

RESEARCH ARTICLE

The Relationship Between Metastasis and MMP-9 in Sheep with Pulmonary Adenocarcinomas

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Abstract: This study aimed to evaluate the role of Matrix Metalloproteinase-9 in the metastasis of ovine pulmonary adenocarcinoma cases by immunohistochemical methods. Lung tissue samples were collected from 26 sheep brought to our department for routine histopathological diagnosis. Lung tissues were fixed in 10% neutral buffered formalin, following routine procedures tissues were embedded in paraffin wax. Sections from tissues were cut into 5 µm thickness and stained with Hematoxylin & Eosin to detect histopathological changes. The sections were examined under a light microscope and photographs were taken. Avidin-Biotin Peroxidase method was used as immunohistochemical staining. Tumoral cells showed acinar, papillary or mixed type growths in ovine pulmonary adenocarcinomas. Only 2 of 20 cases had metastases to lymph nodes, and tumoral cell proliferation in these metastatic areas was similar to primary cancer foci. All ovine pulmonary adenocarcinomas were positive for JSRV CA and Matrix Metalloproteinase-9 immunoreactivity. Any positive expression of JSRV CA and Matrix Metalloproteinase-9 were not detected in the control group. The number of JSRV CA and Matrix Metalloproteinase-9 immune positive cells was statistically increased in the ovine pulmonary adenocarcinoma group compared to the control group. In line with the data of this study, it is thought that Matrix Metalloproteinases play a serious role in tumor metastasis in ovine pulmonary adenocarcinomas, especially Matrix Metalloproteinase-9.

Keywords: Matrix metalloproteinase-9, Metastasis, Ovine pulmonary adenocarcinoma

Pulmoner Adenokarsinomlu Koyunlarda Metastaz ve MMP-9 İlişkisi

Öz: Bu çalışmada, Matriks Metalloproteinaz-9'un koyunların pulmoner adenokarsinom olgularının metastazındaki rolünün immüno-histokimyasal yöntemlerle değerlendirilmesi amaçlandı. Rutin histopatolojik tanı için bölümümüze getirilen 26 koyundan akciğer dokusu örnekleri alındı. Akciğer dokuları %10'luk tamponlu formalinde tespit edildi, rutin prosedürler izlenerek dokular parafin blok içine gömüldü. Dokulardan alınan kesitler 5 µm kalınlığında kesildi ve histopatolojik değişiklikleri saptamak için Hematoksilin & Eozin ile boyandı. Kesitler ışık mikroskopunda incelendi ve fotoğrafları çekildi. İmmünohistokimyasal boyama olarak Avidin-Biotin Peroksidaz yöntemi kullanıldı. Koyunların pulmoner adenokarsinomlarında tümör hücrelerinin asiner, papiller veya miks tip büyüme gösterdiği bulundu. Yirmi olgunun sadece 2'sinde lenf nodlarına metastaz vardı ve bu metastatik alanlardaki tümör hücre proliferasyonu, primer kanser odaklarına benzerdi. Tüm koyun pulmoner adenokarsinomları, JSRV CA ve Matriks Metalloproteinaz-9 immünoreaktivitesi yönünden pozitif. Kontrol grubunda herhangi bir pozitif JSRV CA ve Matriks Metalloproteinaz-9 ekspresyonu tespit edilmedi. JSRV CA ve Matriks Metalloproteinaz-9 immün pozitif hücre sayısı, kontrol grubuna kıyasla koyun akciğer adenokarsinomu grubunda istatistiksel olarak artmıştı. Çalışmanın verileri doğrultusunda Matriks Metalloproteinazların başta Matriks Metalloproteinaz-9 olmak üzere koyun akciğer adenokarsinomlarında tümör metastazında ciddi rol oynadığı düşünülmektedir.

Anahtar sözcükler: Matriks metalloproteinaz-9, Metastaz, Koyun pulmoner adenokarsinomu

INTRODUCTION

Ovine pulmonary adenocarcinoma (OPA), also known as sheep pulmonary adenomatosis or jaagsiekte, is contagious

lung cancer caused by *Jaagsiekte sheep retrovirus* (JSRV; family *Retroviridae*, subfamily *Orthoretrovirinae*, genus *Betaretrovirus*)^[1-3]. OPA, which is seen all over the world except Australia, New Zealand and the Falkland Islands,

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causes significant economic losses (growth rate, carcass weight, milk and wool production) in the sheep industry [4,5]. Since the incubation period is quite long in naturally infected sheep, OPA has been reported generally in 2-4 year old sheep, goats and rarely in 2-month-old lambs [6,7]. Transmission of OPA occurs primarily through the aerosol route, which means that close contact with infected sheep is an important risk factor. The virus has also been detected in the milk and colostrum of infected sheep, which is a potential source of infection for newborn lambs [8]. Clinical signs in animals affected by OPA can be briefly summarized as follows; cough, dyspnea, tachypnoea, nasal discharge, loss of condition, exercise intolerance, and in some cases copious pulmonary fluid production [5,9]. JSRV causes oncogenic transformation mostly in type 2 pneumocytes and less frequently in club cells (Clara cells) and undifferentiated cells [10,11]. The World Health Organization defines OPA as a mixed adenocarcinoma with acinar, papillary, and bronchoalveolar developmental patterns, and OPA is a useful animal model for understanding viral oncogenesis in human lung adenocarcinomas due to its clinical, morphological, pathological, and histopathological similarities [7,12,13]. Metastases to regional lymph nodes occur in 0.3-25% of cases, however, distant tissue metastases are extremely rare [3,14].

Matrix metalloproteinases (MMPs) is known for their ability to impair the extracellular matrix (ECM) and basement membrane, thereby playing a vital role in promoting tumor invasion and metastasis [15,16]. MMP-9, a member of MMPs, can degrade types IV, V, VII, IX and X collagen, elastin, fibrin, fibrinogen and plasminogen, and its overexpression prognostic value for the diagnosis of distant tissue metastasis or local recurrence for human lung cancers such as invasive adenocarcinoma, non-small-cell lung cancer (NSCLC) [17-19].

The aim of the study to evaluate the role of MMP-9 in the metastasis of OPA cases by immunohistochemical methods.

MATERIAL AND METHODS

Ethical Approval

This study was approved by the Kafkas University Animal Experiments Local Ethics Committee (KAU-HADYEK-2021/098).

Animals

Lung tissue samples were taken from 26 sheep (OPA group: 20 sheep and Control group: 6 sheep) brought to our department for routine histopathological diagnosis.

Histopathological Examinations

Lung tissues were fixed in 10% neutral buffered formalin, following routine procedures tissues were embedded in paraffin wax. Sections from tissues were cut into 5 µm

thickness and stained with Hematoxylin & Eosin to detect histopathological changes. The sections were examined under a light microscope and photographs were taken.

Immunohistochemical Examinations

The routine streptavidin-biotin peroxidase complex method was used according to the manual instructions of kit (Thermo Scientific Histostain-Plus IHC Kit, HRP, broad spectrum, REF: TP-125-HL). Primary antibodies MMP-9 (Santa Cruz, sc-393859, Dilution Ratio: 1/100) and JSRV Capsid Protein (JSRV CA, supplied by Prof. Massimo Palmarini, Dilution Ratio: 1/1500) were used after antigen retrieval (the sections were boiled in Citrat Buffer Solution (pH 6) for 25 min in the microwave oven at 800 watt) and nonspecific protein blocking. The reactions were detected with 3,3'-Diaminobenzidine (DAB) chromogen. Counterstainings were conducted using hematoxylin. Then, glass slides were mounted with Entellan and a coverslip. For control sections, instead of the primary antibody, PBS was applied in drops on the sections.

Prepared slides were examined under a light microscope (Olympus Bx53) and photographed via the Cell^P program (Olympus Soft Imaging Solutions GmbH, 3,4). Analyzes of the images were done with Image J Program (1.51j8). MMP-9 and JSRV CA expressions were analysed by examining three representative fields of labelled neoplastic cells with the 40X magnification. Rating systems were designated as negative (-) 0%, low (+) 1-10%, moderate (++) 11-59% or severe (+++) >60% [7].

Statistical Analysis

The significance of the MMP-9 and JSRV CA expression score difference between the OPA and control groups was evaluated with the Mann-Whitney U test. All analyzes were performed in the SPSS® (Version 18.0, Chicago, IL, USA) program. Scorings in groups were given as mean ± standard error (SE). The differences between the groups after statistical analysis were considered significant at the P<0.05 level.

RESULTS

Histopathological Results

In histopathological examinations, tumoral lesions were scattered in the lung and large and small foci of proliferating neoplastic cells in the initial stage OPA cases. In advanced stages of OPA, well circumscribed tumoral growths are seen in large areas. Acinar structures formed as a result of proliferation of alveolar cubic epithelial cells and papillary extensions towards the lumen were detected. While papillary structures were more prominent in some areas, acinar structures were predominant in some areas. In addition to acinar and papillary structures, masses formed by tumoral cells in alveolar lumens were

remarkable. Cell proliferation in the bronchioles in the tumoral areas was also in patterns similar to what we observed in the alveoli. There were very few mitotic figures, anisonucleosis and anisocytosis in these cuboidal-columnar tumor cells. The interstitium of proliferating alveoli and bronchioles was enlarged due to increase in connective tissue and mononuclear cell infiltrations. The presence of large numbers of alveolar macrophages in alveolar lumens around tumoral growths were determined. Bronchopneumonia and large areas of necrosis were other important histopathological findings. In lymph nodes (2 cases in total), quite large tumoral areas showing papillary, acinar or mixed growths similar to primary foci were observed (Fig. 1-A-D).

Immunohistochemical Results

All OPA cases were positive for JSRV CA and MMP-9 immunoreactivity. Any positive expression of JSRV CA and MMP-9 in the control group were not detected (Table 1). The number of JSRV CA and MMP-9 immune positive cells was statistically increased in the OPA group compared to the control group (Table 2). JSRV CA positive reactions were mostly in the apical cytoplasm of tumoral cells and in granular form (Fig. 2). Dark brown-black MMP-9 positive reactions were in both the cytoplasm and nucleus of tumoral cells exhibiting papillary and acinar growths. In two cases that metastasized to the lymph nodes, the reactions were quite severe and concentrated in the nucleus. Parallel to the primary lesions in metastatic lymph nodes, MMP-9 expressions were intranuclear. MMP-

9 immune positivity in advanced OPA cases was similar to metastatic cases. In the initial stages of OPA, the intensity of MMP-9 expressions was rather weak (Fig. 3, Fig. 4).

DISCUSSION

Anamnesis (weight loss, nasal discharge, dyspnea), clinical findings (wheelbarrow test), macroscopic and microscopic findings, immunohistochemical staining, electron microscopy, molecular methods (PCR), ultrasonography and computed tomography are very useful in the diagnosis of OPA [4,6,11,13]. The definitive diagnosis of the disease is made as a result of necropsy and histopathological examinations [4,13,20]. In histopathological examinations, papillary growths extending towards the alveolar and bronchiolar lumens in well-circumscribed tumoral areas were observed, consistent with the literature data [3,14]. In addition to papillary growths, tumoral cells form acinar structures in alveolar lumens were found as previously reported [6-8]. In this study, myxoid foci and cystic growths were also present in some cases, consistent with the literature data [3,12,14]. Similar to previous works, tumoral cells were cuboidal or columnar and mitotic figures were quite low [4,12]. As reported in previous studies, there were quite a number of alveolar macrophages in the alveolar lumens around the tumoral areas [3,13]. In addition, secondary bacterial pneumonia and large areas of necrosis were other important histopathological findings. These findings are similar to as reported by Kiran et al. [6]. In lymph nodes, quite large tumoral proliferations similar

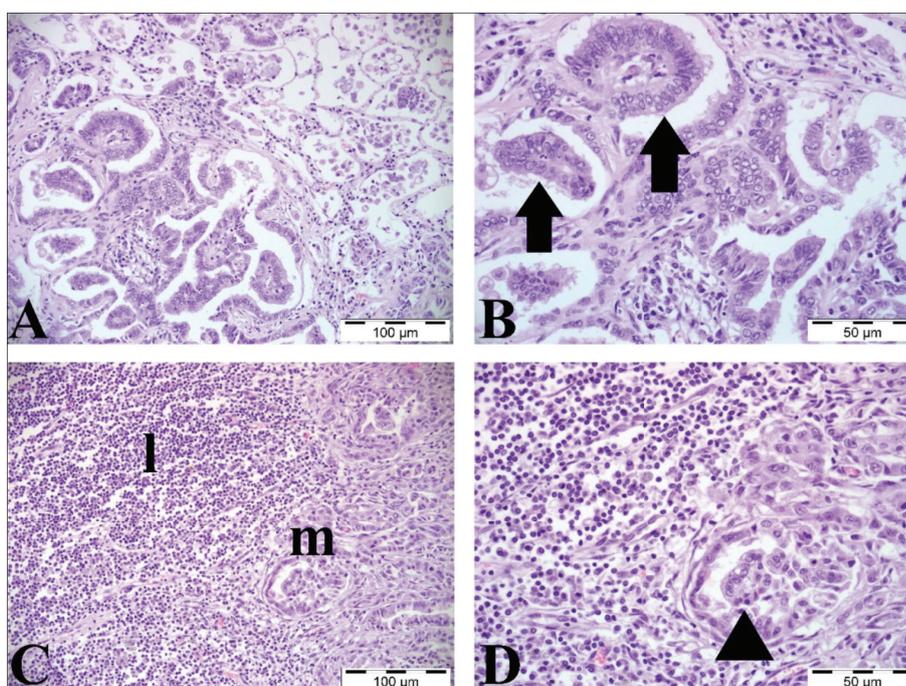


Fig 1. Lung and lymph tissue, H&E, A- Well circumscribed tumoral focus, B- Higher magnification, neoplastic cell proliferation with acinar structures in alveolar lumens (arrows), C- Metastatic focus (m) and lymph node (l), D- Higher magnification, Mass consisting of tumoral cells in the metastatic focus (arrowhead)

Table 1. JSRV CA and MMP-9 immunoreactivity scores of all groups

Case No	JSRV CA	MMP-9	Metastasis
OPA Case 1	+++	+	-
OPA Case 2	+++	++	+
OPA Case 3	+++	++	+
OPA Case 4	++	+	-
OPA Case 5	++	+	-
OPA Case 6	++	+	-
OPA Case 7	++	+	-
OPA Case 8	+++	+	-
OPA Case 9	+++	+	-
OPA Case 10	+++	+	-
OPA Case 11	+++	+	-
OPA Case 12	++	+	-
OPA Case 13	++	+	-
OPA Case 14	++	+	-
OPA Case 15	++	++	-
OPA Case 16	+++	++	-
OPA Case 17	+++	+	-
OPA Case 18	+++	+	-
OPA Case 19	+++	+	-
OPA Case 20	+++	+	-
Control Case 21	-	-	-
Control Case 22	-	-	-
Control Case 23	-	-	-
Control Case 24	-	-	-
Control Case 25	-	-	-
Control Case 26	-	-	-

Table 2. Mean ± SE values of all groups

Groups	JSRV CA	MMP-9
Control	0±0 ^a	0±0 ^a
OPA	2.60±0.11 ^b	1.20±0.09 ^b
P value	<0.001	<0.001

^{a,b} Values within a row with different superscripts differ significantly at P<0.05

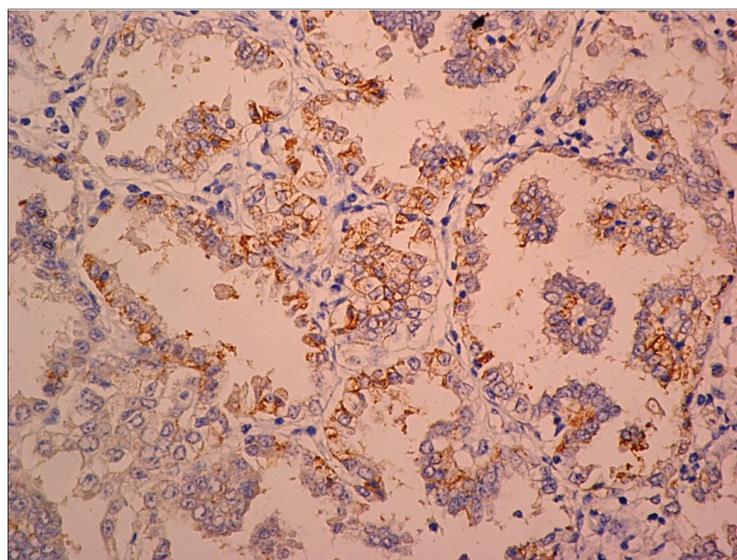


Fig 2. JSRV CA immune positive reaction in the apical cytoplasm of neoplastic cells, IHC, 20X Objective

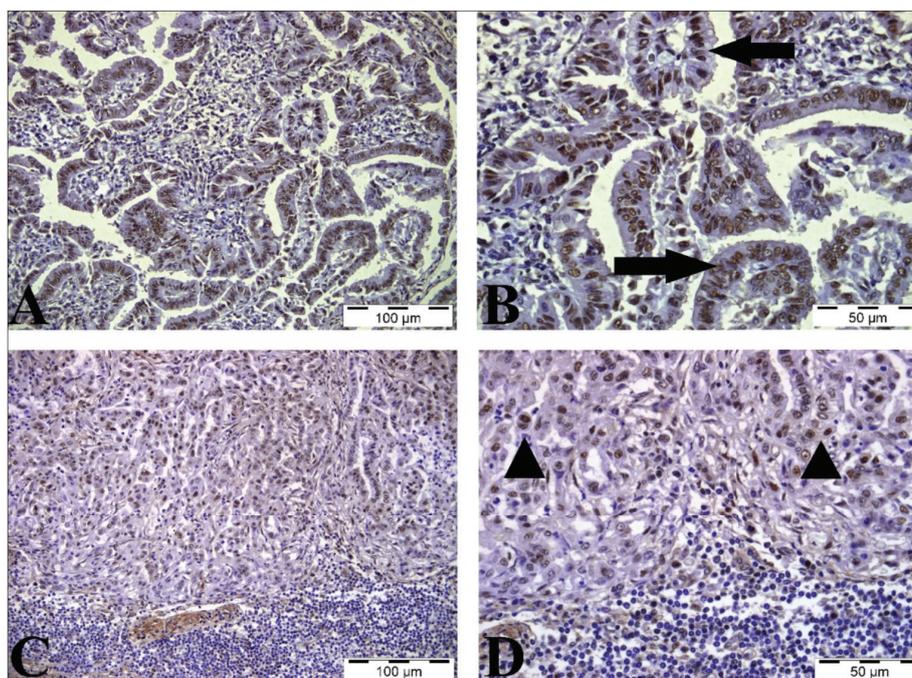


Fig 3. Lung and lymph tissue, MMP-9, IHC, A- Lung, tumoral area, B- Higher magnification, immune positive reactions (arrows) in the nuclei of neoplastic cells forming acinar structures in their alveolar lumens, C- Metastatic focus in the lymph node, D- Higher magnification, intranuclear MMP-9 expressions (arrowheads) in tumoral cells

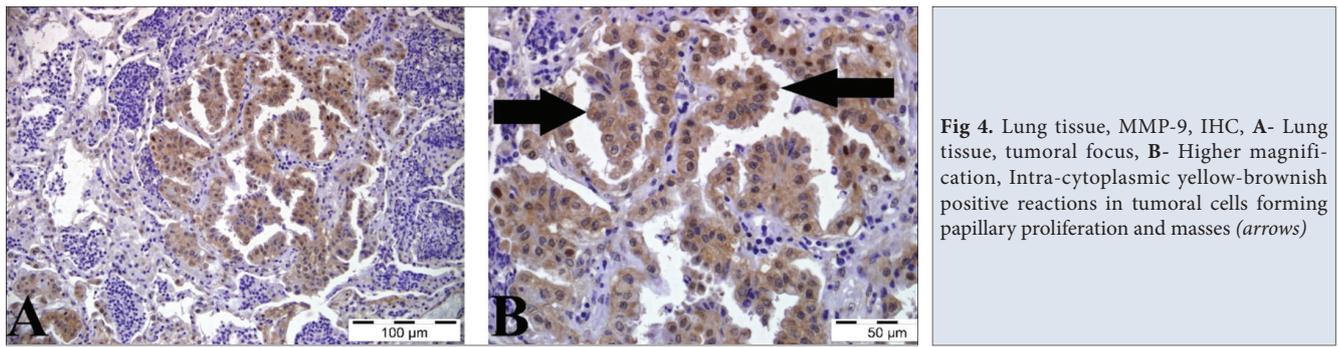


Fig 4. Lung tissue, MMP-9, IHC, A- Lung tissue, tumoral focus, B- Higher magnification, Intra-cytoplasmic yellow-brownish positive reactions in tumoral cells forming papillary proliferation and masses (arrows)

to primary lung foci were detected. These findings were consistent with previous studies [3,6,7,14]. All of the cases were diagnosed as OPA histopathologically were immune positive for JSRV CA expression and these immune positive reactions were in the cytoplasm of neoplastic epithelial cells as reported by different researchers [3,7,12,14].

OPA mostly metastasizes to bronchiolar and mediastinal lymph nodes. Metastasis to distant tissues such as liver, kidney, heart and skeletal muscle is very rare [1,14]. In our study, in parallel with the literature data, only 2 of 20 (10%) OPA cases had metastasis in the mediastinal lymph node [1,14,20]. Metastasis is a complex multistep process involving cell adhesion and proteolytic degradation of the ECM [16,21]. The proteolytic degradation of the ECM plays a key role in the invasion and metastasis of tumor cells [16,18,22]. MMPs, a family of zinc-dependent endopeptidases, are involved in breakdown of ECM and basement membrane, and perform critical tasks in facilitating tumoral invasion and metastasis [15,16,23]. Various researchers have associated increased MMP-9 expression in many malignancies such as NSCLC, lung adenocarcinoma, colon cancer, with cancer progression, invasion, metastasis, pathological grading and staging, and poor prognosis [15,16,24]. The increase in MMP-9 expression is a very valuable marker in the evaluation of recurrence and distant tissue metastasis in NSCLC patients, and it has been demonstrated by cell culture and clinical evaluations that tumoral invasion and metastasis decreased significantly by inhibiting MMP-9 [17,21]. Although there are a large number of studies evaluating the relationship between MMP-9 and metastasis in lung cancers in human medicine in the literature reviews, there are almost no studies evaluating the relationship between OPA and MMP-9 in veterinary medicine [20]. Mishra et al. [20] evaluated MMP-2 expressions in OPA cases by immunohistochemical methods and observed MMP-2 positive reactions in the cytoplasm of tumoral cells and in inflammatory cells. Mishra et al. [20] reported that there was no direct relationship between metastasis of OPA cases and MMP-2 expressions. In another study, Gomes et al. [22] measured MMP-2 and MMP-9 genes in lungs and cultured AECIIs of JSRV-induced pulmonary adenocarcinomas and reported no difference in MMP-2 mRNA levels in cancers

compared to normal lungs. They also noted that only four cancers expressed MMP-9, while normal samples did not express this gene. Chitra et al. [23] measured MMP-9 expressions in JSRV *Env*-mediated lung adenocarcinoma invasive cell line (A549) with gelatin zymogram and noted that MMP-9 expressions were quite high. In current study, MMP-9 expression increased statistically in OPA cases compared to normal lung tissues were determined, similar to the literature data [20,22,23]. MMP-9 expressions were severe especially in metastatic OPA cases and neoplastic cells were positive for MMP-9 immunoreactivity in tumor areas in lymph nodes, similar to primary lung tumor areas. Nuclear MMP-9 staining was also detected in our study. This is a rare finding because MMP-9 expression is predominantly cytoplasmic or extracellular. Nuclear MMPs have functions such as leading to apoptosis, tissue remodeling upon injury, and cancer progression [25]. It has been reported that intranuclear MMP-9 activity degrades nuclear DNA repair proteins and causes accumulation of oxidative DNA damage in various types of cells such as neurons [26,27]. The nuclear localization of gelatinases and their nuclear substrates supports a new role for intranuclear gelatinase activity in an intrinsic apoptotic pathway [26,28], and Hill et al. [27] confirmed the nuclear localization of gelatinases and their substrates in an acute stroke injury model further supporting a novel role for intranuclear gelatinase activity in an intrinsic apoptotic pathway in neurons during acute stroke injury. In line with the data of this study, it is thought that MMPs play a serious role in tumor metastasis and cancer progression in OPA cases, especially MMP-9. In addition, intranuclear MMP-9 expressions may be related to the triggering of the intrinsic apoptosis pathway caused by JSRV and the resulting DNA damage.

In conclusion, in order to fully understand the metastatic potential of OPA cases, it is essential to evaluate all members of the MMP family in a systemic and controlled manner and to evaluate different methods such as gelatin zymogram, PCR and western blot apart from immunohistochemical methods.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that data supporting the study findings are also available to the corresponding author.

FUNDING SUPPORT

None.

COMPETING INTERESTS

Authors declare there are no conflicts of interest in the present study.

AUTHOR CONTRIBUTIONS

EK: Idea, concept and writing the article; HN, AY and EK: Histopathological and immunohistochemical stainings; EB, SD and EK: Histopathological and immunohistochemical analysis.

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