Prognostic value of zymographic gelatinase activity of MMP-2 and MMP-9 in tumor recurrence of canine intrascrotal hemangiomas

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Abstract
There is limited information about intrascrotal hemangioma tumors and their gelatinase activity in the dogs. This study was planned to identify the serum gelatinases in male dogs affected by intrascrotal hemangioma and determine their prognostic value in tumor recurrence. Ten dogs with intrascrotal hemangioma were diagnosed among a total of 65 testicular excision samples. According to the incidence of recurrence, the patients divided into two groups: tumors with recurrence and tumors with no-recurrence. Serum gelatinase activity was assayed by semi-quantitative zymography to determine their prognostic value in canine intrascrotal hemangioma. Both latent and active MMP-9 and only latent MMP-2 appeared in the gels. Gelatinases showed a significant higher serum activity in dogs with hemangioma than those of the normal dogs. There was a significant association between increased serum activity of gelatinase A and gelatinase B and tumor recurrence. The dogs with hemangioma had a shorter disease-free survival time; however, multivariate analysis showed that serum activity of MMP-2 and MMP-9 was not an independent prognostic factor. According to the findings, MMP-2 and MMP-9 may cause a progressive angiogenesis in canine intrascrotal hemangioma and tumor recurrence subsequently. Our results showed that serum activity of MMP-2 and MMP-9 may be used as a non-invasive prognostic marker for tumor recurrence prediction in dogs affected by intrascrotal hemangioma.

Keywords Zymography • Serum gelatinase • Canine intrascrotal hemangioma • Recurrence

Introduction
Genital system neoplasms are rare in dogs; however, tumors of the testis in males are the most frequent (Sapierzyński et al. 2007). Hemangiomas are benign tumors of vascular endothelium (Lee et al. 2008; Meuten 2008; Withrow et al. 2013) that can occur in a variety of sites of the body (Meuten 2008, Withrow et al. 2013). Hemangiomas are commonly seen in dogs, but rarely observed in other domestic animals (Meuten 2008; Pirie et al. 2006). Matrix metalloproteinases (MMPs) are zinc-dependent enzymes that regulate angiogenesis and form new blood vessels (Sang 1998). Gelatinases A and B degrade extracellular matrix (ECM) and increase angiogenesis (Sang 1998; Schnaper et al. 1993; Ray and Stetler-Stevenson 1994; Nagase and Woessner 1999; Pepper 2001). Furthermore, gelatinase activity has a major role in tumor invasion (Nagase and Woessner 1999; Sang 1998; Pepper 2001) and metastases (Sang 1998).

Recently, measuring serum activity of MMPs has been used widely as the prognostic and diagnostic markers for a variety of tumors like canine spontaneous NHL (Gentilini et al. 2005), breast cancer (La Rocca et al. 2004; Wu et al. 2008), human NSCLC (Laack et al. 2002), human patients with sarcomas (Yaman et al. 2008), and canine mammary tumors (Aresu et al. 2011). One study has demonstrated that MMPs assessment in serum can be used as indicator for tumor metastasis or recurrence (Miya et al. 2005).

Since the serum activity of MMPs has not been studied for testicular hemangioma in dogs, we decided to identify the
serum MMP-2 and MMP-9 in male dogs affected by intrascrotal hemangioma, and to determine their prognostic value in tumor recurrence.

**Materials and methods**

**Patients and samples**

Sixty-five breeder-owned dogs with testicular mass were included in this study between September 2013 and September 2016. All the procedures were performed under general anesthesia (Fig. 1). Serum biochemistries, CBC, urinalysis (UA), and thoracic radiographs and abdominal ultrasound were performed to rule out lung and visceral metastases. All of the intrascrotal masses were evaluated with ultrasonography. Blood samples were collected and serum sample of all dogs were sent to a veterinary hospital at Tehran University, Dr. Rastegar’s laboratory. Surgical recommendation was included orchiectomy with intrascrotal ablation (Meuten 2008; Withrow et al. 2013), although according to owners’ preference, just a surgical excision of the masses was supposed to be performed (Figs. 2 and 3). The surgical samples of the intrascrotal tissue were collected and placed in fixative solution of buffered formalin and then embedded in paraffin and divided into sections by 3 μm. Hematoxylin and eosin staining with routine method was used for the sections. Each section was evaluated by two experienced clinical pathologists. Finally, 10 intrascrotal hemangiomas were diagnosed according to published criteria (Morris and Dobson 2008; Meuten 2008; Kennedy and Palmer 2013; Withrow et al. 2013). A prospective study was designed for 10 intrascrotal hemangioma cases with an average age of 3.1 ± 0.99 years (1.6–5.1 years) and an average weight of 23.1 ± 11.2 kg (9.5 to 40.6 kg). In addition, 10 healthy dogs with similar weight and breed to patients were selected as controls, and the same laboratory tests were performed on them.

**Gelatin zymography**

Serum samples were placed at −80 °C quickly to be used later for gelatin zymography. Gelatin zymography was performed using the routine protocol 2 (Loukopoulos et al. 2003; Coughlan et al. 1998). A dilution of 1:5 was prepared with distilled water for all serums. Fifteen microliters of each sample was diluted by sampler buffer (DNAbiotech Co) (1:1 ratio). A volume of 15 μL of the sample dilution was loaded in the gel cavities. A 10% SDS-PAGE gel copolymerized with 0.1% bovine gelatin was used for electrophoresis. Recombinant human gelatinase A and gelatinase B (0.5 ng/lane) (Sigma) and latent MMP-9 and MMP-2 combined solutions (1 ng/lane) (BioRad) were loaded on the separated lanes. Electrophoresis was performed in 20 mA and 125 V under non-reducing condition for 120 min. The gels were incubated twice in 2.5% Triton-100 X at 25 °C for 30 min and in buffer with compounds composed of 0.5 M Tris-HCl in pH = 7.4 with 10 mM CaCl2 at 37 °C for 28 h. Coomassie brilliant blue was used for gel staining, and then the stain was removed by mixture of acetic acid and methanol. The clear bands against a blue background were accepted as MMPs. Gelatin zymography was performed frequently on the serum samples to obtain the apparent bands and quantification of MMPs activity by densitometry subsequently. Molecular weight of all proteins (kDa) was dedicated by the protein ladder (Tris-Glycine 4 ~ 20%, CinnaGen). The 62-, 64-, 66-, and 68-kDa forms were accepted as active MMP-2 (Loukopoulos et al. 2003; Coughlan et al. 1998). The 72-, 88-, and 92-kDa were also identified as latent MMP-2, active MMP-9, and latent MMP-9, correspondingly (Loukopoulos et al. 2003). A Gel Documentation System (Bio-Doc, UPV, USA) was employed to take images of the gels, and the apparent bands were evaluated by densitometric analysis software (NIH ImageJ 1.50q).

**Patients follow-up**

After surgical treatment and ablation of the tumors, all patients affected with intrascrotal hemangioma tumor were followed up. Clinical examinations, abdominal sonography, and chest
Radiography were performed every 5–6 months for all dogs during a 3-year follow-up to identify the existence of tumor recurrences locally and/or distant metastases. Since surgical intervention to the observation of tumor metastasis or recurrence was considered as disease-free survival (DFS) time, this time was calculated for all treated dogs.

Methods of statistical analysis

Zymography results of tumor and healthy dogs were statistically analyzed with SPSS software (version 23). Normal distribution of the data was checked by a Kolmogorov-Smirnov test. The results are presented as a mean value ± SEM, using independent t test. Statistical evaluation was performed using these tests to detect mean differences of hemangioma-affected and healthy dogs’ groups. Also, the means of MMPs serum concentrations of tumor recurrence (recurrence group, n = 5) were compared with no recurrence group (n = 5) by using the independent t test.

Survival curves were obtained from Kaplan-Meier estimates, and the Log-rank analysis obtained the differences between groups (with/no recurrence groups). The data was analyzed in multivariate Cox’s proportional hazard’s method as well. The cut-off points of serum MMPs were defined by the ROC curves included in the intrascrotal hemangioma tumors. A P value less than 0.05 was determined for statistical analysis.

Results

Ten cases were diagnosed as intrascrotal hemangiomas among a total of 65 tissue samples. Tumor infection was diagnosed in seven large breed (three great Danes, two German shepherds and two Dobermans) and three small breed (two terriers and a spitz) dogs. Ultrasound findings of the scrotum of dogs with hemangioma showed enlarged and filled with material of mixed echogenicity. Also, color Doppler showed a high vascularity in the mass. On parasagittal images, both testicles were normal in size and echogenicity (Fig. 3). There were no evidence of the metastasis in thoracic radiography and abdominal ultrasonography in a 3-year longitudinal study after surgical treatment. Serum biochemistries, CBC, and urinalysis (UA) demonstrated healthy condition of the dogs in the follow-up study. Tumor recurrence was observed in five dogs.

Macroscopical and microscopical findings

In some cases, hemorrhagic lesions were seen. The tumors were well-restricted, enclosed, and multilobulated masses that have a different color from dark red to dark brown. In larger scrotal masses, the appearance of the cut surface showed a honeycomb schema of fibrous trabeculae that formed cavities filled with blood (Fig. 4). Many spindle cells were detected that formed vascular structures with a single line of invariable endothelial cell. The vascular structures have variable size that filled with many erythrocytes. Mitotic figures were rarely seen in all types of the cells. According to the vascular channel size, four cavernous and six capillary forms of hemangioma were diagnosed (Fig. 5a–d). Recurred masses did not show different pathologic feature compared with primary lesions.

Fig. 3 Mixed echogenicity (a) and vascularity (color Doppler) (b) in the scrotum ultrasound of a dog with scrotal hemangioma

Fig. 4 Gross photograph of the surgically excised intrascrotal mass from a 2-year-old Great Dane
Matrix metalloproteinases 2 and 9 serum activities

Gelatin zymography showed both latent forms of MMP-9 and MMP-2 bands in serum samples of all control and tumor cases. Three gelatinolytic bands with molecular masses of 92, 82, and 72 kDa were detected that represented as latent MMP-9, active MMP-9, and latent MMP-2, respectively. Control serums just exhibited inactive forms of gelatinase A and gelatinase B. Three bands for inactive MMP-2 and MMP-9 and active MMP-9 appeared in serum of the intrascrotal hemangioma-affected dogs. Serum gelatin zymography did not detect any active form of gelatinase A in all dogs. The recurrent hemangiomas exhibited more intense bands rather than no recurrent cases (Fig. 6).

Semi-quantitative evaluation of serum metalloproteinases activity revealed a higher activity of pro-MMP-2 in dogs with recurrent hemangiomas compared to those without recurrent cases. The results were analyzed using a t-test, with a p-value < 0.05 indicating significance.

![Fig. 5](image1) Sections of intrascrotal hemangiomas; a, b Proliferation of variably sized blood vessels (arrows). c, d Stromal tissue cells with spindle-shaped and large and hyperchromatic nuclei (arrows). The neoplastic cells forming small blood-filled spaces (a: ×40 objective, b: ×40 objective, c and d: ×100 objective)

![Fig. 6](image2) Zymography of intrascrotal hemangiomas and normal serums with 10% polyacrylamide gel combined with 0.1% gelatin. Bands of latent MMP-2 (72 kDa), latent MMP-9 (92 kDa), and active MMP-9 (82 kDa) are seen. Lane C1, recombinant human active MMP-2 and active MMP-9; lane C2, latent MMP-2, 9 mixture solutions; lane N, control dog; S1 and S2, serum samples of two dogs with recurrent intrascrotal hemangioma; S3 and S4, serum samples of two dogs with no recurrent intrascrotal hemangioma

![Fig. 7](image3) Serum latent MMP-2 and MMP-9 concentrations in normal male dogs and with hemangiomas. t test (p value < 0.05, error bar: 95% of confidence interval)
intrascrotal hemangioma \((n = 10; 1.85 \pm 0.16 \, \text{ng/lane})\) than the control dogs \((n = 10; 0.87 \pm 0.08 \, \text{ng/lane})\), and this finding was statistically significant \((P = 0.0001)\) (Fig. 7). Furthermore, a total MMP-9 activity was increased significantly \((P = 0.0001)\) from intrascrotal hemangioma-affected dogs \((3.18 \pm 0.26 \, \text{ng/lane})\) to the control dogs \((0.82 \pm 0.09 \, \text{ng/lane})\). Serum activity of both inactive and active gelatinase B and inactive gelatinase A showed significantly higher levels in intrascrotal tumor group in comparison with control group \((P < 0.05)\) (Table 1). Pro-MMP-2 \((2.25 \pm 0.19 \, \text{ng/lane})\) and total MMP-9 \((3.92 \pm 0.15 \, \text{ng/lane})\) in tumor-recurrent cases demonstrated higher activities than in non-tumor-recurrent cases with significant levels \((P < 0.05)\). Also, significant differences were obtained for latent gelatinase A and active gelatinase B serum activities in recurrent patient against not recurrent patient \((P < 0.05)\) (Table 2).

**Patients follow-up**

Ten dogs followed post-surgically had median disease-free survival time of 18 months (with range of 6–36 months). Intrascrotal hemangioma was recurred in five dogs during the follow-up study. Complete orchiectomy was performed for all recurred tumors. There was a statistically significant relationship between increased levels of inactive MMP-2 and active MMP-9 with a short disease-free survival time according to the Kaplan-Meier survival curves (Figs. 8 and 9). High serum activity of pro-MMP-2 (up to 1.6 ng/lane) and active-MMP-9 (up to 2 ng/lane) shows a significant relation with shorter DFS time. These findings revealed the prognostic value of serum activity of these enzymes. The multivariate Cox’s proportional hazard’s analysis illustrated that MMP activity in serum had no independency as a prognostic parameter.

**Discussion**

Early surgical castration is the most important reason for low frequency of testicular tumors in the dogs (Sapierzyński et al. 2007). The testicles are the second most common area for a variety of tumors in dogs; therefore, castration is the best choice for prevention (Reichler 2009). In our study, 10 intrascrotal hemangiomas were diagnosed in breeder-owned outdoor dogs that are exposed to the sunlight most of the times. High prevalence of the testicular tumors was observed in canine commercial breeding establishments. The breeder-owned dogs were used as genetic reservoir in the kennels, and the owners preferred to preserve their fertility potential. The incidence of tumor recurrence may be influenced by surgical complications because the orchiectomy was refused by the owners. However, we recommended orchiectomy with scrotal ablation for intrascrotal hemangiomas to diminish the possibility of recurrence after surgical treatment.

Determination of gelatinase activity in serum of canine intrascrotal hemangioma was preformed using a semi-quantitative gelatin zymography. The results of this study demonstrate gelatinase A and B serum activity in dogs affected with intrascrotal hemangioma. Serum levels of latent

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<th>Table 1</th>
<th>Mean values of MMPs serum activity in intrascrotal hemangioma-infected and healthy dogs</th>
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<tr>
<td>Parameters (ng/lane)</td>
<td>Control group ((n = 10))</td>
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<tr>
<td>ProMMP-2</td>
<td>0.87 ± 0.08b</td>
</tr>
<tr>
<td>ProMMP-9</td>
<td>0.82 ± 0.09</td>
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<tr>
<td>Active MMP-9</td>
<td>0</td>
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<tr>
<td>Total MMP-2</td>
<td>0.87 ± 0.08</td>
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<tr>
<td>Total MMP-9</td>
<td>0.82 ± 0.09</td>
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\(a)\) These data are significantly different 
\(b)\) All data are expressed as mean ± SEM, statistical test: independent \(t\) test

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<tr>
<th>Table 2</th>
<th>Mean values of MMPs serum activity in intrascrotal hemangioma-infected dogs with recurrence and no-recurrence groups</th>
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<tr>
<td>Parameters (ng/lane)</td>
<td>Recurrence group ((n = 10))</td>
</tr>
<tr>
<td>ProMMP-2</td>
<td>2.25 ± 0.19b</td>
</tr>
<tr>
<td>ProMMP-9</td>
<td>1.16 ± 0.14</td>
</tr>
<tr>
<td>Active MMP-9</td>
<td>2.76 ± 0.14</td>
</tr>
<tr>
<td>Total MMP-2</td>
<td>2.25 ± 0.19</td>
</tr>
<tr>
<td>Total MMP-9</td>
<td>3.92 ± 0.15</td>
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</tbody>
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\(a)\) These data are significantly different 
\(b)\) All data are expressed as mean ± SEM, statistical test: Independent \(t\) test
MMP-2 and MMP-9 and active-MMP-9 showed significant higher activity in hemangiomas versus in normal cases. These findings may be suggestive of MMPs function in tumor angiogenesis and biologic features of intrascrotal hemangioma in dogs. In recent years, many researchers showed serum activity of MMPs in vast tumors of dog and human (Gentilini et al. 2005; La Rocca et al. 2004; Laack et al. 2002; Yaman et al. 2008; Aresu et al. 2011; Miyae et al. 2005).

La Rocca suggested measurement of gelatinase serum activity as a useful parameter to subgrouping human breast cancer (La Rocca et al. 2004). In addition, serum MMP-9 levels by enzyme-linked immunosorbent assay have been used as a predicting biomarker for prognosis of breast cancer. They found an association between high serum levels of MMP-9 and tumor metastasis to the lymph nodes, higher tumor progression. Furthermore, breast cancer-affected cases with increased serum level of MMP-9 showed faster tumor recurrence and death (Wu et al. 2008).

Two papers reported serum MMP concentrations for prognosis of canine non-Hodgkin’s lymphoma (Gentilini et al. 2005; Hazar et al. 2004). Previously, Miyae et al. demonstrated high serum activity of gelatinases A and B in metastatic or recurrent malignant tumors in dogs. They introduced serum activity of gelatinases A and B as a reliable marker for prognosis of canine malignancies (Miya et al. 2005).

In agreement with their findings, the gelatinase activity in recurrent tumors was significantly higher than non-recurrent tumors.

Furthermore, patients that developed recurrent hemangiomia had a tendency to exhibit higher angiogenesis that is may-be associated with high serum activity of gelatinases. Gelatinzymography is a practical method for gelatinase activity assay, and it can be non-aggressive by serum sampling. A positive correlation between serum samples and tumor extract has been previously discussed (Miya et al. 2005). To our knowledge, this paper presents the first data about gelatinase activity in serum of dogs affected with intrascrotal hemangioma.

Conclusions

The higher gelatinase activity in serum of dogs with recurrent intrascrotal hemangioma was an interesting finding. Serum gelatinase activity can be used as a useful marker in tumor-affected cases in veterinary. Although, other abnormalities which can increase serum level of MMPs must be ruled out. We found that the serum activity of gelatinases of intrascrotal hemangioma could be associated with DFS and recurrence of the tumor. The breeding dogs can be involved by a variety of the testicular and scrotal tumors, and estimation of serum gelatinase activity would be helpful for prognosis and development of hemangioma tumors. Pro-MMP-2 and active-MMP-9 serum activity may have prognostic value in intrascrotal hemangiomas and maybe in other types of hemangioma. There is a need for more extensive studies to determine prognostic value of serum activity of MMP-2 and MMP-9 as follow-up markers in hemangiomas that may occur in various sites of the body.

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Compliance with ethical standards

Conflict of interest  The authors declare that they have no conflict of interest.

Ethical approval  All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with animals performed by any of the authors.

Informed consent  Informed consent was obtained from all individual participants included in the study. Additional informed consent was obtained from all individual participants for whom identifying information is included in this article.

References


