Journal of Health Sciences Institute, 8(2): 248-256, 2023
DOI: 10.51754/cusbed.1309299

Journal of Health Sciences Institute

| cusbed.cumhuriyet.edu.tr Founded: 2016 Available online, ISSN: 2587-0874 Publisher: Sivas Cumhuriyet Üniversitesi

Intermediate Filaments, P53 Gene, Cellular Proliferation, Metastasis and Apoptosis in Feline Squamous Cell Carcinomas

Emin Karakurt[1,a,*], Serpil Dağ[1,b], Özgür Aksoy[2,c], Enver Beytut[1,d], Celal Şahin Ermutlu[2,e], Ayfer Yıldız[1,f], Uğur Yıldız[2,g], Ersin Tanrıverdi[2,h], Hüseyin Koç[1,i], Mehmet Turan[2,j]

[1]*Department of Pathology, Faculty of Veterinary Medicine, Kafkas University, Kars,Türkiye*
[2]*Department of Surgery, Faculty of Veterinary Medicine, Kafkas University, Kars,Türkiye*
**Corresponding author*

Research Article

History

Received: 03/06/2023
Accepted: 16/07/2023

ABSTRACT

In this study, intermediate filaments, Pan Cytokeratin (Pan CK), Vimentin, Desmin and S-100 were evaluated to reveal the cellular origin of feline SCCs. Alpha-Smooth Muscle Actin (α-SMA) for cancer-related fibroblasts (CAFs) in the tumor microenvironment, and p53, a tumor suppressor gene, were investigated. Proliferating Cell Nuclear Antigen (PCNA) expression was evaluated for the cell proliferation index. Matrix Metalloproteinase-9 (MMP-9) immunoreactivity was evaluated for the metastasis and invasion capacity. In addition, it is aimed to reveal the expressions of proapoptotic Bax gene, antiapoptotic Bcl-2 gene, caspase-dependent pathway Caspase-3 and caspase-independent pathway Apoptosis Inducing Factor (AIF) for apoptosis mechanism. Biopsy samples taken from 7 cats brought to Department of Pathology for routine histopathological examination were used in this study. Tumor tissue samples were fixed in 10% formaldehyde solution. Serial sections of 5 µm thickness were taken from the paraffin blocks prepared after routine tissue follow-up procedures. Hematoxylin & Eosin (H&E) staining was performed on the sections. Avidin-Biotin Peroxidase-Technique (ABC) was used as immunohistochemical staining. It was determined that the tumors had epithelial-mesenchymal transition, exhibited a very high proliferation index, had p53 mutation, and showed low metastasis/high invasion capacity. It was revealed that Bax/Bcl-2 ratio increased in favor of proapoptotic Bax, and caspase-independent apoptosis was more dominant than caspase-dependent apoptosis.

Keywords: Apoptosis, Cellular proliferation, Feline squamous cell carcinoma, Intermediate filaments, Metastasis

[a] *mehmeteminkarakurt@hotmail.com* *https://orcid.org/0000-0003-2019-3690*
[b] *serpildag06@hotmail.com* *https://orcid.org/0000-0001-7667-689X*
[c] *drozguraksoy@hotmail.com* *https://orcid.org/0000-0002-4800-6079*
[d] *enverbeytut@hotmail.com* *https://orcid.org/0000-0003-3360-2940*
[e] *sahinermutlu@hotmail.com* *https://orcid.org/0000-0002-8923-7682*
[f] *yildizayfer360@gmail.com* *https://orcid.org/0000-0002-6569-5435*
[g] *uguryildiz94@gmail.com* *https://orcid.org/0000-0002-4782-1012*
[h] *ersintanriverdii@hotmail.com* *https://orcid.org/0000-0001-8502-2070*
[i] *huseyinkoc@ktu.edu.tr* *https://orcid.org/0000-0002-9031-6505*
[j] *mehmetturan0236@gmail.com* *https://orcid.org/0000-0001-7724-583X*

How to Cite: Karakurt E, Dag S, Aksoy O, Beytut E, Ermutlu S, Yildiz A, Yildiz U, Tanriverdi E, Koc H, Turan M (2023) Intermediate Filaments, P53 Gene, Cellular Proliferation, Metastasis and Apoptosis in Feline Squamous Cell Carcinomas, Journal of Health Sciences Institute, 8(2): 248-256

Introduction

Squamous cell carcinoma (SCC) is a malignant tumor originating from epidermal cells that differentiate into keratinocytes (Simčič et al., 2021). SCCs can be divided into three subclasses according to their origin: oral SCC, ocular SCC, and cutaneous SCC (Gudenschwager-Basso et al., 2022). Cutaneous and oral SCCs are the most common malignant tumors observed in cats (Kabak et al., 2020). Pinnae, eyelids, and nasal planum are regions where cutaneous SCCs are frequently seen (Layne and Graham, 2016). Oral SCCs are mostly located on the base of the tongue, mandible and maxilla in cats. These tumors are typically locally invasive and cause destruction of underlying bone tissue (Olmstedt et al., 2016). However, the rate of metastasis in feline SCCs is quite low. If the tumor metastasizes, regional lymph nodes and lungs are the organs most affected by this condition (Simčič et al., 2021). Tumors are mostly seen in older cats and the average age range is 12 years (Layne and Graham, 2016). Prolonged exposure to ultraviolet radiation, lack of pigment in the skin, sparse hair, and papillomaviruses are the main risk factors for the development of cutaneous SCCs (Sanz Ressel et al., 2021; Gudenschwager-Basso et al., 2022). Although there are different therapeutic applications for SCCs, surgical excision has been used as the most successful treatment method to date (Sanz Ressel et al., 2021). It has been reported that the survival time for cats with SCC is only 2-4 months and the 1-year survival rate is less than 10% (Olmstedt et al., 2016). Human cutaneous, head and neck SCCs are similar to feline SCCs in many respects, which makes feline SCCs a very suitable animal model for humans (Gudenschwager-Basso et al., 2022).

In this study, intermediate filaments such as Pan Cytokeratin (Pan CK), Vimentin, Desmin and S-100 were evaluated to reveal the cellular origin of cutaneous, ocular and oral SCCs in cats. In addition, the expression of Alpha-Smooth Muscle Actin (α-SMA) for cancer-related fibroblasts (CAFs) in the tumor

microenvironment, and p53, an important tumor suppressor gene, were investigated. Proliferating Cell Nuclear Antigen (PCNA) expression was evaluated for the cell proliferation index of cancer. Matrix Metalloproteinase-9 (MMP-9) immunoreactivity was evaluated for the detection of metastasis and invasion capacity. In addition, it is aimed to reveal the expressions of various markers such as proapoptotic Bax gene, antiapoptotic Bcl-2 gene, caspase-dependent pathway Caspase-3 and caspase-independent pathway Apoptosis Inducing Factor (AIF) for controlled cell death-apoptosis mechanism by immunohistochemical methods.

Material and Methods

Animals

In this study, biopsy samples taken from 7 cats brought to Kafkas University, Faculty of Veterinary Medicine, Department of Pathology for routine histopathological examination between 2019-2022 were used. Information on animals is detailed in Table 1.

Table 1. Information about cats with SCC

Case No	Age	Gender	Breed	Location	Macroscopy	Metastasis	Recurrence	Differentiation
1	6	Male	Cross	Nasal Planum	Ulcerative Nodular	-	-	Poorly
2	5	Male	Cross	Ear Pinnae, Nasal Planum	Ulcerative Nodular Papillomatous	-	-	Well
3	6	Male	Cross	Periocular	Ulcerative Crateriform	-	-	Moderately
4	5	Female	Cross	Periocular	Hemorrhagic Crateriform	-	-	Well
5	1	Male	Cross	Oral Cavity	Hemorrhagic Nodular	-	-	Moderately
6	3	Female	Cross	Ear Pinnae	Hemorrhagic Papillomatous	-	+	Poorly
7	2	Female	Cross	Periocular	Hemorrhagic Crateriform	-	-	Moderately

Ethical Approval

The ethics committee report of the study was approved by Kafkas University Animal Experiments Local Ethics Committee (KAU-HADYEK/2022-059).

Histopathological Examinations

Tumor tissue samples taken from cats were fixed in 10% formaldehyde solution. Serial sections of 5 micron thickness were taken from the paraffin blocks prepared after routine tissue follow-up procedures. Hematoxylin & Eosin (H&E) staining was performed on the sections in order to reveal the histopathological changes in the tissues. Sections were evaluated under the light microscope by at least two different pathologists, and the histopathological changes detected were photographed.

The degrees of differentiation of SCC cases were determined based on the presence of keratin pearls, the size of tumoral islands, and squamous differentiation (Kabak et al., 2020).

Immunohistochemical examinations

Serial sections taken from paraffin blocks prepared from tumoral tissues at 4 micron thickness, using Avidin-Biotin Peroxidase-Technique (ABC), Pan Cytokeratin, Vimentin, Desmin, α-SMA, S-100, PCNA, p53, MMP-9, Bax, Bcl-2, Caspase 3 and AIF commercial antibodies were stained according to the manufacturer's procedures. Information on the primary antibodies used in the study is given in Table 2. Thermo Scientific Histostain IHC Kit (HRP, broadspectrum, REF: TP-125-HL) was used to conduct all immunostaining. Amino ethyl carbazole (AEC, Thermo Scientific, REF: TA-125-HA) was applied as the chromogenic substrate and incubated for 15 minutes. Slides were rinsed with distilled water for 5 minutes and coated with AEC mount after staining with Mayer Hematoxylin.

After coating, the prepared preparations were observed under a light microscope (Olympus Bx53) and the sections were photographed using the Cell ^P program (Olympus Soft Imaging Solutions GmbH, 3,4). Detailed analyzes of the photographs were made using the Image J program (1.51j8).

Analysis of immunohistochemical staining results; the immune positive reactions were made with a grading system based on the number of positive cells (tumoral cells, tumor microenvironment, etc.) For the quantification of immune-positive reactions in tissues, the analysis was started on the basis of high-intensity reaction areas. In each tumoral tissue, 3 different areas were evaluated with a 40X objective. The numbers of positively stained cells in each area were recorded separately, and the average of these 3 areas was accepted as the mean positive cell number of that case. Grading system was designated as; (-) = no immunoreactivity, (+) = weak, 1-10% positive, (++) = moderate, 11-59% positivity, and (+++) = severe positivity over 60% (Karakurt et al., 2023).

Table 2. Information on primary antibodies used in immunohistochemical studies

Primary Antibodies	Company and Catalog Numbers	Dilution	Incubation Condition
Pan CK	Novus Biologicals, PCK-26	1/400	Over night, 4 °C
Vimentin	Thermo Scientific, RM-9120-R7	Ready to use	Over night, 4 °C
α-SMA	Thermo Scientific, MS-113-R7	Ready to use	Over night, 4 °C
Desmin	Leica Biosystems, PA0032	Ready to use	Over night, 4 °C
S-100	Thermo Scientific, MS-296-P1	Ready to use	Over night, 4 °C
PCNA	Santa Cruz, sc-56	1/100	Over night, 4 °C
p53	ABclonal, A3185	1/100	Over night, 4 °C
MMP-9	Santa Cruz, sc-393859	1/100	Over night, 4 °C
Bax	Santa Cruz, sc-80658	1/100	Over night, 4 °C
Bcl-2	ABclonal, A19693	1/100	Over night, 4 °C
Caspase-3	ABclonal, A2156	1/100	Over night, 4 °C
AIF	ABclonal, A2568	1/100	Over night, 4 °C

Results

Macroscopic Findings

All of the cases were crossbreed stray cats. 4 of the cats were male and 3 were female. The average age was 4. 6 of 7 cases were cutaneous and 1 was oral SCC. Of the cutaneous SCCs, 2 were localized in the nasal planum, 2 in the ear pinnae, and 3 in the periocular areas. Only one case of cutaneous SCC showed multiple localization (ear pinnae + nasal planum). It was found that solitary or multiple tumoral masses exhibiting nodular, papillomatous or crateriform growths could range from a few mm to 2-3 cm in diameter, and their surfaces were highly hemorrhagic and ulcerative. In addition, some of the SCC cases were found to have a pinkish and irregular appearance, covered with a purulent exudate. In only one case, recurrence of the tumoral mass was recorded after the operation. One cat died shortly after treatment. In the examinations, no metastases were detected in regional lymph nodes or distant tissues such as lungs in all 7 cases (Figure 1 a-b-c-d).

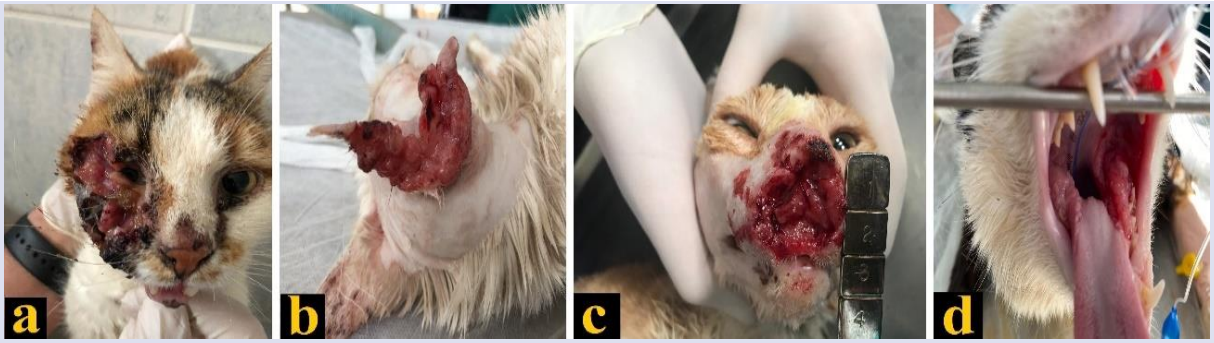

Figure 1. Macroscopic views of tumoral masses taken from different regions such as **(a)** periocular, **(b)** ear pinnae, **(c)** nasal planum, **(d)** oral cavity.

Microscopic Findings

Two of the cases were well-differentiated, three were moderately-differentiated, and two were poorly-differentiated. In well-differentiated SCCs, it was noted that keratin pearls were quite numerous and large, tumoral islands were large and squamous differentiation was evident (Figure 2 a). It was determined that the number and size of keratin pearls decreased in moderately-differentiated SCCs compared to well-differentiated SCCs. In addition, tumoral islands were also smaller in size than well-differentiated SCCs and an increase in the number of poorly-differentiated cells (Figure 2 b). In poorly-differentiated SCCs, keratinization was not either a single cell or remarkable level. Tumor islands were found to be quite small in size compared to well and moderately-differentiated cases, and pleomorphic areas were found to increase significantly (Figure 2 c).

The nuclei of large and oval-shaped neoplastic cells were highly hyperchromatic, especially in poorly differentiated-SCCs. It was observed that the nuclei of the tumoral cells were very prominent and the number of nuclei increased from place to place. The nucleus cytoplasm ratio was increased in favor of the nucleus. Significant anisocytosis was noted in tumoral cells. Mitotic activity was found to be quite low in well, moderately and poorly-differentiated SCCs. In addition, the presence of abnormal mitotic figures, albeit few, was detected. In some cases, intercellular bridges called multiple desmosomes were present in the membrane of tumoral cells. In many cases, it was found that the tumoral mass invaded the adjacent cartilage and muscle tissues. The inflammatory reaction, mostly caused by mononuclear cells located between the tumor islands, was remarkable.

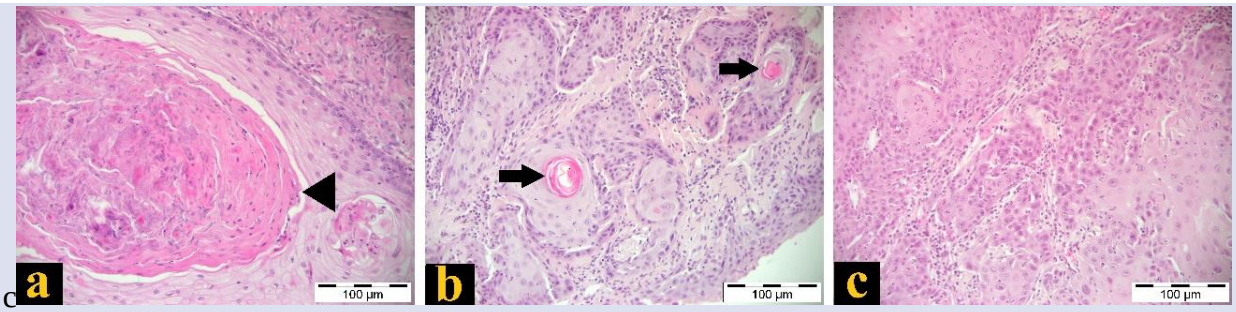

Figure 2. **(a)** Well-differentiated SCC, keratin pearl (arrowhead). **(b)** Moderately-differentiated SCC, keratin pearls (arrows). **(c)** Poorly-differentiated SCC, H&E, Bar=100 µm.

Immunohistochemical Findings

The immunopositivity scores of feline SCCs are given in Table 3. All cases were immunopositive for Pan CK expression. Immune positive reactions were much more pronounced in well-differentiated SCCs compared to moderately and poorly-differentiated SCCs. These reactions were observed especially in tumoral islands and intense intracytoplasmic staining was observed in neoplastic cells. All SCCs showed vimentin positive staining. Vimentin positive reactions were especially concentrated in mesenchymal stroma cells such as fibrocytes and fibroblasts. In addition to mesenchymal stroma cells, vimentin immunoreactivity was found in the cytoplasm of a few tumoral cells in poorly-differentiated SCCs. Well, moderately and poorly-differentiated SCCs were immune positive for α-SMA. However, the staining was detected in mesenchymal stroma cells, such as fibroblasts and myofibroblasts, located in the tumor microenvironment and in the periphery of tumoral islets rather than tumoral cells. While no desmin positive staining was observed in tumoral cells, desmin immunoreactivity was found in the invaded muscle tissue adjacent to the cancerous tissue. The tumor microenvironment showed a negative reaction for desmin, similar to tumor cells. S-100 staining was not detected in any of the SCCs (Figure 3 a-b-c-d).

Table 3. Scores of all immunohistochemical markers and cell types showing positive staining

Case	Pan CK	Vimentin	α-SMA	Desmin	S-100	PCNA	p53	MMP-9	Bax	Bcl-2	Caspase-3	AIF
1 Poorly	TC +	M -TC +	MC +++	MuC ++	-	TC +++	TC ++	TC +	TC ++	TC +	TC +	TC +++
2 Well	TC +++	MC +++	MC +	-	-	TC ++	-	-	TC +++	TC +	TC +	TC +++
3 Moderately	TC ++	MC +++	MC ++	-	-	TC ++	TC +	TC +	TC ++	TC +	TC +	TC +++
4 Well	TC +++	MC +++	MC +	-	-	TC ++	TC +	-	TC +++	TC +	TC +	TC +++
5 Moderately	TC ++	MC +++	MC +++	MuC +	-	TC +++	-	-	TC +++	TC +	TC +	TC +++
6 Poorly	TC +	M-TC ++	MC +++	MuC +++	-	TC +++	TC ++	TC ++	TC ++	TC +	TC+	TC ++
7 Moderately	TC ++	MC ++	MC +	MuC +	-	TC ++	TC ++	TC +	TC +++	TC +	TC +	TC ++

***TC:** Tumoral cells, **MC:** Mesenchymal cells, **MuC:** Muscle cells, **M-TC:** Mesenchymal and tumoral cells, (-) = no immunoreactivity, (+) = weak, 1-10% positive, (++) = moderate, 11-59% positivity, and (+++) = severe positivity over 60%*

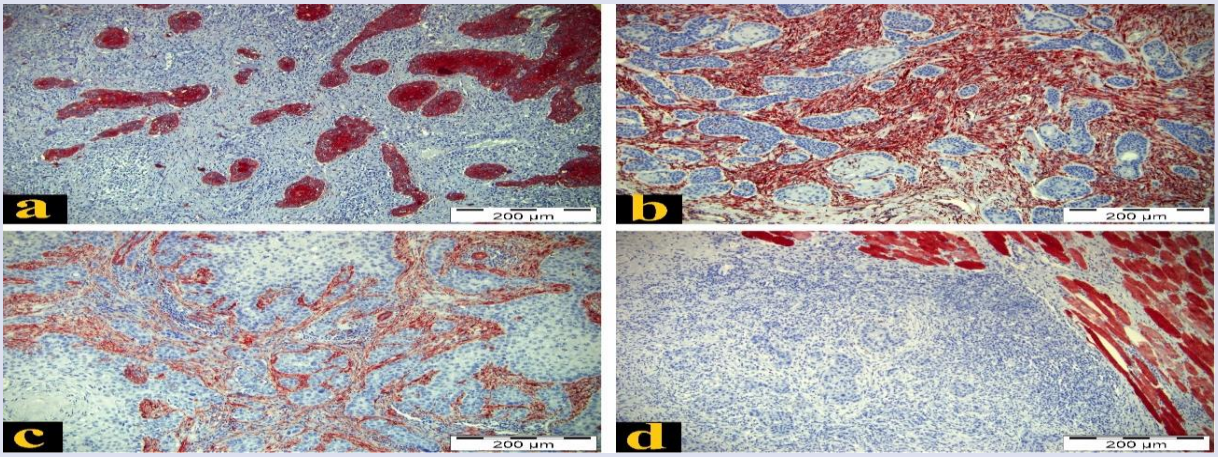

Figure 3. (a) Uniform dense intracytoplasmic Pan CK immunopositive reactions in tumoral islands. **(b)** Vimentin positive staining in cells of mesenchymal origin in the stroma between tumoral cords. **(c)** Expression of α-SMA, especially in fibroblasts and vascular endothelial cells, which are located between trabecular structures formed by neoplastic cells. **(d)** Intense desmin positive staining in muscle cells adjacent to the tumoral tissue, IHC, Bar= 200 µm.

All cases gave a positive reaction for PCNA expression. PCNA positive reactions were detected in the nuclei of tumoral cells. It was observed that the intensity of staining was stronger in areas where pleomorphism was evident. No PCNA positive reaction was detected in the tumoral stroma. It was observed that PCNA immunoreactivity was not significantly different in well, moderately and poorly-differentiated SCCs. Contrary to PCNA results, 5 of 7 SCCs were immunopositive for p53. It was determined that p53 immunoreactivity was more intense in the nuclei of neoplastic cells in the periphery of tumor islands. It was determined that the intensity of p53 expression in cancer cells decreased significantly from the periphery to the keratin pearl in the center of the tumoral islands. Similar to PCNA immunoreactivity, no significant difference was noted between well, moderately and poorly-differentiated SCCs in terms of p53 positive staining. 4 out of 7 SCCs showed MMP-9 positive staining. Very weak intracytoplasmic reactions were detected in the cytoplasm of tumoral cells (Figure 4 a-b-c).

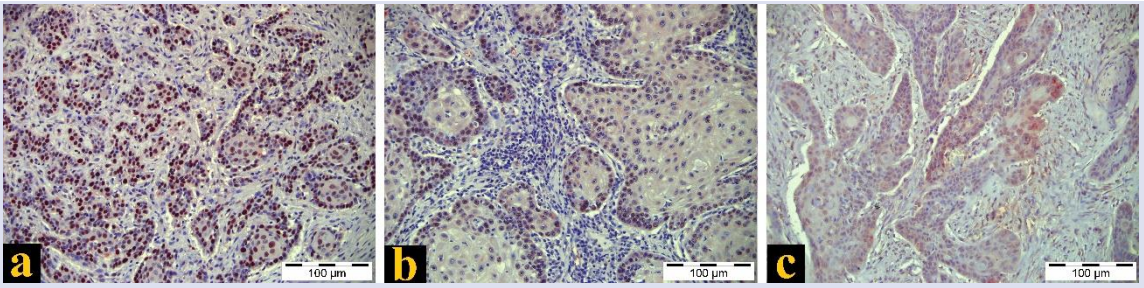

Figure 4. (a) Severe intranuclear PCNA positive reactions in cells in tumoral nests. **(b)** Weak intranuclear p53 immunoreactivity in neoplastic cells located in the periphery of tumoral islands. **(c)** Intracytoplasmic MMP-9 staining in tumoral cords, IHC, Bar= 100 µm.

All SCCs were positive for Bax, Bcl-2, Caspase-3 and AIF immunoreactivity. Proapoptotic Bax expressions were much more pronounced compared to antiapoptotic Bcl-2 expressions. Bax positive staining was observed in the cytoplasm of tumoral cells in a granular form and quite intensely. Bcl-2 positive staining was detected intracytoplasmically similar to Bax expressions. However, the intensity of the staining was quite weak. Regardless of the degree of differentiation, it was noted that proapoptotic Bax expressions were higher than apoptosis-inhibiting Bcl-2 expressions on a case-by-case basis. Caspase-3 immunopositive reactions were detected in both the cytoplasm and nucleus of epithelial cells in tumoral islands. Compared to caspase-3 immunoreactivity, AIF positive staining was found to be significantly increased. AIF positive reactions were granular form in the cytoplasm of tumoral cells in trabecular structures. Accordingly, it was revealed that the apoptosis mechanism in cats with SCC is triggered by a caspase-independent pathway rather than a caspase-dependent pathway (Figure 5 a-b-c-d).

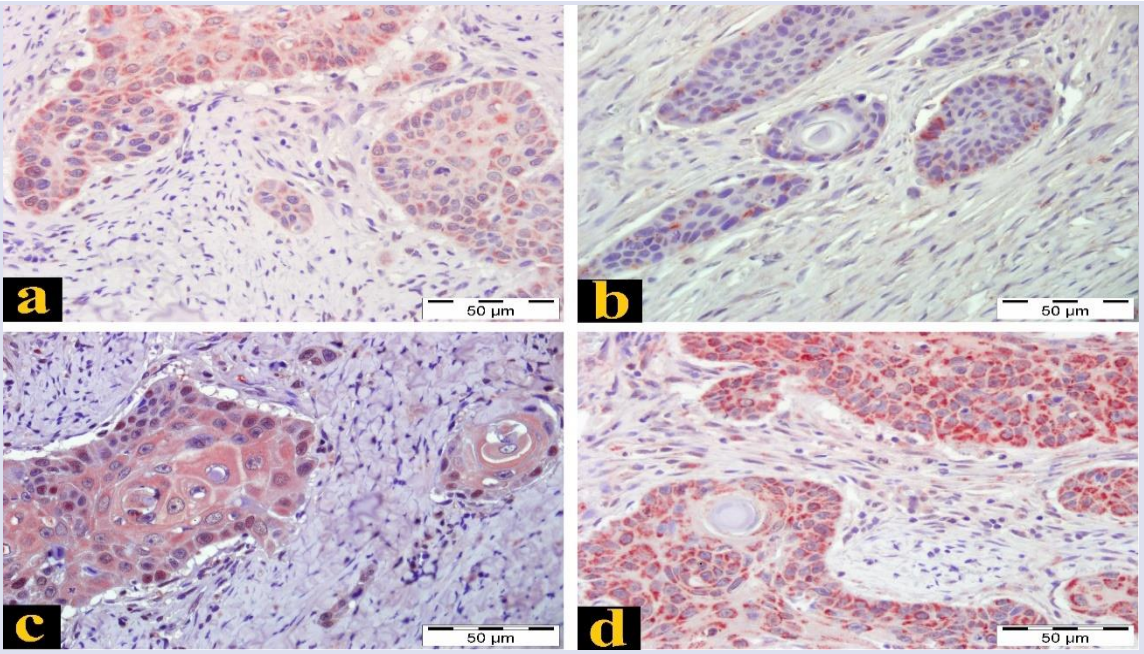

Figure 5. (a) Intense granular Bax positive staining in the cytoplasm of tumoral cells. **(b)** Very weak intracytoplasmic Bcl-2 immunoreactivity. **(c)** Caspase-3 positive reactions in both the cytoplasm and nucleus of neoplastic cells in trabecular structures. **(d)** Very severe and intracytoplasmic granularity in tumoral islets AIF staining, IHC, Bar= 50 µm.

Discussion

Intermediate filaments that form part of the cytoskeleton are different types of proteins. Although their functions are not fully known, they are thought to be involved in the regulation of cellular and intracellular movements, as well as providing cellular integrity, shape and organelle positions as components of the cytoskeleton (Milli et al., 2000). Cytokeratin filaments are found in epithelial cells, vimentin filaments in mesenchymal cells, desmin filaments in muscle cells, neurofilaments in nerve cells, and glial fibrillary acidic protein filaments in astrocytes (Martín de las Mulas et al., 1995). In addition, microfilaments such as α-SMA are also used to detect cell type, except for intermediate filaments. α-SMA microfilaments are determinative for myoepithelial cells (Milli et al., 2000). In this study, markers such as Pan CK, Vimentin, α-SMA, Desmin and S-100 were evaluated immunohistochemically in detecting the cellular origins of well, moderately and poorly-differentiated SCCs. As expected, Pan CK, an important epithelial tumor marker, gave a positive reaction in all SCCs, especially in well-differentiated (Rodríguez Guisado et al., 2021). Pan CK immunoreactivity was detected in the cytoplasm of neoplastic epithelial cells, consistent with literature data (Martín de las Mulas et al., 1995; da Conceicao et al., 2016; Sparger et al., 2018; Barbosa et al., 2019). Different researchers reported that they did not find vimentin immunoreactivity in tumoral foci in feline SCCs (Conceicao et al., 2016; Rodríguez Guisado et al., 2021). In addition, they reported that vimentin-positive staining was observed especially in the connective tissue around the tumoral islands (Barbosa et al., 2019). In the current study, vimentin expression was predominantly detected in mesenchymal cells in the tumor stroma, similar to previous studies (Conceicao et al. 2016; Barbosa et al., 2019; Rodríguez Guisado et al., 2021). However, especially in poorly differentiated-SCCs, in addition to mesenchymal cells, epithelial tumor cells were also found to be positive for vimentin expression, albeit in very few numbers. This increased expression of vimentin in SCCs, which is an epithelial tumor, led to the conclusion that it may be related to the epithelial-mesenchymal transition (EMG) of tumors (Harris et al., 2019). In human medicine, it has been reported that EMG is associated with the metastatic process in many different types of epithelial malignant tumors such as breast cancer and SCC. In small animal medicine, it has been suggested that EMG occurs during the metastatic process of canine prostate carcinoma and canine mammary carcinoma (Furusawa et al., 2021). On the other hand, other studies have reported that EMG contributes to the aggressiveness of oral SCCs in cats (Harris et al., 2019). Although no metastasis data could be obtained due to the fact that the cats with SCC used in this study were stray animals, it is thought that the invasive and aggressive character of the cases confirms this increase in vimentin expression. As expected, epithelial cancer cells were negative for Desmin and S-100 staining in all cases (Martín de las Mulas et al., 1995; Guisado et al., 2021). Desmin positive reactions were detected only in the muscle tissue adjacent to the tumoral tissues.

α-SMA, a specific marker for myofibroblasts, is also widely used in the detection of cancer-associated fibroblasts (CAFs) (Yoshimoto et al., 2017). CAFs, also known as activated fibroblasts or myofibroblasts, are important components of the tumor microenvironment and increase cancer cell invasion, proliferation, and growth rate. CAFs promote angiogenesis, regulate inflammatory cell infiltration and expression of extracellular matrix proteases, and reduce cancer cell death (Klobukowska and Munday, 2016). In this study, intense α-SMA positive staining was found in fibroblasts in the tumor stroma as previously reported (Conceicao et al. 2016; Klobukowska and Munday, 2016). Klobukowska and Munday (2016) reported that cats with CAFs positive-feline oral SCCs had a shorter average survival time compared to cats with CAFs negative-feline oral SCCs. Based on the results of the study, they suggested that the presence of CAFs in the tumor stroma is an important prognostic marker for oral feline SCCs (Klobukowska and Munday, 2016). In the current study, it was determined that only one case died in a short time (approximately 1 month) after the operation due to secondary bacterial infections. Unfortunately, we do not have any information regarding the survival of the other 6 cases. In addition, no significant difference was found between the deceased case and the other 6 cases in terms of α-SMA expression.

Mitotic dysregulation and uncontrolled cell growth are strong indicators for potential malignant transformation (Mestrinho et al., 2017b). PCNA is a 36-kDa non-histonic nuclear peptide required for DNA replication (Mestrinho et al., 2017a). PCNA is a specific marker for cell division, and its expression has been found to be associated with cancer progression and malignancy (Martano et al., 2016). PCNA is used in human medicine to predict histological stage, recurrence rate, and prognosis in cancers such as oral SCC (Mestrinho et al., 2017a). It has been found that increased PCNA expression in canine oral squamous cell carcinomas is associated with poor mean survival and advanced cancer stages (Martano et al. 2016; Mestrinho et al., 2017b; Mestrinho et al., 2017a). In the literature review, it was seen that the cell proliferation index in feline SCCs was mostly evaluated through Ki67 expression. In only one study, it was found that a high PCNA index in feline cutaneous SCCs was associated with a poor response to radiotherapy used in cancer treatment (Théon et al., 1995). In this study, it was determined that PCNA positivity was almost the same in well, moderately and poorly-differentiated cases, including cases that recurrenced, died shortly after the surgecial operation, and showed invasive character, and there was no significant difference between SCCs. Although an effective cancer marker for SCCs in humans and dogs, PCNA has not been shown to be a very reliable marker for detecting malignancy, progression, and prognosis in feline SCCs. The

lack of a remarkable difference in PCNA staining results in SCCs with advanced histological stage, recurrent and invasive character was interpreted as the small sample size.

The p53 protein, which acts as a tumor suppressor, has functions such as maintaining genetic integrity and triggering apoptosis against DNA damage (Munday and Aberdein, 2012). Cells with any deficiencies in the p53 gene cannot be repaired or undergo apoptosis despite DNA damage. These cells continue to divide so that genetic damage continues to accumulate in these cells and they eventually undergo malignant transformation (Renzi et al., 2019). In more than 50% of cases of head and neck cancers in humans, either a mutation in the p53 gene or an increase in the p53 protein is observed. This has also been associated with poor clinical outcomes and tumor progression (Supsavhad et al., 2016). In addition, prolonged exposure to sunlight, especially ultraviolet light B (UV B), is an important epidemiological risk factor for skin SCCs in humans. It has been determined that these UV rays also cause mutations in the p53 gene (Nasir et al., 2000; Munday and Aberdein, 2012). Altamura et al. 2018 reported that Felis catus Papilloma Virus Type 2 E6 oncoprotein causes degradation of the p53 gene in cats with SCC, similar to humans, and possibly contributes to tumor development. Five of the seven cats used as material in this study gave an intranuclear positive reaction for the mutant p53 gene. Such a mutation in the p53 gene is very likely due to the fact that cats live in Kars, a high altitude region, and are exposed to sunlight for a long time. Various researchers have noted that p53 is an important prognostic marker for feline oral SCCs (Supsavhad et al., 2016; Renzi et al., 2019). In the current study, although an increase in p53 immunoreactivity was observed in moderately and poorly differentiated-SCCs compared to well-differentiated SCCs, this increase was not significant. In this respect, similar to Munday et al. (2019) reported, p53 was not thought to be a very effective predictor of survival and prognosis for feline SCCs.

Matrix Metalloproteinases (MMPs) are zinc-dependent endopeptidases that degrade the extracellular matrix, facilitating tumor invasion and regulating tumor-associated angiogenesis (Nasry et al., 2018). Remodeling of the extracellular matrix and basement membrane by cancer cells is an important step in terms of metastasis and invasiveness (Altamura et al., 2020). A subset of MMPs, also known as gelatinases (MMP-2 and MMP-9), has been shown to be associated with prostate, breast, gallbladder, lung and breast cancers in humans (Jankowski et al., 2002). In addition, it has been found that MMPs are expressed in many cancer types such as meningiomas, breast cancers, and lymphomas in cats (Nasry et al., 2018). In the literature review, it was noted that there are few studies evaluating MMP expressions in cats with SCC (Jankowski et al., 2002; Altamura et al., 2020). In a study, Jankowski et al., (2002) reported that MMP-2 and MMP-9 levels were significantly increased in cancer tissue compared to healthy control tissues and tumor stroma in many cat tumors such as squamous cell carcinoma, fibrosarcoma, mammary gland adenocarcinomas. Altamura et al., (2020) showed that MMP-1, MMP-2 and MMP-9 are expressed at both gene and protein level in feline oral SCC cell lines. They suggested that this increase in MMP level may also be related to the invasive potential of cancer cells. Although invasion into adjacent tissues was observed in most of the cases, no lymph node or distant tissue metastases were observed. MMP-9 immunoreactivity was observed in moderately and poorly-differentiated SCCs rather than well-differentiated SCCs. Invasive cases were found to have a more severe reaction in terms of MMP-9. In this respect, it is undeniable that MMP-9 has the potential to be a useful marker in detecting tumor aggressiveness in feline SCCs.

Apoptosis is an important form of cell death for normal development, host defense and suppression of carcinogenesis (Madewell et al., 1999). Tumor development is a localized generalized increase in cell number. It is also a result of cell gain by proliferative activity and cell loss by apoptosis and necrosis (Madewell et al., 2001). Failure to maintain the proper balance in cell number is a distinctive feature for neoplasms. Although the increase in cellular proliferation seems to be a characteristic feature for many tumors, the disorder in the apoptosis mechanism is a much more key factor for cancer development (Madewell et al., 1999). Bcl-2 and Bcl-2-associated X protein (Bax) are important genes of the Bcl-2 family. Bcl-2 is antiapoptotic and Bax is proapoptotic (Altamura et al., 2021). Bax/Bcl-2 cross-regulation regulates apoptosis, cell survival and cell proliferation (Dos Anjos et al., 2019). Caspases play an important role in apoptosis. Caspase-mediated apoptosis involves two pathways. The first of these is the extrinsic pathway and is initiated by the activation of caspase-8 by cell surface receptors. Another pathway is the intrinsic pathway and is organized by mitochondria with the activation of caspase-9. Caspase-3 is effective in both the extrinsic and intrinsic pathways. There is also a caspase-independent Apoptosis-inducing factor (AIF)-signed pathway apoptosis mechanism released by mitochondria (Ozkaraca et al., 2022). There are very few studies on the mechanisms of apoptosis in cats with SCC (Madewell et al., 1999; Madewell et al., 2001; Dos Anjos et al., 2019). Madewell et al., (1999) investigated the topographic distribution of Bcl-2 protein in healthy and neoplasia cat tissues and reported that 12 cases with cutaneous SCC were negative for Bcl-2 expression. In another similar study, Madewell et al., (2001) reported that 14 cats with cutaneous SCC were negative for Bax and Bcl-2 positive reactions. Altamura et al., (2016) suggested that the 2016 E6 and E7 oncogenes inhibit the accumulation of UV B-triggered proapoptotic markers such as p53, p21 and Caspase-3, Bax and Bak, and that UV exposure and virus have a synergistic effect in tumor pathogenesis. Contrary to these studies, Dos Anjos et al., (2019) recorded Bax and Bcl-2 expression in the cytoplasm of tumor cells. In the current study, Bax and Bcl-2 positive stainings were detected in the cytoplasm of tumor cells similar to Dos Anjos et al., (2019) reported.

Proapoptotic Bax expressions were observed to be stronger than antiapoptotic Bcl-2 expressions. Compared to caspase-3 immunoreactivity, AIF positive staining was found to be significantly increased. In the light of the study data, it was noted that the caspase-independent pathway is much more effective than the caspase-dependent apoptosis mechanism in feline SCCs.

Conclusion

In conclusion, it was concluded that Pan CK is a very useful marker in revealing the cellular origin of feline SCCs. In addition, CAFs in the tumor microenvironment were found to be quite high. α-SMA was very effective in detecting the presence of these cells, which is a valuable parameter about the prognosis of cancer. All of the SCCs gave a positive reaction in terms of vimentin staining, which showed that the tumors had EMT. Although as tumor differentiation increased, PCNA and p53 immunoreactivity increased in parallel with differentiation, there was no significant difference between cases in terms of these markers. It has been observed that MMP-9 has the potential to be a useful marker in demonstrating the cancer aggressiveness of MMP-9 in invasive cases. It was determined that the Bax/Bcl-2 ratio in the cases was in favor of the proapoptotic Bax gene and the caspase-independent pathway was more dominant in the apoptosis mechanism.

Declaration of Conflicting Interests

The Authors declare that there is no conflict of interest.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

References

Altamura, G., Corteggio, A., Pacini, L., Conte, A., Pierantoni, G. M., Tommasino, M., Accardi, R., & Borzacchiello, G (2016). Transforming properties of Felis catus papillomavirus type 2 E6 and E7 putative oncogenes in vitro and their transcriptional activity in feline squamous cell carcinoma in vivo. Virology, 496, 1-8. https://doi.org/10.1016/j.virol.2016.05.017

Altamura, G., Degli Uberti, B., Galiero, G., De Luca, G., Power, K., Licenziato, L., Maiolino, P., & Borzacchiello, G (2021). The small molecule BIBR1532 exerts potential anti-cancer activities in preclinical models of feline oral squamous cell carcinoma through inhibition of telomerase activity and down-regulation of TERT. Frontiers in Veterinary Science, 7, 620776. https://doi.org/10.3389/fvets.2020.620776

Altamura, G., Power, K., Martano, M., Degli Uberti, B., Galiero, G., De Luca, G., Maiolino, P., & Borzacchiello, G (2018). Felis catus papillomavirus type-2 E6 binds to E6AP, promotes E6AP/p53 binding and enhances p53 proteasomal degradation. Scientific Reports, 8(1), 17529. https://doi.org/10.1038/s41598-018-35723-7

Barbosa, F. M. S., Santos, JdeJCdos., Gois, DDde., Costa Lima, Rda., Souza, MdeFde., Silva Lima, LHda., Souza E Silva, AAde., & Lucena, RBde (2019). Squamous cell carcinoma of the lung with small intestine metastasis in cat. Acta Scientiae Veterinariae, 47, 406. https://doi.org/10.22456/1679-9216.94711

da Conceicao, M. E. B. A. M., Martins, D. M., Bertolo, P. H. L., Bezerra, D. K. D., da Silva, C. D. C. F., de Aguirra, L. R. V. M., Vasconcelos, R.D., & Pereira, W. L. A. (2016). Squamous cell carcinoma in third eyelid of cat. Acta Scientiae Veterinariae, 44(Suppl 1), 170. https://doi.org/10.22456/1679-9216.82863

Dos Anjos, D. S., Bueno, C., Magalhães, L. F., Magalhães, G. M., Mattos-Junior, E., Pinto, M. M. R., De Nardi, A. B., Brunner, C. H. M., Leis-Filho, A. F., Calazans, S. G., & Fonseca-Alves, C. E Electrochemotherapy induces tumor regression and decreases the proliferative index in canine cutaneous squamous cell carcinoma. Scientific Reports, 9(1), 15819. https://doi.org/10.1038/s41598-019-52461-6

Furusawa, Y., Takahashi, M., Shima-Sawa, M., Hatai, H., Miyoshi, N., Yamato, O., & Yabuki, A (2021). Immunocytochemical evaluation of epithelial-mesenchymal transition in epithelial tumors of dogs and cats. The Journal of Veterinary Medical Science, 83(9), 1363–1368. https://doi.org/10.1292/jvms.21-0021

Gudenschwager-Basso, E. K., Stevenson, V., Sponenberg, D. P., Cecere, T. E., & Huckle, W. R. (2022). Characterization of the expression of angiogenic factors in cutaneous squamous cell carcinoma of domestic cats. Veterinary Sciences, 9(7), 375. https://doi.org/10.3390/vetsci9070375

Harris, K., Gelberg, H. B., Kiupel, M., & Helfand, S. C. (2019). Immunohistochemical features of epithelial-mesenchymal transition in feline oral squamous cell carcinoma. Veterinary Pathology, 56(6), 826–839. https://doi.org/10.1177/0300985819859873

Jankowski, M. K., Ogilvie, G. K., Lana, S. E., Fettman, M. J., Hansen, R. A., Powers, B. E., Mitchener, K. M., Lovett, S. D., Richardson, K. L., Parsley, L., & Walton, J. A. (2002). Matrix metalloproteinase activity in tumor, stromal tissue, and serum from cats with malignancies. Journal of Veterinary Internal Medicine 2002; 16 (1): 105–108. https://doi.org/10.1892/0891-6640(2002)016<0105:mmaits>2.3.co;2

Kabak, Y. B., Sozmen, M., Devrim, A. K., Sudagidan, M., Yildirim, F., Guvenc, T., Yarim, M., Gulbahar, Y. M., Ahmed, I., Karaca, E., & Inal, S. (2020) Expression levels of angiogenic growth factors in feline squamous cell carcinoma. Acta Veterinaria Hungarica, 68(1), 37–48. https://doi.org/10.1556/004.2020.00005

Karakurt, E., Nuhoğlu, H., Dağ, S., Çelebi, Ö., Büyük, F., Beytut, E., Yıldız, A., Kuru, M., & Akça, D. (2023) Immunohistochemical investigation of TNF-α and IFN-γ expressions in sheep fetuses with brucellosis. Pakistan Veterinary Journal, 43(1), 85-90. http://dx.doi.org/10.29261/pakvetj/2022.088

Klobukowska, H. J., & Munday, J. S. (2016). High numbers of stromal cancer-associated fibroblasts are associated with a shorter survival time in cats with oral squamous cell carcinoma. Veterinary Pathology, 53(6), 1124–1130. https://doi.org/10.1177/0300985816629713

Layne, E. A., & Graham, M. (2016). Cutaneous squamous cell carcinoma manifesting as follicular isthmus cysts in a cat. JFMS Open Reports, 2, 2055116915625356. https://doi.org/10.1177/2055116915625356

Madewell, B. R., Gandour-Edwards, R., Edwards, B. F., Walls, J. E., & Griffey, S. M. (1999). Topographic distribution of bcl-2 protein in feline tissues in health and neoplasia. Veterinary Pathology, 36(6), 565–573. https://doi.org/10.1354/vp.36-6-565

Madewell, B. R., Gandour-Edwards, R., Edwards, B. F., Matthews, K. R., & Griffey, S. M. (2001). Bax/bcl-2: cellular modulator of

apoptosis in feline skin and basal cell tumours. Journal of Comparative Pathology, 124(2-3), 115–121. https://doi.org/10.1053/jcpa.2000.0437

Martano, M., Restucci, B., Ceccarelli, D. M., Lo Muzio, L., & Maiolino, P. (2016). Immunohistochemical expression of vascular endothelial growth factor in canine oral squamous cell carcinomas. Oncology Letters, 11(1), 399-404. https://doi.org/10.3892/ol.2015.3847

Martín de las Mulas, J., Espinosa de los Monteros, A., Carrasco, L., van Niel, M., & Fernández, A. (1995). Immunohistochemical distribution pattern of intermediate filament proteins in 50 feline neoplasms. Veterinary Pathology, 32(6), 692–701. https://doi.org/10.1177/030098589503200611

Mestrinho, L. A., Faísca, P., Peleteiro, M. C., & Niza, M. M. (2017a). PCNA and grade in 13 canine oral squamous cell carcinomas: association with prognosis. Veterinary and Comparative Oncology, 15(1), 18–24. https://doi.org/10.1111/vco.12134

Mestrinho, L.A., Pissarra, H., Carvalho, S., Peleteiro, M.C., Gawor, J., & Niza, M. M. R. E. (2017b) Comparison of histological and proliferation features of canine oral squamous cell carcinoma based on intraoral location: 36 cases. Journal of Veterinary Dentistry, 34(2), 92–99. https://doi.org/10.1177/0898756417713979

Milli, U., Haziroglu, R,, Aydin, Y., & Gulbahar, M. (2000). Köpek meme tümörlerinde sitokeratin, vimentin ve alfa-düz kas aktin intermedier ve mikro filamentlerinin immunohistokimyasal lokalizasyonu. *Turkish Journal of Veterinary & Animal Sciences, 24(1), 81-92.*

Munday, J. S., & Aberdein, D. (2012). Loss of retinoblastoma protein, but not p53, is associated with the presence of papillomaviral DNA in feline viral plaques, Bowenoid in situ carcinomas, and squamous cell carcinomas. Veterinary Pathology, 49(3), 538–545. https://doi.org/10.1177/0300985811419534

Munday, J. S., He, Y., Aberdein, D., & Klobukowska, H. J. (2019). Increased p16CDKN2A, but not p53, immunostaining is predictive of longer survival time in cats with oral squamous cell carcinomas. Veterinary Journal, 248, 64–70. https://doi.org/10.1016/j.tvjl.2019.04.007

Nasir, L., Krasner, H., Argyle, D. J., & Williams, A. (2000). Immunocytochemical analysis of the tumour suppressor protein (p53) in feline neoplasia. Cancer Letters, 155(1), 1–7. https://doi.org/10.1016/s0304-3835(00)00337-2

Nasry, W. H. S., Wang, H., Jones, K., Dirksen, W. P., Rosol, T. J., Rodriguez-Lecompte, J. C., & Martin, C. K. (2018). CD147 and cyclooxygenase expression in feline oral squamous cell carcinoma. Veterinary Sciences, 5(3), 72. https://doi.org/10.3390/vetsci5030072

Olmsted, G. A., Farrelly, J., Post, G. S., & Smith, J. (2017). Tolerability of toceranib phosphate (Palladia) when used in conjunction with other therapies in 35 cats with feline oral squamous cell carcinoma: 2009-2013. Journal of Feline Medicine and Surgery, 19(6), 568–575. https://doi.org/10.1177/1098612X16638118

Ozkaraca, M., Ozdemir, S., Comakli, S., & Timurkan, M. O. (2022). Roles of apoptosis and autophagy in natural rabies infections. Veterinární Medicína–Czech, 67(1), 1-12. https://doi.org/10.17221/221/2020-VETMED

Renzi, A., De Bonis, P., Morandi, L., Lenzi, J., Tinto, D., Rigillo, A., Bettini, G., Bellei, E., & Sabattini, S. (2019). Prevalence of p53 dysregulations in feline oral squamous cell carcinoma and non-neoplastic oral mucosa. PloS One, 14(4), e0215621. https://doi.org/10.1371/journal.pone.0215621

Rodríguez Guisado, F., Suárez-Bonnet, A., & Ramírez, G. A. (2021) Cutaneous spindle cell squamous cell carcinoma in cats: clinical, histological, and immunohistochemical study. Veterinary Pathology, 58(3), 503–507. https://doi.org/10.1177/0300985820985126

Sanz Ressel, B. L., Massone, A. R., & Barbeito, C. G. (2021). Persistent activation of the mammalian target of rapamycin signalling pathway in cutaneous squamous cell carcinomas in cats. Veterinary Dermatology, 32(6), 675–e180. https://doi.org/10.1111/vde.13001

Simčič, P., Pierini, A., Lubas, G., Lowe, R., Granziera, V., Tornago, R., Valentini, F., Alterio, G., Cochi, M., Rangel, M. M. M., de Oliveira, K. D., Ostrand Freytag, J., Quadros, P. G., Sponza, E., Gattino, F., Impellizeri, J. A., & Torrigiani, F. (2021). A retrospective multicentric study of electrochemotherapy in the treatment of feline nasal planum squamous cell carcinoma. Veterinary Sciences, 8(3), 53. https://doi.org/10.3390/vetsci8030053

Sparger, E. E., Murphy, B. G., Kamal, F. M., Arzi, B., Naydan, D., Skouritakis, C. T., Cox, D. P., & Skorupski, K. (2018). Investigation of immune cell markers in feline oral squamous cell carcinoma. Veterinary Immunology and Immunopathology, 202, 52–62. https://doi.org/10.1016/j.vetimm.2018.06.011

Supsavhad, W., Dirksen, W. P., Hildreth, B. E., & Rosol, T. J. (2016). p16, pRb, and p53 in feline oral squamous cell carcinoma. Veterinary Sciences, 3(3), 18. https://doi.org/10.3390/vetsci3030018

Théon, A. P., Madewell, B. R., Shearn, V. I., & Moulton, J. E. (1995). Prognostic factors associated with radiotherapy of squamous cell carcinoma of the nasal plane in cats. The American Veterinary Medical Association, 206(7), 991–996.

Yoshimoto, S., Hoshino, Y., Izumi, Y., & Takagi, S. (2017). α-Smooth muscle actin expression in cancerassociated fibroblasts in canine epithelial tumors. Japanese Journal of Veterinary Research, 65(3), 135-144. https://doi.org/10.14943/jjvr.65.3.135.