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RESEARCH ARTICLE

Determination of Apoptosis and Autophagy in Bovine Ocular Squamous Cell Carcinomas by Immunohistochemistry

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A B S T R A C T

Ocular squamous cell carcinoma (OSCC) is one of the most common neoplasms in cattle in Kars province, Turkey. This study was performed on 45 cases of OSCC sent by the Kafkas University Faculty of Veterinary Medicine Research Hospital and private veterinary clinics to the university pathology department between the years 2012 and 2021. The removed tumor masses were macroscopically different in size and in hemorrhagic degree, and their surfaces were smooth or cauliflower-like. The removed masses were stained with hematoxylin–eosin for the histopathological examination, which showed neoplastic formations characterized by differentiation of atypical keratinocytes from the epidermis to the dermis. The cases were divided into three groups according to the density and number of tumor pearls, mitotic activity and their pleomorphism status: well differentiated, moderately differentiated, and poorly differentiated. Caspase-3 and apoptosis-inducing factor (AIF) as apoptosis markers and protein light chain 3 (LC3B) as autophagy markers were stained by immunohistochemical methods to determine the role of apoptosis and autophagy in OSCC cases. A statistically significant difference was observed between the groups separated according to the degree of differentiation in caspase-3 and LC3B immunostaining; however, non-significant difference in AIF immunostaining was found among three groups. The histopathological and immunohistochemical evaluations showed that caspase-dependent apoptosis was more predominant than caspase-independent apoptosis in OSCC cases of cattle, and autophagy and apoptosis might play a common role in tumor aggressiveness and progression.

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INTRODUCTION

Ocular squamous cell carcinoma (OSCC), also called "cancer eye," is the most common tumor seen in the eye after basal cell carcinoma. OSCCs are found especially in cattle, but also in other animal species such as cats, dogs, sheep, and goats (Marà *et al.,* 2005; Taş *et al.,* 2009). They are seen in cattle breeds such as Hereford, Hybrid Hereford, and Holstein-Friesian with a light-colored and weakly pigmented eye area, and mostly in cattle older than 5 years of age (Tsujita and Plummer, 2010; Mathewos *et al.,* 2020). The disease causes significant economic losses in cattle due to a 12.6% reduction in the carcass weight, shortening of life span, and treatment costs (Taş *et al.,* 2009).

The balance called "homeostasis" between new cells produced by mitosis and dead cells that have no function is achieved by mechanisms such as apoptosis and autophagy in the body (Soengas *et al.,* 1999; D'Arcy, 2019). Apoptosis is the best described form of programmed cell death and basically occurs in two main ways. These are the extrinsic pathway mediated by death receptors and the intrinsic pathway mediated by mitochondria. Caspase-3 needs to be fully activated to determine the occurrence of apoptosis (Crowley and Waterhouse, 2016). The apoptosis-inducing factor (AIF) is the main mediator of caspase-independent cell death (Srivastava *et al.,* 2007). The presence of AIF in cells is an important parameter in determining the role of caspases in cell death (Modjtahedi *et al.,* 2006).

Another process important in maintaining cellular metabolism is autophagy. In cases of nutrient deficiency, the cells increase autophagic activation to survive (Mizushima *et al.,* 2011; D'Arcy, 2019). The synthesis and processing of the protein light chain 3 (LC3B) increases during autophagy, which makes the LC3B

protein an important parameter in detecting autophagy in cells (Li *et al.*, 2017).

This study aimed to evaluate the role of apoptosis and autophagy in cattle with OSCC by histopathological and immunohistochemical methods.

MATERIALS AND METHODS

The study was conducted on 45 cases of OSCC aged 8-16 years sent by Kafkas University Faculty of Veterinary Medicine Research Hospital and private veterinary clinics to the university pathology department between the years 2012 and 2021. The ethics committee approval of the study was obtained from Kafkas University Animal Experiments Local Ethics Committee.

Histopathological examinations: Tumoral masses removed by surgery were subjected to routine laboratory procedures. Hematoxylin and eosin staining was performed to detect histopathological changes by taking 5-µm-thick sections from the prepared paraffin blocks, and the sections were evaluated under a light microscope (Olympus Bx53). Photographs were taken using the Cell ^P program (Olympus Soft Imaging Solutions GmbH, 3, 4). The criteria described earlier by Carvalho *et al*. (2005), Gharagozlou *et al*. (2007) and Sözmen *et al*. (2019) were used as the basis for differentiation degrees and mitotic index of the OSCC cases; t The cases were categorized as well, moderately, and poorly differentiated. Keratin pearls are large and numerous, tumor islands are large, squamous differentiation is prominent, keratin pearls are small to medium in number in the moderately differentiated group, tumor islands are small to medium in size, squamous differentiation increases in the number of poorly differentiated cells, keratin pearls in the poorly differentiated group at the level of individual cells, tumor islands are very small, pretty bad and completely differentiated.

Immunohistochemical examination: For immuno-histochemical staining, 3-µm-thick sections were cut from paraffin blocks onto adhesive slides coated with poly-L-lysine. Caspase-3 (SantaCruz, sc-56053) and AIF (Aviva Systems Biology AVARP02013_P050) were used as apoptosis markers, while LC3B (Abclonal, A7198) was used as an autophagy marker. Immunohistochemical staining was performed following the manufacturer's protocols.

For analyzing caspase-3, AIF and LC3B, immunohistochemical staining was performed using a grading system based on the number of positive cells, for which immune-positive reactions were examined and which most strongly reflected the staining characteristics. For each tumoral tissue, 5 different areas were evaluated in 100 lenses, and the mean of these 5 areas was considered as the mean number of positive cells of that animal.

Statistical analyses: Statistical analysis of the results was performed using the SPSS (SPSS 18.0, IL, USA) program. The normal distribution order of the groups according to the caspase, AIF and LC3B mean positive cell numbers was evaluated visually using a histogram and Shapiro–Wilk test. The data showed normal distribution, and hence the groups were compared with parametric tests. Statistical difference between the grades of the antibody was evaluated using one-way analysis of variance, and post-hoc comparison was made using the Tukey HSD test. The obtained results were expressed as mean ± standard deviation. A P value <0.05 indicated a statistically significant difference.

RESULTS

Macroscopic findings: Of the 45 OSCC cases in cattle, 24 had lesions in the left and 21 in the right eye. Further, 4 lesions were formed in the third eyelid, 5 in the upper eyelid, 6 in the lower eyelid, 6 in the lateral canthus, and 24 spread all over the eye. Deformations and atrophies occurred in the affected eyes as a result of the pressure exerted by the masses. The nodular, papillomatous, or cauliflower-like tumoral masses had varying sizes, their surfaces were highly hemorrhagic and ulcerative, and they sometimes had irregular pink hemorrhagic patches covered with a purulent exudate (Fig. 1).

Histopathological findings: In the tumoral area, neoplastic cells with ground-glass appearance and eosinophilic cytoplasm and tumoral islets formed by these cells were found. A comparison made according to the degrees of differentiation showed that the keratin pearls were quite numerous and large in well-differentiated OSCC cases, and the tumoral islets were large. In moderately differentiated OSSC cases, the number and size of keratin pearls decreased compared with well-differentiated cases. In poorly differentiated OSCC cases, keratinization was either single-celled or absent. Tumoral islets were quite small compared with well- and moderately differentiated cases; also, the pleomorphic areas increased significantly. The mitotic figures increased significantly in well, moderately, and poorly differentiated cases (Fig 2a, 2b, 2c). The mitotic index increased as the degree of differentiation worsened (Table 1). In the pleomorphic areas, apoptotic cells were observed as a spherical structure with purple-colored eosinophilic cytoplasm and scattered chromatin material. Tumoral islets, formed by neoplastic cells and inflammatory cell infiltrates among the trabecular or cord structures, where plasmocytes, lymphocytes, or histiocytes were predominant, were also seen.

Immunohistochemical findings: Statistical comparisons between caspase-3, AIF, and LC3B immunoreactivities in well, moderate, and poorly differentiated OSCC cases are shown in Table 2. As a result of immunostaining, high caspase-3 immunoreactivity was observed in the cytoplasm of tumor cells. periphery of keratin pearls in the well-differentiated group (Fig. 3a-b). However, in the moderately (Fig. 3c-d) and poorly differentiated (Fig. 3e-f) groups, a more severe immune reaction occurred in the cytoplasm of neoplastic cells in tumoral islets, especially in cells located in areas of increased pleomorphism. Caspase-3 immunoreactivity was found to be more severe and intense in the poorly differentiated group than in the other two groups. As a result of positive cell count, a non-significant difference was found between

well and moderately differentiated groups, but a significant increase was observed in the poorly differentiated group compared to the other two groups (P<0.05). AIF immunoreactivity examination showed that the positive reaction in all three groups was formed in the cytoplasm of tumor cells, in fine granular form and very weakly (Fig. 4a-b, 4c-d, 4e-f). The mean values of AIF-positive cell numbers were not significantly different between the three groups (P>0.05). On LC3B staining, a marker of autophagy, tumor cells surrounding keratin pearls in the

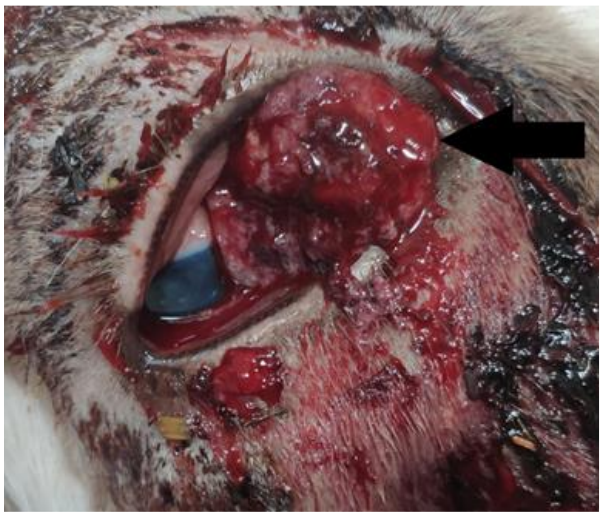

Fig. 1: Photograph of a bleeding tumoral mass on the third eyelid; surfaces are highly hemorrhagic and ulcerative, and show irregular pink hemorrhagic patches covered with purulent exudate.

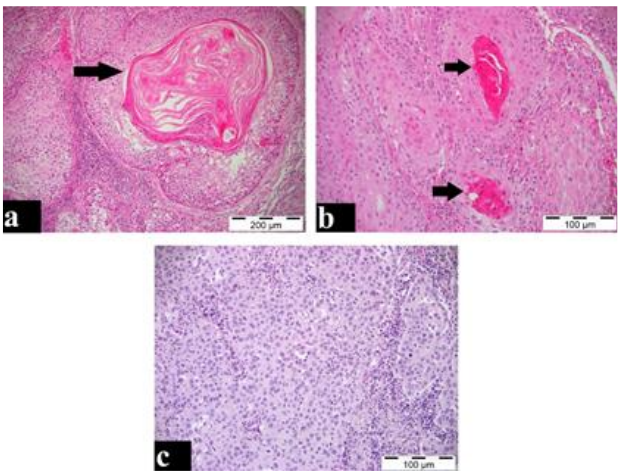

Fig. 2: (a) Cases of well-differentiated OSCC showing fairly large keratin pearls, H&E, Bar= 200 µm, (b) Moderately differentiated OSCC cases exhibiting small to moderate keratin pearls and tumor islets, H&E, Bar= 100 µm, (c) Cases of poorly differentiated OSCC with marked pleomorphism, H&E, Bar=100 µm.

Table 1: Mean ± SD mitosis rates between well, moderately and poorly differentiated groups

Degree of differentiation	Mitotic index
Well differentiated	4.2875±0,33566[a]
Moderately differentiated	4.8393±0,41517[b]
Poorly differentiated	5.7821±0,59364[c]
P value	<0.001

[a-b-c] represents the statistical difference between groups (P<0.05).

Table 2: The mean ± SD values of the statistical analyzes between well, moderate and poorly differentiated groups

	Caspase-3	AIF	LC3B
Well differentiated	409.29±11.23[a]	314.86–16.16[ab]	408.07±9.790[a]
Moderately differentiated	441.40±6.75[a]	325.40–10.71[ab]	442.07±11.384[a]
Poorly differentiated	476.53±11.06[b]	337.53–15.41[ab]	482.80±13.827[b]
P value	0.0001	0.537	0.0003

[a-b] Values with different superscripts within a column differ significantly (*P*<0.05).

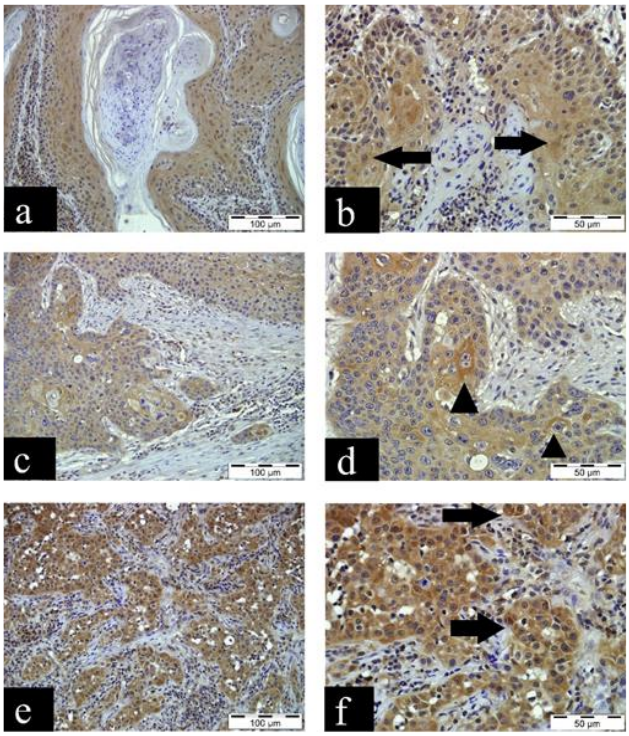

Fig. 3: Cases of OSCC, Caspase-3 IHC, ab: Well-differentiated OSCC (arrows), a: Bar= 100 µm, b: Bar= 50 µm. cd: Moderately differentiated OSCC (arrowheads), c: Bar= 100 µm, d: Bar = 50 µm. e-f: Poorly differentiated OSCC (arrows), e. Bar= 100 µm, f: Bar= 50 µm.

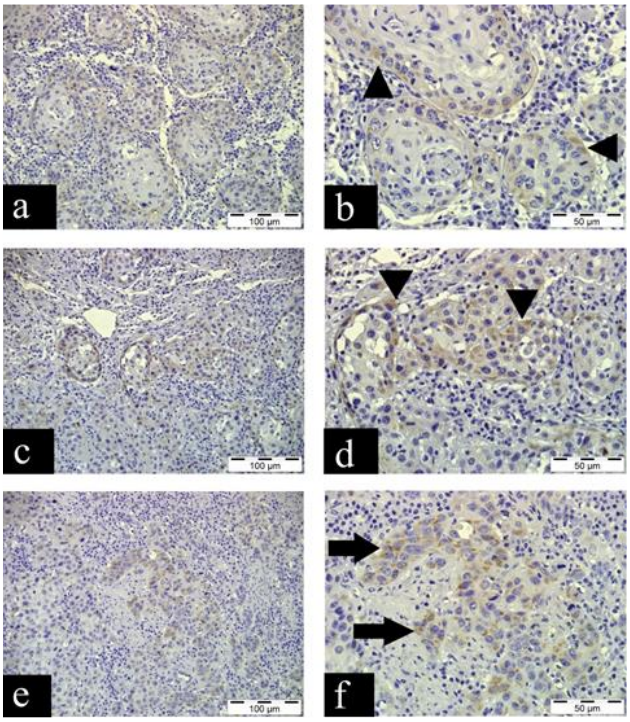

Fig. 4: OSCC cases, AIF positive immune reaction ab: Well-differentiated OSCC (arrowheads), a: Bar= 100 µm, b: Bar= 50 µm. cd: Moderately differentiated OSCC (arrowheads), c: Bar= 100 µm, d: Bar= 50 µm. e-f: Poorly differentiated OSCC (arrows), e: Bar= 100 µm, f: Bar= 50 µm.

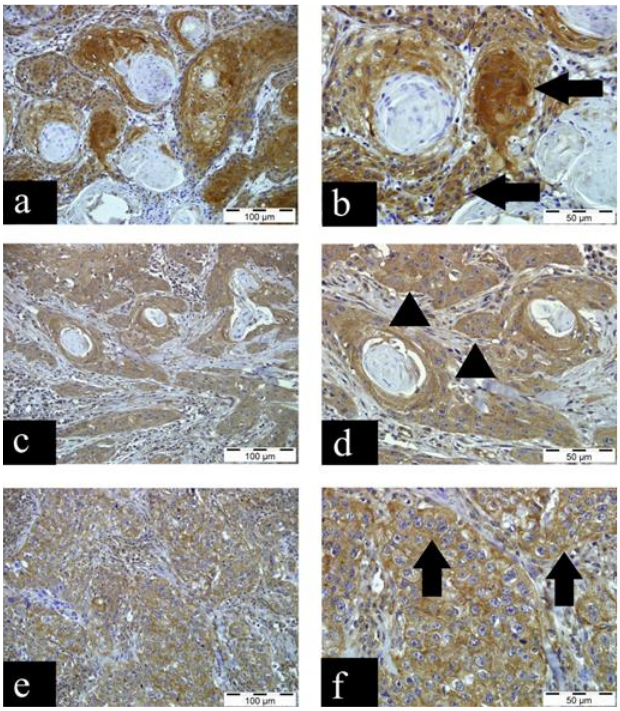

Fig. 5: OSCC states, LC3B IHC, a-b: Well differentiated OSCC (arrows), a: Bar= 100 µm, b: Bar= 50 µm. c-d: Moderately differentiated OSCC (arrowheads), c: Bar= 100 µm, d: Bar= 50 µm. e-f: Poorly differentiated OSCC (triangles), e. Bar= 100 µm, f: Bar= 50 µm.

well-differentiated group showed a severe cytoplasmic positive immune reaction (Fig. 5a-b). In the moderately differentiated group, a positive immune reaction was observed both in the tumor cells around the keratin pearls and in the cytoplasm of the neoplastic cells in the tumor islets (Fig. 5c-d). However, in the poorly differentiated group, the reaction was widely distributed in the cytoplasm of neoplastic cells in tumor islands (Fig. 5e-f). The mean number of cells staining LC3B-positive was significantly higher ($P<0.05$) in the poorly differentiated group compared to the moderately and poorly differentiated groups. However, the difference in mean positive cell counts between well and moderately differentiated groups was not statistically significant (Table 2).

DISCUSSION

OSCC is one of the most important and most common neoplasms of the eye in cattle (Marà *et al.,* 2005; Taş *et al.,* 2009). The etiopathogenesis of the disease is multifactorial, including causes such as UV rays, ocular pigmentation deficiency, and viral agents such as bovine papilloma virus and bovine herpes virus; also, genetic make-up plays an important role in the development of the tumor (Marà *et al.,* 2005; Gharagozlou *et al.,* 2007). Previous studies found that ocular pigment deficiency and UV rays were the two most important factors for OSCC (Gharagozlou *et al.,* 2007; Tsujita and Plummer, 2010). OSCC was mostly found in the lateral limbus of the eye, the third eyelid, the cornea, the conjunctiva, and the part of the skin adjacent to these structures, and less common in the vulva and the perineal area (Yavuz and Yumuşak, 2017; Sözmen *et al.,* 2019; Mathewos *et al.,* 2020). The present study showed that 36 of 45 OSCC cases were present in the Simmental cattle breed and 8 in the Simmental cross-bred cattle, which was consistent with the results in the literature (Gharagozlou *et al.,* 2007). Of the 45 neoplastic lesions, 24 were in the left eye and 21 in the right eye. Further, 23 of the lesions covered the whole eye, 6 were located under the eyelid, 6 were in the lateral cauntus, 4 in the third eyelid, and 5 in the upper eyelid. Gharazoglou *et al*. (2007), Taş *et al*. (2009), Stratigos *et al*. (2015) and Podarala *et al*. (2020) also reported nodular or cauliflower-like tumoral masses of varying sizes with hemorrhage, which caused deformation and atrophy in the eyes.

Histopathological examination revealed that the density and size of the keratin pearls in OSCC cases varied according to the differentiation degree of the tumoral structure, as reported previously (Gharagozlou *et al.,* 2007; Yakan *et al.,* 2017). In well-differentiated OSCC cases, the keratin pearls were high in number and quite large, the intercellular connections were clear, and slight pleomorphism was observed. The number and size of keratin pearls were found to be less in moderately differentiated OSCC cases than in well-differentiated OSCC cases. In addition, pleomorphism was dominant in the tumoral area, the number of keratin pearls was very low or absent, and single-cell keratinization was noted in poorly differentiated OSCC cases, unlike in the other two grades. Previous studies reported that the intercellular connections were lost and mitotic and apoptotic figures were seen in the tumoral area (Gharagozlou *et al.,* 2007; Stratigos *et al.,* 2015; Sözmen *et al.,* 2019; Podarala *et al.,* 2020).

Previous studies reported that neoplastic grading was based on the number of mitoses present in the neoplastic area (Carvalho *et al.,* 2005; Yavuz and Yumuşak, 2017). Carvalho *et al*. (2005) found that the neoplastic area was classified as well-differentiated OSSC when the number of mitoses was 6 or higher; as moderately differentiated when the number was between 2 and 6, and as poorly differentiated when the number was between 0 and 2. Sözmen *et al*. (2019) counted the mitotic figures in the top 10 tumoral areas and made a scoring according to the count results. Their evaluation was as follows: 0–2 = "+"; 3–6 = "++"; and 6 and higher = "+++." The present study also showed that the number and size of keratin pearls were larger in well-differentiated OSSC cases compared with moderately and poorly differentiated OSCC cases, and a positive relationship was observed between differentiation degree and pleomorphism. Similarly, the top five areas in tumor formation were determined, the mitotic figures in this area were counted, and their mean was accepted as the mean mitotic index of that case. Poorly differentiated OSCC cases had a higher mitotic activity compared with well- and moderately differentiated cases.

Apoptosis, which causes disturbance of the balance between cell death and cell proliferation, plays an important role in the pathogenesis of many diseases, including acquired immunodeficiency syndrome, Alzheimer's, Parkinson's, autoimmune diseases, and cancers (Elmore 2007). Basically, two main pathways initiate apoptosis. These are called the intrinsic (mitochondrial) and extrinsic pathways (Crowley and Waterhouse, 2016). Caspase-3 activity is required for most of the morphological and biochemical events

associated with apoptosis and is therefore generally considered an important marker of apoptosis (Crowley and Waterhouse, 2016). Therefore, the detection of caspase-3 in the tumor is a very important parameter to determine the role of apoptosis in tumor development (Zhou *et al.,* 2018). Koomägi and Volm (2000) examined 135 small cell lung carcinomas to determine whether a relationship existed between caspase-3 expression and clinical outcome. It was found that cases with high caspase-3 expression had a longer mean survival than cases with low caspase-3 expression. Contrary to Koomägi and Volm (2000), Takata *et al*. (2001) immunohistochemically examined the clinical significance of caspase-3 expression and its relationship with the incidence of apoptosis in resected nonsmall cell lung cancer (NSCLC). They reported that strong caspase-3 immunoreactivity was an important factor in predicting poor prognosis in NSCLC. The results of the present study contradicted those of Koomägi and Volm (2000) and supported the findings of Takata *et al*. (2001). We found that the immunoreactivity of caspase-3 increased with the increase in tumor aggressiveness. A stronger caspase-3 immune reactivity was found, especially in areas with high pleomorphism. In addition, previous studies showed that AIF played a role in cell homeostasis and tumor development (Wang *et al.,* 2019). Skyrlas *et al*. (2011) compared the expression of AIF in resection samples of SCC (N = 23) and keratoacanthoma (N = 29) and reported that AIF expression was more intense in SCC cases, especially in well-differentiated areas. Wang *et al*. (2019) reported a negative relationship between tumor grades and AIF expression. Their histopathological grading showed intense expression of AIF in grade 1 tumor lesions and a lesser reaction in grade 3 tumors compared with grades 1 and 2. Unlike the results of Skyrlas *et al*. (2011) and Wang *et al*. (2019), we found that AIF immunoreactivity increased in poorly differentiated OSSC cases compared with moderately and well-differentiated cases. However, the difference in the mean number of positive cells among three groups was statistically non-significant. The fact that the resulting AIF reaction occurred at a much lower level than the immune reaction of caspase-3 suggested that caspase-dependent apoptosis played a more dominant role than caspase-independent apoptosis in OSCC cases in cattle.

Autophagy acts as a central regulator in controlling homeostasis in the body, from regulating basic metabolic functions inside cells to various diseases such as aging, cancer, neurodegenerative disorders, and lysosomal disorders (Mehrpour *et al.,* 2010). Autophagy is shaped by three different mechanisms: macroautophagy, microautophagy, and chaperone-mediated autophagy (Nakamura and Yoshimori, 2017). The level of LC3-II, an autophagosomal marker, reflects autophagic activity. The detection of LC3 activation is used as a reliable parameter to monitor autophagy and autophagy-related processes, including autophagic cell death (Candé *et al.,* 2004; In cancer biology, however, the role of autophagy is complex; it contributes to tumor development, suppression, or cancer cell development and proliferation (Yun and Lee, 2018) Sivridis *et al.* (2011) evaluated LC3 expression by the immunohistochemical method to determine the relationship between autophagic activity and aggressiveness of SCC. They concluded that LC3 induced two different reactions, diffuse cytoplasmic and nuclear, and that LC3 was an important parameter in demonstrating the aggressiveness of SCC. In the present study, we evaluated the immunoreactivity of LC3B to determine the relationship between tumor aggressiveness and autophagy in OSCC. As a result, similar to Sivridis *et al*. (2011), we found that LC3B immunoreactivity was higher in poorly differentiated OSCC cases than in well- and moderately differentiated cases and showed an increase consistent with tumor aggressiveness. In addition, when the immunoreactivity of LC3B was evaluated in all three groups, no statistical difference was found between well- and moderately differentiated groups but a statistically significant increase was observed in poorly differentiated cases than the other two groups.

Conclusions: In conclusion, the present study revealed that the levels of caspase-3and LC3B were significantly increased in poorly differentiated OSCC cases compared to well differentiated and moderately differentiated cases. When the study data were evaluated, it was speculated that apoptosis and autophagy play a common role on tumor aggressiveness and development. These two cell death pathways are thought to have a mechanism that favors the development of each other in OSSC cattle cases.

Declarations

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Conflicts of interest: The authors declare that they have no conflicts of interest with regard to the work presented in this report.

Authors contribution: Ayfer YILDIZ performed the laboratory work of the study and conducted the literature review and writing up of the manuscript. Emin KARAKURT carried out the literature review, interpretation of tumoral tissue and photography.

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