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# RESEARCH ARTICLE

## The immunohistochemical evaluation of the expression of intermediary filaments, PCNA, p53 and MMP-9 in feline fibrosarcomas

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## Kedilerin fibrosarkomlarında intermedier filamentler, PCNA, p53 ve MMP-9 ekspresyonlarının immunohistokimyasal olarak değerlendirilmesi

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**Öz**

**Amaç:** Güncel çalışmada, kedilerin fibrosarkomlarında hücre proliferasyon indeksi, p53 tümor baskılayıcı gen durumu, tümor metastazı, invazyon kapasitesi ile hücresel orjinin ortaya konulması amacıyla Prolifere olan hücre nükleus antijeni (PCNA), Matriks Metalloproteinaz-9 (MMP-9), Vimentin, Alfa-Düz Kas Aktini (α-SMA), S-100, Desmin ve Pan- Sitokeratin (Pan-SK) gibi kanser markerları ve intermedier filamentlerin ekspresyonlarının immunohistokimyasal olarak değerlendirilmesi amaçlanmıştır.

**Gereç ve Yöntem:** Bu çalışmada, Patoloji Anabilim Dalı'na rutin histopatolojik inceleme amacıyla getirilen 7 adet kediden alınan tümoral doku örnekleri kullanılmıştır. Histopatolojik değişikliklerin değerlendirilebilmesi amacıyla kesitlere Hematoksilen & Eozin boyaması uygulandı. İmmunohistokimyasal boyamalarda streptavidin-biotin peroksidaz kompleksi yöntemi kullanıldı.

**Bulgular:** 7 spontane kutanöz fibrosarkom vakasının 5'i evre I, 1'i evre II ve 1'i ise evre III olarak saptandı. Tümöral dokulardaki belirgin kollajen bantlarının varlığı Masson Trikrom boyama ile ortaya konuldu. Vimentin ve α-SMA boyanmaları yönünden tüm vakalar pozitifti. Pan SK, S-100 ve Desmin boyanmaları yönünden tümör hücreleri negatif reaksiyon verdi. Tüm vakalar PCNA yönünden pozitifti. Sadece 2 vaka p53 pozitif boyanma gösterdi. Bu 2 vaka, ileri evrelerdi. Sadece bir fibrosarkom vakasında (Evre III) tümör tipi dev hücrelerinde intrasitoplazmik MMP-9 ekspresyonları tespit edildi.

**Öneri:** Sonuç olarak, çalışmada kullanılan tümör örneklem sayısı az olsa da, PCNA, p53 ve MMP-9'un kedilerin spontane fibrosarkomlarında hücre proliferasyonunun belirlenmesi, malignitenin derecelendirilmesi, tümör agresifliği ve davranışı ile prognozun saptanmasında oldukça kullanışlı markerlar olduğunu ortaya koymuştur.

**Anahtar kelimeler:** Fibrosarkom, hücre proliferasyonu, intermedier filamentler, kedi

**Abstract**

**Aim:** The present study was aimed at the immunohistochemical evaluation of the expression of cancer indicators; including the Proliferating Cell Nucleus Antigen (PCNA), Matrix Metalloproteinase-9 (MMP-9), vimentin, alpha-smooth muscle actin (α-SMA), S-100, desmin and pan-Cytokeratin (Pan-CK), as well as intermediary filaments, with a view to demonstrate the cell proliferation index, p53 tumour-suppressor gene status, tumour metastasis, invasion capacity and cellular origin of feline fibrosarcomas.

**Materials and Methods:** The study material comprised of tumoral tissue samples from 7 cats, which were referred to the Pathology Department for routine histopathological examination. In order to evaluate the histopathological changes, the sections were stained with Haematoxylin and Eosin. Streptavidin-biotin peroxidase complex method was used for immunohistochemical staining.

**Results:** Out of the seven spontaneous cutaneous fibrosarcoma cases, five were in stage I, one was in stage II, and one was in stage III. The presence of conspicuous collagen bands in the tumoral tissues was demonstrated by Masson's trichrome (MT) staining. All cases stained positively for vimentin and α-SMA. The tumoral cells reacted negatively for pan-CK, S-100 and desmin. All cases were immunopositive for PCNA. Two cases stained positively for p53. These two cases were advanced stage. Only a single case of fibrosarcoma (stage III) presented with intracytoplasmic MMP-9 expression in neoplastic giant cells.

**Conclusion:** Despite only very few tumour samples having been examined in the present study, it was concluded that PCNA, p53 and MMP-9 served as highly useful indicators for determining cell proliferation, grading malignancy, detecting tumour aggressiveness and behaviour, as well as predicting prognosis in spontaneous feline fibrosarcomas.

**Keywords:** Cellular proliferation, feline, fibrosarcoma, intermediary filaments

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## Introduction

Soft tissue sarcomas (STSs) are a group of tumours, which are frequently diagnosed in domestic cats. STSs include fibrosarcomas, nerve sheath tumours originating from the dermis or subcutis, myxosarcomas, leiomyosarcomas, liposarcomas, rhabdomyosarcomas, perivascular wall tumours and fusiform cell tumours/sarcomas (Dobromylskyj 2022). Feline injection-site sarcomas (FISSs) have proven to be a very useful model to better understand soft tissue tumours (Patruno et al 2021). FISSs are mostly localized to the neck, thorax, lumbar region, flanks and legs (Ishtiaq and Sozmen 2021). The risk of developing these tumours is reported to increase with the number of injections performed in a given region (particularly in the intrascapular region) (Zabielska et al 2012). The pathogenesis of FISSs is still under discussion. However, according to the most widely accepted hypothesis, neoplastic transformation is triggered by the excessive expression of growth factors (acidic and basic fibroblast growth factor) in relation to an inflammatory response against the rabies and feline leukaemia viruses, as well as by oncogene activation (Zabielska-Koczywąs et al 2017, Cecco et al 2019). Nonetheless, various cytokines and mutations of tumour-suppressor genes are also detected in this type of cancer (Carneiro et al 2019). These locally invasive tumours are reported to metastasize to the lungs in approximately 20% of all affected cats (Bing et al 2018).

Tumour diagnosis is based on clinical examination, anamnesis and histopathological examination. Treatment options include among others, surgical intervention, radiotherapy and chemotherapy (Zabielska-Koczywas et al 2017). Most FISSs are fibrosarcomas (72%). Nevertheless, other histological variants including histiocytic sarcomas, leiomyosarcomas, liposarcomas, myxosarcomas and rhabdomyosarcomas, are also encountered (Zanuncio et al 2021). Fibrosarcomas originating from fibroblasts, myofibroblasts and immature proliferous fibroblasts are the most common malignant mesenchymal tumours in cats and comprise 14-28% of all feline skin tumours with a mean host age of 12 years (Patruno et al 2021). In addition to the skin, subcutaneous tissues and the oral cavity are other common sites of this malignancy. There is no known breed or sex predisposition for the development of these tumours (Ahmed and Sozmen 2020). Recurrence is common in cases of fibrosarcoma and metastases to distant organs may result in the death of the animal (Patruno et al 2021).

The present study was aimed to demonstrate the cell proliferation index, p53 tumour-suppressor gene status, tumour metastasis, invasion capacity and cellular origin of feline fibrosarcomas.

## Material and Methods

### *Animals*

The study material comprised of tumoral tissue samples from 7 cats, which were referred to the Pathology Department of Kafkas University Faculty of Veterinary Medicine for routine histopathological examination between the years 2018-2022. Detailed information pertaining to the animals is presented in Table 1.

### *Ethics Board Approval*

This study was approved by the Local Ethics Board for Animal Experiments of XXX University (Decision number: KAU-HADYEK- 2022/079).

### *Histopathological Examination*

The tumoral tissue samples taken from the cats were firstly fixed in 10% buffered formalin solution. Following routine tissue processing, five-micron-thick serial sections were cut from the paraffin blocks. In order to evaluate the histopathological changes, the sections were stained with haematoxylin and eosin (H&E). The preparations were examined in detail under a light microscope by at least two different pathologists, and any changes were imaged with a camera. The staging of the tumours was performed according to the criteria described by Couto et al 2002 and Haddad et al 2010 (cellular differentiation, presence and distribution of necrosis within the neoplasm, mitotic rate, presence of multinucleate giant cells). The criteria and scoring system used for this purpose are shown in Table 2.

### *Masson's Trichrome Staining*

Staining was performed in accordance with the manufacturer's instructions (Facepath, Barcode Number: 8681065132824).

### *Immunohistochemical Examination*

Four-micron-thick serial sections, cut from the paraffin blocks of the tumoral tissue samples, were stained with commercial antibodies against vimentin, Alfa-smooth muscle actin (α-SMA), desmin, S-100, Pan-Cytokeratin (pan-CK), Proliferating Cell Nucleus Antigen (PCNA), p53 and Matrix Metalloproteinase-9 (MMP-9), in accordance with the manufacturer's instructions and by using the avidin-biotin-peroxidase (ABC) technique. Detailed information on the primary antibodies used in this study are given in Table 3. All immunostainings were performed using the Thermo Scientific Histostain IHC Kit (HRP, broad spectrum, REF: TP-125-HL). Amino-ethyl-carbazole (AEC, Thermo Scientific, REF: TA-125-HA) was used as a chromogenic substrate and

Table 1.Information pertaining to the animals included in the study.

| Case Number | Species | Breed | Age | Sex | Localization | Microscopy | Metastasis | Recurrence | Stage |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Cat | Crossbreed | 8 | Male | Jaw and Fore Leg | Fibrosarcoma | - | - | I |
| 2 | Cat | Crossbreed | 2 | Male | Waist | Fibrosarcoma | - | - | I |
| 3 | Cat | Crossbreed | 12 | Female | Jaw | Fibrosarcoma | - | - | I |
| 4 | Cat | Crossbreed | 6 | Male | Flank | Fibrosarcoma (with giant cells) | - | + | III |
| 5 | Cat | Crossbreed | 10 | Female | Hip | Fibrosarcoma | - | - | I |
| 6 | Cat | Crossbreed | 10 | Female | Upper Thigh (Femur) | Fibrosarcoma | - | - | II |
| 7 | Cat | Crossbreed | 8 | Male | Jaw | Fibrosarcoma | - | - | I |

Table 2.Tumour staging system.

| | 1 | 2 | 3 |
|---|---|---|---|
| **Differentiation Score** | Sarcomas resembling adult mesenchymal tissue | Displaying a described histological phenotype | Poorly differentiated tumours |
| **Mitotic Rates** | 1-9 mitotic figures in 10 areas examined at 400X | 10-19 mitotic figures in 10 areas examined at 400X | 20 or more mitotic figures in 10 areas examined at 400X |
| **Necrosis** | Necrosis not observed | Rate of necrosis less than 50% of the total area of the sample | Rate of necrosis more than 50% of the total area of the sample |

*** Final scores of 3 or 4 were assigned as stage 1; final scores of 5 or 6 were assigned as stage II; and final scores of 7, 8 or 9 were assigned as stage III.

Table 3.Information pertaining to the primary antibodies used in the immunohistochemical examinations.

| Primary Antibodies | Manufacturer and Catalogue Number | Dilution | Incubation |
|---|---|---|---|
| Anti-Pan-CK | Novus Biologicals, PCK-26 | 1/400 | Overnight at 4 °C |
| Anti-Vimentin | Thermo Scientific, RM-9120-R7 | Ready for use | Overnight at 4 °C |
| Anti-α-SMA | Thermo Scientific, MS-113-R7 | Ready for use | Overnight at 4 °C |
| Anti-Desmin | Leica Biosystems, PA0032 | Ready for use | Overnight at 4 °C |
| Anti-S-100 | Thermo Scientific, MS-296-P1 | Ready for use | Overnight at 4 °C |
| Anti-PCNA | Santa Cruz, sc-56 | 1/100 | Overnight at 4 °C |
| Anti-p53 | ABclonal, A3185 | 1/100 | Overnight at 4 °C |
| Anti-MMP-9 | Santa Cruz, sc-393859 | 1/100 | Overnight at 4 °C |

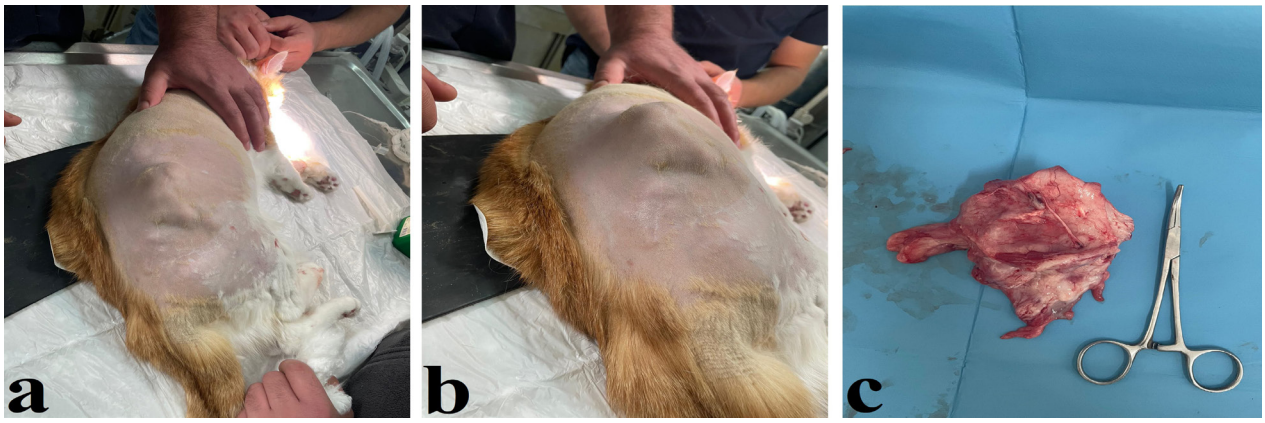


Figure 1. **a-b-c:** Macroscopic appearance of a tumoral mass before and after surgery.

was left for incubation for 15 minutes. Next, the sections were washed in distilled water for 5 min, stained with Mayer's haematoxylin, and covered with AEC mounting solution.

After being mounted, the preparations were examined under a light microscope (Olympus Bx53) and were imaged with the use of the Cell ^P software (Olympus Soft Imaging Solutions GmbH, 3.4). The detailed analyses of the images were performed using the Image J software (1.51j8).

When analysing the results of the immunohistochemical stainings, a scoring system, which was based on the number of positive tumoral cells in the regions with the strongest staining that were identified by immunopositive reactions, was used. In each tumoral tissue, 3 different regions were evaluated at 400X magnification. The number of immunopositively stained cells was recorded for each region, and the mean value of the 3 regions was noted as the mean number of positive cells for the given case. Scoring was performed as follows: (-) absent, no immunoreactivity in the cells; (+) weak, immunopositivity in 1-10% of the cells; (++) moderate, immunopositivity in 11-59% of the cells, and (+++) severe, immunopositivity in more than 60% of the cells (Beytut 2017).

## Results

The macroscopic examination of the tumoral masses revealed a rather hard consistency and greyish white colour of the cross sections. The exterior of some of the masses were severely haemorrhagic and necrotic (Figure 1 a-b-c). Distant tissue metastases were not found in any of the cases.

Out of the seven spontaneous cutaneous fibrosarcoma cases, five were in stage I, one was in stage II, and one was in stage III. Histopathological examination showed that the tumour cells had merged with the collagen matrix and formed spiral structures extending in various directions. The neoplastic cells had highly irregular borders, a pale eosinophilic cytoplasm, and a thin elongated nucleus. Most of the tumoral cells were of a fusiform structure. The nuclei of these cells were observed to be hyperchromatic. Furthermore, they displayed marked nuclear and cytoplasmic pleomorphism. Their nucleoli were basophilic and ranged from 1 to 3 in number. Some regions, particularly in the advanced cases (which were in stages II and III) presented with very abundant mitotic figures. Moreover, peculiar neoplastic giant cells with an appearance as if their nuclei were on top of each other were detected in large numbers (stage III). In addition, the tumoral tissues contained large areas of haemorrhage and necrosis. Another histopathological finding was inflammatory cell infiltration, mostly composed of lymphocytes and macrophages (Figure 2 a-b-c).

The presence of conspicuous collagen bands in the tumoral tissues was demonstrated by Masson's trichrome (MT) staining. The cytoplasm of well-differentiated fibrocytes and fibroblasts stained blue with MT. Staining was much stronger in the cases that were in the early stage, compared to those in the advanced stages (Figure 3a).

The immunoreactivity scores of the cats with fibrosarcoma are presented in Table 4. All cases stained positively for vimentin and α-SMA. Vimentin expression in the large number of tumoral cells with spiral structures extending in different directions was determined to be intracytoplasmic. The vimentin-positive reactions were diffuse and highly strong. On the other hand, intracytoplasmic α-SMA reactions were localized to the peripheral bundles (myofibroblast-like cells) and particularly to the blood vessels. The tumoral cells reacted negatively for pan-CK, S-100 and desmin. All cases were immunopositive for PCNA. PCNA expression was localized to the nucleus of the tumoral cells. Stainings were much stronger in the advanced stage cases, compared to the cases in the early stage. Only 2 cases stained positively for p53. These two cases were advanced stage cases and reactions were mostly observed intranuclearly in multinucleate giant

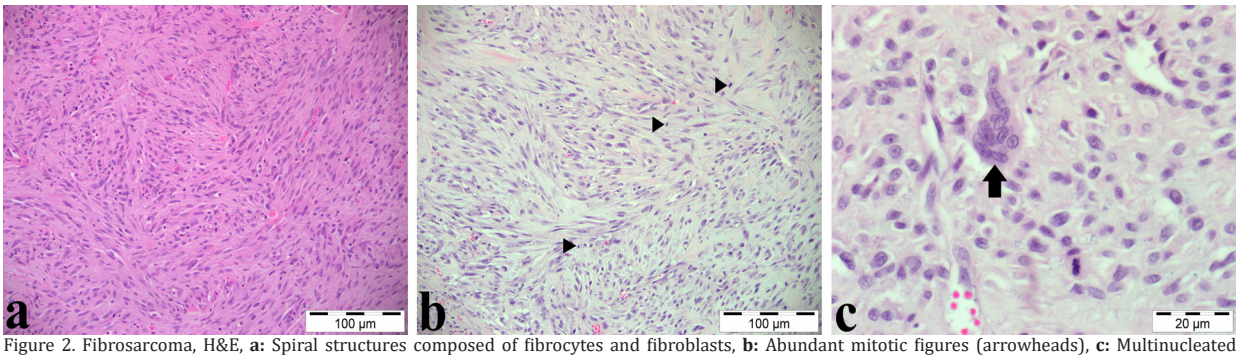


Figure 2. Fibrosarcoma, H&E, **a:** Spiral structures composed of fibrocytes and fibroblasts, **b:** Abundant mitotic figures (arrowheads), **c:** Multinucleated neoplastic giant cell (arrow).

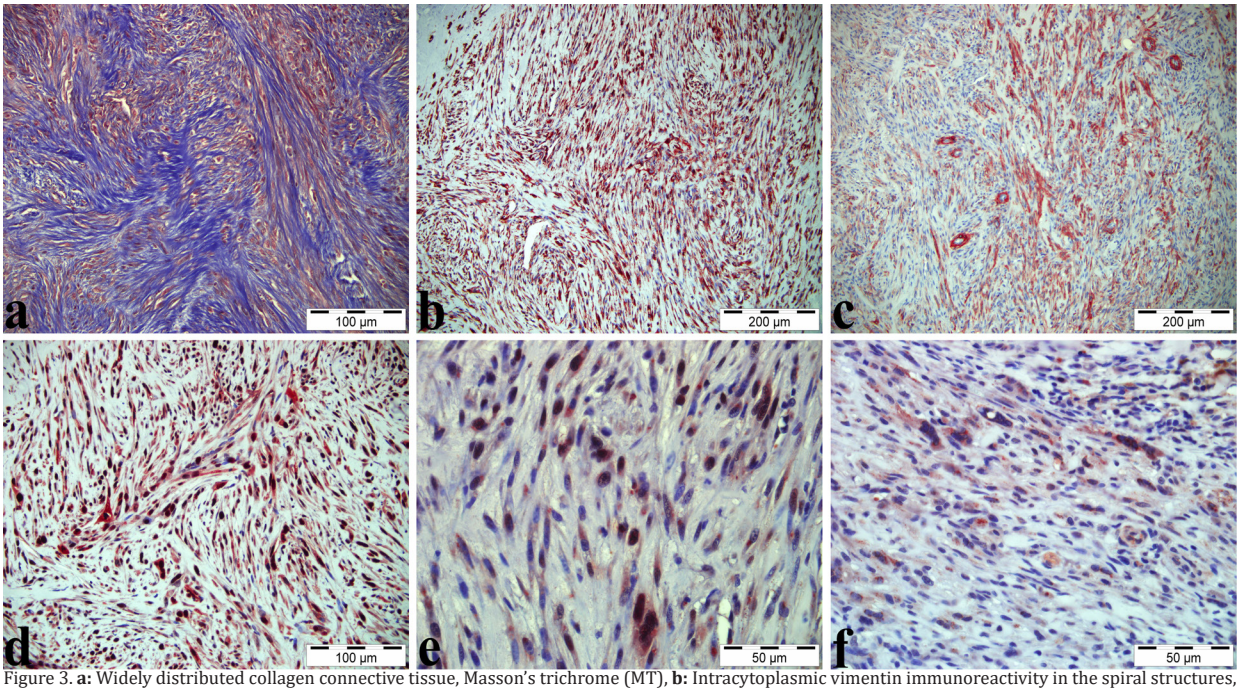


Figure 3. **a:** Widely distributed collagen connective tissue, Masson's trichrome (MT), **b:** Intracytoplasmic vimentin immunoreactivity in the spiral structures, **c:** α-SMA-positive staining localized to the periphery of the tumour, and particularly to the blood vessels, **d:** Intense intranuclear PCNA expression in the neoplastic cells, **e:** p53-positive reactions in the nuclei of the multinucleate giant cells, **f:** Intracytoplasmic MMP-9 staining in the neoplastic giant cells.

Table 4.Immoreactivity scores of all cases

| Case Number | Masson's Trichrome (MT) | Pan-CK | Vimentin | α-SMA | Desmin | S-100 | PCNA | p53 | MMP-9 |
|---|---|---|---|---|---|---|---|---|---|
| 1 (Stage I) | +++ | - | +++ | + | - | - | + | - | - |
| 2 (Stage I) | +++ | - | +++ | + | - | - | ++ | - | - |
| 3 (Stage I) | +++ | - | +++ | + | - | - | + | - | - |
| 4 (Stage III) | + | - | ++ | ++ | - | - | +++ | +++ | ++ |
| 5 (Stage I) | +++ | - | +++ | + | - | - | + | - | - |
| 6 (Stage II) | + | - | ++ | ++ | - | - | +++ | + | - |
| 7 (Stage I) | +++ | - | +++ | ++ | - | - | + | - | - |

cells. Only a single case of fibrosarcoma (stage III) presented with intracytoplasmic MMP-9 expression in neoplastic giant cells.

## Discussion

In the present study, the presence of intermediary filaments, including pan-cytokeratin, vimentin, α-SMA, desmin and S-100 were evaluated immunohistochemically with a view to demonstrate the cellular origin of fibrosarcomas in different stages. As was expected, all fibrosarcomas produced negative reactions for pan-cytokeratin, which is a major epithelial tumour indicator (Ozmen et al 2015, Cora et al 2017, Carneiro et al 2019). This data confirmed that the tumours were not of epithelial origin (Zabielska et al 2012). All of the tumours reacted positively for vimentin (Ishtiaq and Sozmen 2021). This confirmed that the tumours were of mesenchymal origin (Zabielska et al 2012, Zanuncio et al 2021). The expression of vimentin was particularly more distinct in the well-differentiated cases (Martín de las Mulas et al 1995, Martano et al 2012). Staining with Masson's trichrome demonstrated fibroblast proliferation in the tumoral regions (Kadam et al 2021). In agreement with literature data, all of the cases were positive for α-SMA expression (Vascellari et al 2003, Aberdein et al 2007, Zabielska et al 2012). As reported in previous studies (Carminato et al 2011, Ishtiaq and Sozmen 2021), staining was mostly concentrated in the periphery of the tumours. All cases reacted positively for both vimentin and α-SMA. These immunohistochemical findings were in support of the myofibroblastic differentiation of the tumours (Couto et al 2002, Daly et al 2008, Carneiro et al 2019). Similar to the reports of several researchers, all of the cases displayed negative staining for desmin (Couto et al 2002, Vascellari et al 2003, De Man and Ducatelle 2007) and S-100 (Ozmen et al. 2015, Cora et al 2017, Ishtiaq and Sozmen 2021).

PCNA is a well-preserved protein involved in DNA replication, DNA repair, chromatin remodelling and the regulation of the cell cycle (Ahmed and Sozmen 2020). PCNA concentrations are directly related to the proliferation of neoplastic tissues (Zabielska-Koczywąs et al 2017). Moreover, PCNA expression is also proportionate to the malignancy of various canine and feline tumours (Ahmed and Sozmen 2020). However, literature review has shown that there are only very few studies available on PCNA expression in the spontaneous and injection-site fibrosarcomas of cats. In 2015, Ozmen et al. evaluated PCNA expression, as an indicator of malignancy, in cases of feline fibrosarcoma and determined positive reactions in these tumours. Zabielska-Koczywąs et al (2017) suggested PCNA as a highly useful indicator particularly for tumour staging. In a similar study by Ahmed and Sozmen (2020), it was reported that in canine and feline spontaneous and injection-site fibrosarcomas, PCNA-reactivity was positively correlated with both the mitotic index and tumour stage. In their case study on a cutaneous fibrosarcoma in a tiglon, Kadam et al (2021) detected strong PCNA-positive reactivity for neoplastic fibroblasts in both the primary mass and the secondary metastatic tumoral mass in the lungs. In agreement with literature reports, in the present study, PCNA-positive stainings in the neoplastic fibroblasts and multinucleate giant cells were observed to show intranuclear localization (Ozmen et al 2015, Zabielska-Koczywąs et al 2017, Ahmed and Sozmen 2020, Kadam et al 2021). In the present study, although the tumoral samples examined were few in number, PCNA expressions were observed to be particularly stronger in the advanced stage cases (which were poorly differentiated and contained giants cells) and recurrent cases (which were in stages II and III). Based on these findings, it is considered that PCNA serves as a highly useful indicator for the determination of cell proliferation, grading of malignancy, and prediction of prognosis in spontaneous feline fibrosarcomas.

When activated, p53, which is a major protein involved in the cellular response against DNA damage, induces the termination of the cell cycle and triggers DNA repair and/or cell death (Hershey et al 2005, Bing et al 2018). p53 induces apoptosis in the event of unrepairable structural DNA damage (Nieto et al 2003, Mucha et al 2012). p53 mutations are detected in more than 50% of human soft tissue sarcomas (Nasir et al 2000, Nambiar et al 2001). These mutations are more common in metastatic sarcomas and advanced stage lesions. Contrary to human cases, p53 mutations are not very common in feline neoplasms (Mayr et al 2000, Bing et al 2018). However, excessive p53 expression has been determined in feline cases of injection-site sarcomas. In fact, the somatic alterations of the p53 gene have been reported to be correlated with the prognosis of FISSs (Mucha et al 2012). Different from these studies, Nasir et al (2000) reported not to have detected any immunoreactivity for p53 in feline fibrosarcomas. Indeed, in the present study, p53-positive immunostaining was detected in only two out of the seven fibrosarcomas. These were advanced stage cases and one had recurred shortly after surgery. These results show that p53 is a highly useful prognostic indicator for feline fibrosarcomas. Hershey et al (2005) reported that FISS cases displaying cytoplasmic p53 staining recurred within a shorter period, compared to FISS cases displaying nuclear p53 staining. However, these researchers did not detect any statistically significant difference between these cases for survival rate. In the present study, in agreement with the reports of various researchers (Nambiar et al 2001, Nieto et al 2003), all stainings were determined to be intranuclear and in particular localized to neoplastic multinucleated neoplastic giant cells. Contrary to the report of Hershey et al (2005), no cytoplasmic reaction was detected in the present study.

Matrix metalloproteinases (MMPs) are enzymes involved in cancer invasion, metastasis and angiogenesis (Fathipour et al 2018). The active forms of MMP-2 (Gelatinase-A) and MMP-9 (Gelatinase-B) cause the proteolytic degradation of the extracellular matrix components (Loukopoulos et al 2003). Increased levels of these gelatinases in the tumour tissue or serum have been reported to be associated with tumour aggressiveness (Porcellato et al 2017). Loukopoulos et al (2003) reported increase in the active and inactive forms of MMP-2 and MMP-9 in many canine tumours. In a similar study by Fathipour et al (2018), it was determined that; when compared to healthy subjects, dogs with various cutaneous tumours, including fibrosarcoma, presented with significantly increased MMP-2 and MMP-9 levels. Our literature review revealed only one study on the investigation of matrix metalloproteinase levels in cases of feline fibrosarcoma. In this study, Porcellato et al (2017) made a comparative evaluation of the levels of matrix metalloproteins in cases of FISS with and without recurrence, and confirmed the role of these enzymes in tumour invasiveness. Nonetheless, they did not determine any statistically significant difference between the groups. On the other hand, in the present study, only one case produced positive reactions for MMP-9 staining. This case was in an advanced stage and characterized by the presence of giant cells and a high mitotic index. No staining was detected in early stage cases that had not recurred. In this respect, MMP-9 is considered to be an important indicator for the determination of recurrence capacity and tumour aggressiveness in cases of feline fibrosarcoma. Moreover, in the present study, similar to the findings reported by Porcellato et al (2017), MMP-9-positive reactions were mostly localized to the cytoplasm of multinucleated neoplastic giant cells.

## Conclusion

In conclusion, despite only very few tumour samples having been examined in the present study, it was concluded that PCNA, p53 and MMP-9 served as highly useful indicators for determining cell proliferation, grading malignancy, detecting tumour aggressiveness and behaviour, as well as predicting prognosis in spontaneous feline fibrosarcomas.

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## Conflict of Interest

The authors did not report any conflict of interest or financial support.

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## Author Contributions

Motivation/Concept: EK; Design: EK; Control/Supervision: EB, SD; Data Collection and/or Processing: OA, UA, UY, ABK, HK, AYA MA; Analysis and / or Interpretation: EK, EB, SD; Literature Review: EK; Writing the Article: EK; Critical Review: EK

## Ethical Approval

This study was approved by the Local Ethics Board for Animal Experiments of Kafkas University (Decision number: KAU-HADYEK-2022/079).