was obtained. Thus, the MMP-2:TIMP-2 and MMP-9:TIMP-1 mRNA ratios might be useful for predicting the behaviour of MCTs.

Angiogenesis is crucial for the development of solid tumours and it is known that tumour-associated ves-sels can supply oxygen and nutrients to tumour cells for several millimetres (Uchida *et al.,* 2008). VEGF is a major regulator of angiogenesis and a potential autocrine growth factor for neoplastic cells (Kut *et al.,* 2007). Recently, VEGF-A distribution has been evaluated in normal canine tissues (Uchida *et al.,* 2008) and in canine mammary tumours, menin-giomas, lymphomas and MCTs (Wolfesberger *et al.,* 2007, 2008; Qiu *et al.,* 2008; Matiasek *et al.,* 2009; Millanta *et al.,* 2010; Mederle *et al.,* 2010). Several studies have demonstrated significant correlations between tumour grade and angiogenic factors and/ or microvessel density in canine mammary gland tumours, basal cell tumours and squamous cell carcinomas (Maiolino *et al.,* 2000; Restucci *et al.,* 2002). Furthermore, intratumoural microvessel density has been evaluated in canine MCT and has been associated with tumour recurrence and mortality (Preziosi *et al.,* 2004). In the present study, VEGF-A was measured in MCT samples by means of IHC and, for the first time, by qPCR. Both methods confirmed that neoplastic mast cells constitutively ex-pressed VEGF-A at both the mRNA and protein levels, as described by Rebuzzi *et al.* (2007). Addition-ally, increasing VEGF-A mRNA and protein expression according to histological grade was observed. A similar association was described by Patruno *et al.* (2009). The results obtained here might form the basis for development of new therapeutic strategies for canine MCT. For example, the use of an anti-angiogenic compound in association with other chemotherapeutic agents might be beneficial in the management of non-resectable grade 2 and 3 MCTs. As an example, tyrosine kinase inhibitors with both anti-angiogenic and direct anti-neoplastic activities (inhibition of c-KIT tyrosine kinase activ-ity) might be employed. In this respect, surprisingly, transcriptional data obtained here for VEGF-A in grade 1, 2 and 3 MCTs were significantly correlated with c-KIT mRNA levels (data not shown).

In conclusion, the results of the present study have shown the involvement of MMP-9 and VEGF-A in the progression and malignancy of canine MCT. These markers may be new novel therapeutic targets, but future studies should collect clinical outcome data to further understand the potential prognostic roles of these markers.

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Conflict of Interest Statement

The authors declare that they have no competing interests.

Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.jcpa.2012.01.011.

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