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Research Article

Immunohistochemical determination of the matrix metalloproteinase-2 and -7 expression in transmissible venereal tumor in dogs

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ABSTRACT

Transmissible venereal tumor (TVT) is a sexually transmitted, naturally occurring tumor of the canine family and often occurs in tropical and subtropical countries. The matrix metalloproteinases (MMPs) are endogenous proteases accountable for the degradation of extracellular matrix (ECM) components, such as collagen and other proteins including the basement membrane. MMPs play a vital role in the tumor metastasis and angiogenesis. Both MMP-2 and -7 strongly associated with the invasion and metastasis of different cancer types. This study aims to investigate the MMP-2 and -7 expression in naturally occurring TVT in 20 dogs using immunohistochemical methods. Immunohistochemically, we observed increased MMP-2 and -7 expressions in tumor cells. In addition, a positive correlation was determined between the tumor size and immunoexpressions of the markers indicating that both MMP-2 and -7 participate in the TVT pathogenesis.

Köpek transmissible venereal tümörlerinde mmp-2 ve mmp-7 aktivitesinin immunohistokimyasal olarak belirlenmesi

ÖZ

Transmissible venereal tümör (TVT), çiftleşmeyle bulaşan, kanidae ailesindeki hayvanlarda doğal olarak şekillenen, genellikle tropikal ve subtropikal ülkelerde gözlenen bir tümördür. Matriks metalloproteinazlar (MMP)'ler endojen peptidazlar olup, ekstraselüler matriksin (ECM) kollagen ve bazal membran gibi diğer proteinlerini parçalama özelliğine sahiptirler. MMP'ler tümör metastaz ve angiogenezinde önemli rol oynarlar. MMP-2 ve MMP-7 değişik tip kanserlerde invazyon ve metastaz ile ilişkili bulunmuşlardır. Bu çalışmanın amacı, MMP-2 ve MMP-7'nin 20 adet köpekte doğal olarak şekillenmiş TVT olgusunda immunhistoimyasal olarak ekspresyonlarının incelenmesidir. İmmunohistokimyasal olarak TVT'yi oluşturan tümör hücrelerde MMP-2 ve MMP-7 aktivitesinde artış şekillendiği gözlendi. Ayrıca tümör büyüklüğü ile immunoekspresyonlar arasında pozitif korelasyon saptandı, bu sonuç, MMP-2 ve MMP-7'in TVT patojenezinde önemli bir rol oynadığını gösterdi.

INTRODUCTION

Transmissible Venereal Tumor (TVT) is a contagious round-cell neoplasm that is transmitted from one dog to another during mating. Transmission might occur when abraded skin is exposed to the tumor of an infected animal. Although TVTs affect both sexes, regardless of breed and age, females are infected more often than males because one infected male often mates with numerous females (1,2). In addition, TVT is most common during the period of the maximum sexual activity in dogs, and animals are mainly at the highest risk when females exhibit the oestrus signs (3). While this neoplasm has spread in dogs worldwide, it is most frequently reported in tropical and subtropical countries. Primarily, the tumoral masses grows in the genital organs (4,5). Typically, initial lesions are small and superficial, pink to red, which then form hemorrhagic multiple nodular and larger friable masses (6). Moreover, tumors bleed easily, and while growing, the masses are ulcerated and contaminated with bacteria (7). Although TVT is locally invasive and rarely metastatic, metastasis has been reported in different tissue and organs such as the skin, lymph nodes, tonsils, eyes, brain, nose, tongue, lips, mammary glands, and thoracic and abdominal viscera (4,8,9). The definitive diagnosis of TVT is based on cytological and histopathological findings (10).

The most crucial function of the extracellular matrix (ECM) is to maintain tissues with their specific mechanical and biochemical properties (11). The ECM degradation comprises various proteases, but the major are matrix metalloproteinases (MMPs) called matrixins (12). The MMP family comprises 23 enzymes that are characterized by their zinc dependence and neutral endopeptidase activities. Initially, these peptidases were associated with the cleavage of ECM molecules, especially collagen (13). The apparent correlation between the MMPs activity and the cancer development indicates the possibility of various strategies connected with blocking the activity of these enzymes. The MMPs play a pivotal role in the normal development, as well as the pathology of inflammatory diseases and cancer (14,15). Of note, the MMPs are frequently overexpressed in various human cancer types. Furthermore, enhanced expressions of the MMPs have been related to an

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aggressive malignant phenotype and adverse prognosis in patients with cancer (16,17).

MMP-2 (gelatinase A) is an enzyme that is speculated to play a vital role in the invasion to the basement membrane, and it belongs to the gelatinases group and digests the denatured collagens, gelatins (18). Notably, cancerous tissues with a high expression of active MMP pose a risk of metastasis. Hence, the activation rate of pro-MMP-2 and active MMP-2 is used as an indicator of cancer metastasis (19). Conversely, MMP-7 (Matrilysin, pump-1) is the smallest known member of the MMP family and is capable of degrading various ECM proteins and supports the tumorigenesis and progression *in vitro* in the animal model (20). MMP-7 is elevated in numerous human primary cancer types (21). As only one study is available about the MMP-2 expression in TVT, the knowledge about the MMP-7 reaction in TVT is lacking (22).

Hence, this study aims to investigate the expression of MMP-2 and -7 in TVT of dogs using immunohistochemical methods.

MATERIAL and METHODS

In this study, we collected TVT samples from the archive of the Department of Pathology. We selected the paraffin blocks from 12 female and 8 male dogs with naturally occurring TVT. Dogs aged between 6 months and 2 years and were of different breeds. In addition, we collected data and notes about clinical symptoms, gross lesions, and anamnesis of tumors. Notably, ethical approval was not required for this study.

For histopathological and immunohistochemical examinations, we considered three serial sections from the paraffin blocks of TVT. We stained one of these sections with hematoxylin–eosin (H&E) and examined under light microscope (23).

The remained two sections were immunostained with MMP-2 [anti-MMP-2 antibody (ab110186, 1:100 dilution)] and MMP-7 [anti-MMP-7 antibody (ab5706)] (ab10600, 1:100 dilution)] per the manufacturer's instructions using a routine streptavidin–biotin peroxidase technique. We used expose Mouse and Rabbit Specific HRP/DAB Detection IHC Kit (ab80436; Abcam, Cambridge, UK) as the secondary antibody. Primary antibodies were not applied to the negative controls for immunohistochemistry. We performed the morphometric evaluation using the Database Manual Cell Sens Life Science Imaging Software System (Olympus Corporation, Tokyo, Japan). In addition, immunoexpressions were evaluated as 0 = negative; 1 = slight; 2 = moderate; and 3 = marked positive reactions. Furthermore, all dogs were divided into two groups with tumor size $<2.9\ cm^3$ and $\geq 3cm^3$ to evaluate the correlation between the tumor size and immunoexpressions.

We used the one-way analysis of variance test to determine significant differences between the groups. In addition, the expression of markers was compared using the Student's *t*-test. Using the Spearman's rank-difference coefficient of correlation, we evaluated the immunoexpression of MMP-2 and MMP-9 in the tumor tissue. All statistical analyses were performed using the SPSS 18.0 program. Furthermore, we considered $P < 0.05$ as the level of significance in this study.

RESULTS

Based on the necropsy data, all dogs aged 6 months to 2 years. Of all dogs in the study cohort, 12 were females and 8 males. We observed no metastasis and recurrence 2 years postoperatively in any dog.

The size of tumors changed from 1 cm × 1 cm × 1 cm to 8 cm × 5 cm × 6 cm in diameter (Fig. 1). The tumoral masses were soft, in various sizes, and usually comprised some hemorrhagic areas on the upper surface. In addition, necrotic areas were observed in some large tumors. Tumors originated in the epithelial layer and subadjacent stroma as one or multiple nodular proliferative masses in the external genital organs of both sexes. We observed a positive correlation between the tumor size and MMP-2 and -7 expression scores.

The microscopic assessment of tumors revealed oval, rounded/polyhedral, vesicular, and large nucleated cells with indistinct boundaries and poorly stained or clear cytoplasm. Typically, the nuclei were large and single, contained well-defined nucleolus with plenty of chromatin granules. In some tumors, we noted the enhanced mitotic activity in cells; necrosis and bleeding were common in the tumoral mass. In small tumors, especially, vessels were highly hyperemic, and we frequently noted inflammatory cells from mononuclear series (Fig. 2).

The microscopic examination of immunohistochemically stained sections with MMP-2 revealed the increased expression, especially in large masses. In addition, tumor cells markedly expressed MMP-2 in their cytoplasm. We observed homogenous staining both in the cell cytoplasm and throughout the tumoral mass (Fig. 3). However, no staining was detected in primary antibody–omitted negative controls.

In this study, tumoral cells revealed the increased MMP-7 expression in their cytoplasm. Notably, the expression did not exhibit homogenity throughout the mass. Interestingly, inflammatory cells markedly expressed MMP-7 in TVT cases (Fig. 4). We detected no staining in the primary antibody–omitted negative controls. Furthermore, both MMP-2 and -7 were expressed in TVT cases, whereas MMP-2 staining was more intense than MMP-7 staining in most cases.

DISCUSSION

Recent years have witnessed an upsurge in the tumor incidence in animals and humans alike. Thus, studies on the formation and treatment of tumors are increasing rapidly. Lately, people have become more inclined to keep pet animals, such as cats and dogs, in their homes. Hence, the pathogenesis of animal diseases or tumors is being extensively investigated at present. This study determined the MMP-2 and -7 activity of TVT, a common problem in dogs, and investigated the role of these markers in the pathogenesis of this tumor.

TVT is a contagious, neoplastic, sexually transmitted disease commonly observed in street dogs living in tropical and subtropical regions and typically affects the penis and vaginal mucosa (6). TVT primarily affects young dogs (2–5 years), and the disease is commonly diagnosed in females than in males. In this study, TVT cases were found only in the genital organs, the masses localized in the vagina and vulva of females, and

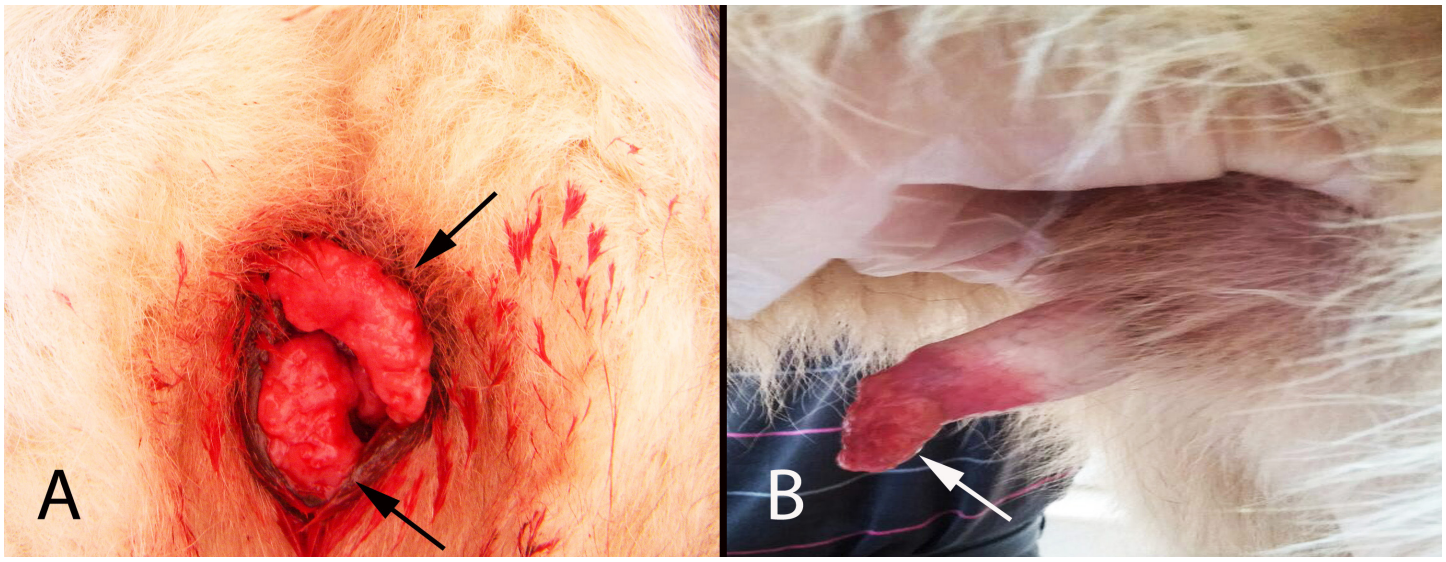

Figure 1 The gross appearance of the tumoral mass (arrows) on the vulva-vagina (A) and penis (B).

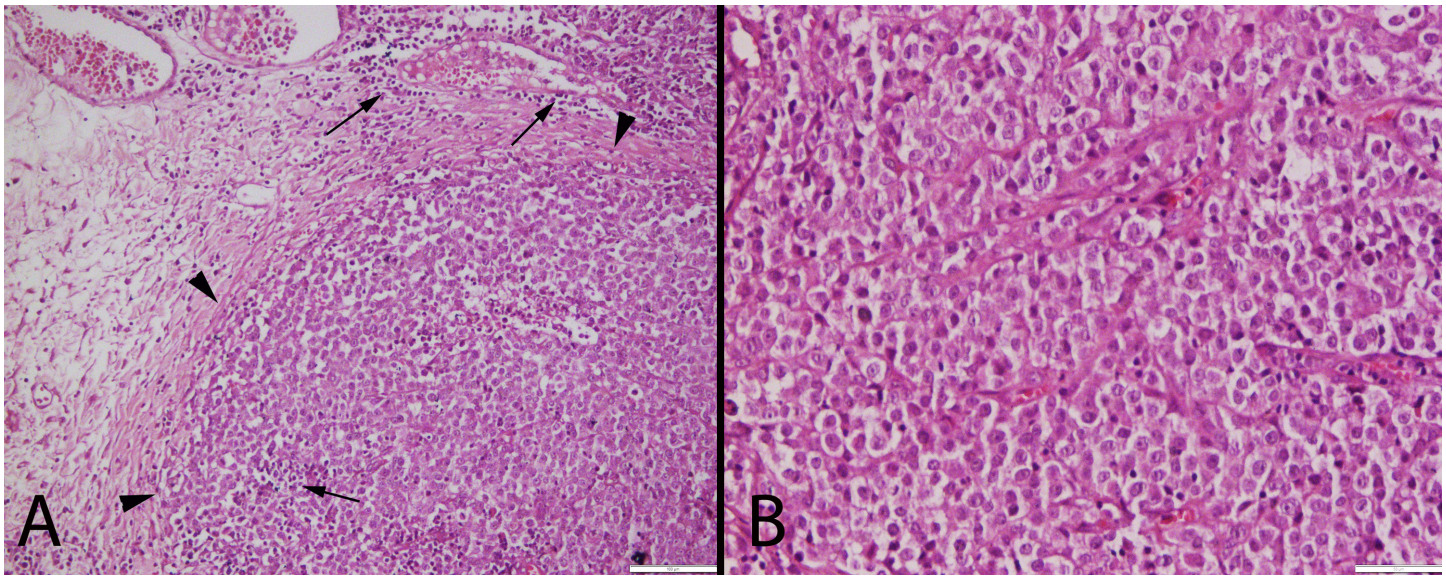

Figure 2 (A) The histopathological appearance of the tumoral mass (arrowheads) and inflammation around the tumor (arrows). Hematoxylin–eosin (HE): bar, 100 µm. (B) Higher magnification of the tumor. HE: bar, 50 µm.

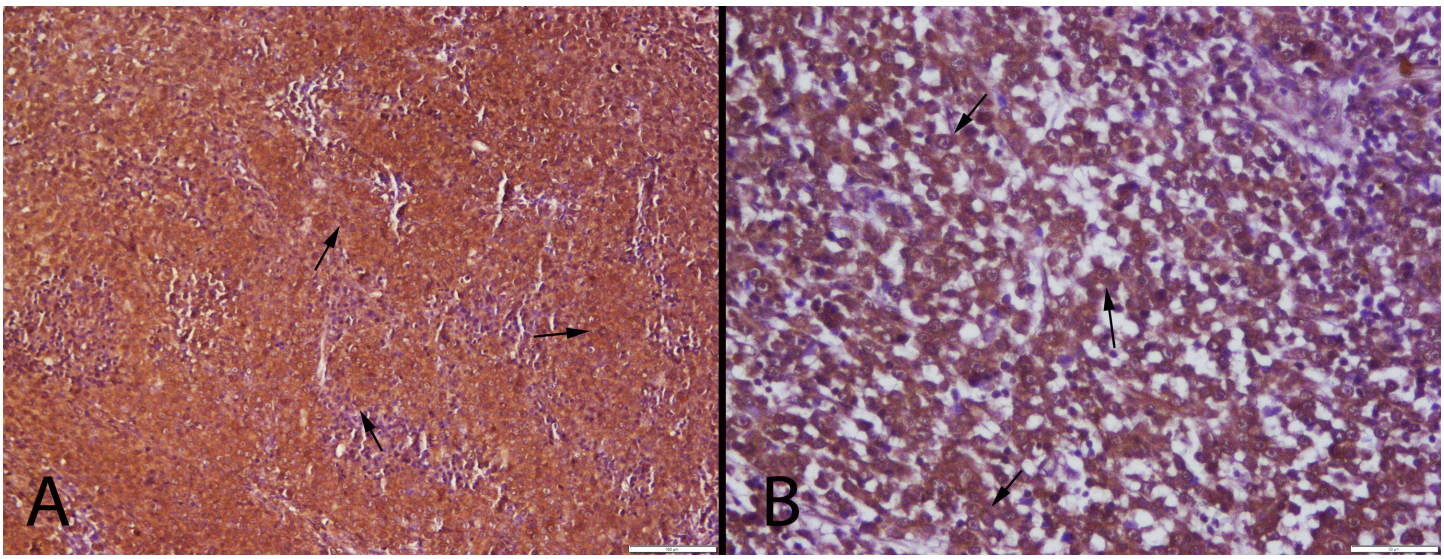

Figure 3 (A) Severe and homogenous MMP-2 expression in tumoral cells (arrows) and tumoral mass. Bar, 100 µm. (B) Higher magnification of the homogeneous expression in tumoral cells cytoplasms (arrows). Bar, 50 µm, the streptavidin–biotin peroxidase method.

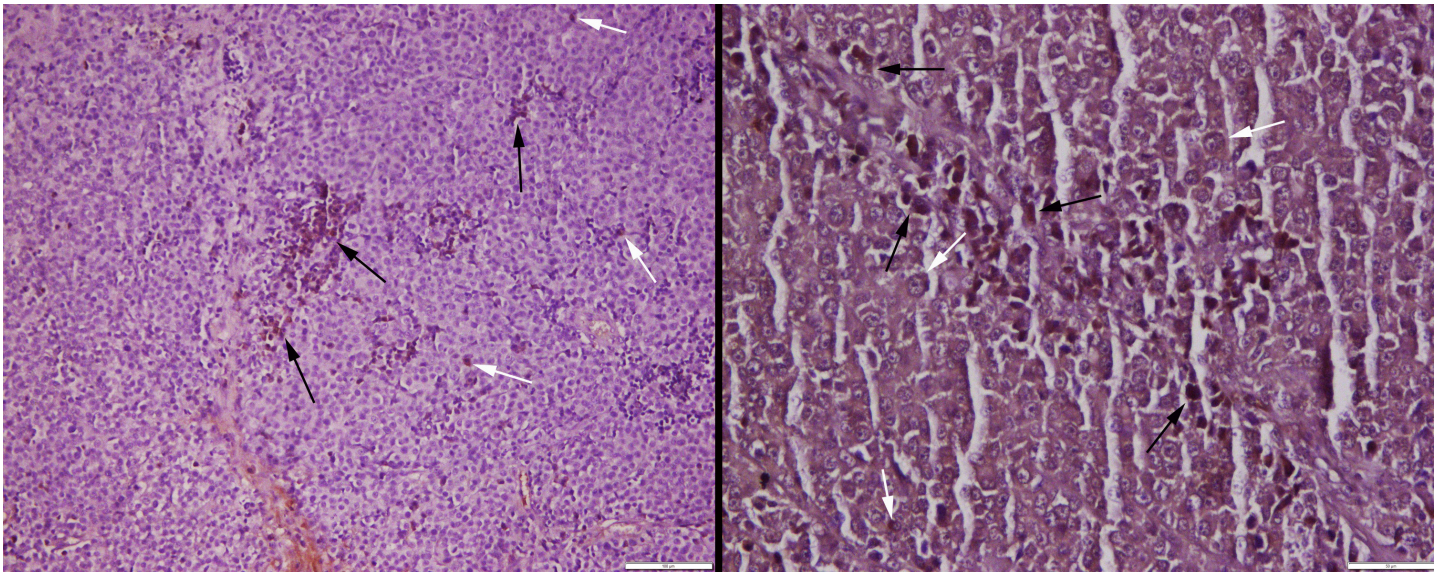

Figure 4 (A) Mild and non-uniform MMP-7 immunoreaction in TVT, moderate expression in tumoral cells (white arrows), and inflammatory cells (black arrows). Bar, 100 µm. (B) Higher magnification of another tumoral mass. Bar, 50 µm, the streptavidin–biotin peroxidase method.

Table 1 The statistical analysis results of MMP-2 and MMP-7 immunoexpression scores.

	<2,9 cm^3 (n=14)	≥3.0 cm^3 (n=6)	P
MMP-2	1.14±0.66	2.55 ±0.54	<0.001
MMP-7	0.78±0.69	1.66±0.81	<0.05

*: Values expressed as mean ± SD.

the penis and prepuce in males. In addition, the dimensions of tumors ranged from 1 cm × 1 cm × 1 cm to 8 cm × 5 cm × 6 cm in diameter. The tumoral masses were soft and usually exhibited hemorrhagic areas on the surface; especially in large tumors, necrotic areas were found in the mass. Of note, the general characteristics of the masses examined in this study corroborated the literature (6,7,9).

In this study, no metastases or recurrence were observed in any dog 2 years postoperatively, which could be attributed to the extragenital localization and early diagnosis because of the

Table 2 Correlations between the tumor size and immunoexpressions of MMP-2 and MMP-7.

	MMP-2	MMP-7
r	0.719**	0.502*
p	0.000	0.024

** : Correlation is significant at the 0.01 level (2-tailed)

* : Correlation is significant at the 0.05 level (2-tailed)

localization. In fact, tumors in most cases (14 dogs) were <3 cm^3, and the early diagnosis and treatment caused complete amelioration.

Traditionally, the biological roles of the MMPs have been related to the degradation of most ECM components. The ECM degradation by the MMPs removes the physical barriers for a growing tumor. In invasive cancer cells, for example, actin-rich protrusions of the plasma membrane can be associated with the ECM degradation (24). In addition, the MMPs are associated with cancer cells' survival and expansion; these are synthesized by cancer cells and are involved in all steps of the carcinogenesis (25). Lately, MMP-2 has garnered attention by its correlation with the tumor invasion and formation of metastases (26). A recent study reported the presence of MMP-2 and -9 in the TVT tissue (22). The findings of this study supported the previous study and demonstrated that MMP-2 was strongly expressed by TVT cells.

Notably, MMP-7 is the smallest known member of the MMP family and can degrade various ECM proteins, including proteoglycans, fibronectin, entactin, laminin, gelatin, and elastin (20). In particular, MMP-7 exhibits the highest activity against insoluble elastin and is 11-fold more active than MMP-3 (27). MMP-7 was initially cloned from some human carcinomas (28). However, knowledge about the reaction of MMP-7 in TVT is limited. This study revealed that MMP-7 is expressed from TVT and inflammatory cells and plays a role in the pathogenesis of the tumor. Furthermore, inflammatory cells, which are accountable for maintaining a local inflammatory response and stromal degradation, might be a crucial source of MMP-7 in TVT.

This study describes the intense expression of MMP-2 and MMP-7 in TVT and demonstrates the implication of these MMPs in the tumor progression and invasion. In addition, a positive correlation exists between the tumor size and immunoexpression of markers. Nevertheless, an enhanced understanding of the molecular mechanisms underlying the activation of MMP-2 and MMP-7 might lead to a new therapeutic strategy for TVT.

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