



RESEARCH ARTICLE

Apoptosis in bovine ocular squamous cell carcinomas

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Received:04.01.2022, Accepted: 19.05.2022

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Sığırların oküler yassı hücreli karsinomlarında apoptozis

Eurasian J Vet Sci, 2022, 38, 2, 131-136

DOI: 10.15312/EurasianJVetSci.2022.374

Öz

Amaç: Hücre ölümü özellikle de apoptozis hücre konusunda en çok çalışılan konular arasında yer almaktadır. Hastalıklardaki apoptozis mekanizmasının anlaşılması sadece hastalıkların patogenezinin anlaşılmasına değil hastalıkların tedavisine de yardımcı olacaktır. Bu çalışmada sığırlarda gözlenen oküler yassı hücreli karsinomların (Bovine ocular squamous cell carcinoma, BOSCC) derecelendirilmesi ve farklı anaplastik özellikler gösteren bu tümörlerde apoptozisin değerlendirilmesi hedeflendi.

Gereç ve Yöntem: BOSCC'lerde apoptozisin incelenmesi amacıyla, formalinde tespit edilmiş 10 adet kötü differensiyasyon, 10 adet orta differensiyasyon ve 10 iyi differensiyasyon olan toplam 30 adet tümör dokusu kullanıldı. Histopatolojik inceleme için hematoksilin ve eozin (H&E); apoptozisin tespit için tüm BOSCC'lerde TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) boyaması yapıldı.

Bulgular: BOSCC'lerde malignite arttıkça apoptozisin azaldığı saptandı ($p<0.001$). İncelenen tüm tümörler malign olmasına rağmen iyi differensiyasyon tipte kontrollü hücre ölümü olan apoptozis şiddetli olduğu ancak orta ve kötü differensiyasyon tiplerde apoptozisin hafif şiddette olduğu saptandı.

Öneri: Sığırlarda gözlenen bu durumun kanser patogenezi ve tedavisine yönelik çalışmalarda göz önünde bulundurulması gerektiği kanısına varıldı.

Anahtar kelimeler: Apoptozis, BOSCC, diferensiyasyon, sığır oküler yassı hücreli karsinom

Abstract

Aim: Cell death, especially apoptosis, is one of the most studied topics regarding cells. Understanding the mechanism of apoptosis in diseases will help understand the pathogenesis of diseases, as well as the treatment. This study aims to grade the Bovine ocular squamous cell carcinoma (BOSCC) and to evaluate apoptosis in tumors with different anaplastic features.

Materials and Methods: A total of 30 formalin-fixed tumor tissues consisting of 10 poorly-differentiated, 10 moderately-differentiated, and 10 well-differentiated tissues were used to examine apoptosis in BOSCCs. Hematoxylin & eosin (HE) was performed for histopathological examination and TUNEL staining was performed for detection of apoptosis in all BOSCCs.

Results: It was determined that apoptosis decreased as the malignancy increased in BOSCCs ($p<0.001$). Although all the tumors examined were malignant, apoptosis, i.e. controlled cell death, was found to be severe in well-differentiated type and mild in moderate and poorly-differentiated types.

Conclusion: It was concluded that this case observed in bovines should be considered in studies on the pathogenesis of cancer and treatment.

Keywords: Apoptosis, BOSCC, differentiation, bovine ocular squamous cell carcinoma



Introduction

Bovine ocular squamous cell carcinoma (BOSCC), also called 'cancer eye', is a tumor originating from keratinocytes, which is formed in the ocular tissues, especially the eyelid. The tumor, which is mostly observed in bovines and causes the most economic loss, has a high incidence rate across the World (Carvalho et al 2002, Tsujita et al 2010, Fornazari et al 2017, Sözmen et al 2019, Vala et al 2020). According to a study, 73% of ocular tumors in bovines were squamous cell carcinoma (Ceylan et al 2012). Genetic susceptibility, age, altitude, regional conditions, circumocular apigmentation, and viral factors are among the causes of BOSCC (Mara et al 2005, Aksoy et al 2006, Ferreira et al 2008, Taş et al 2009, Tsujita et al 2010, Fornazari et al 2017).

Organ homeostasis in multicellular organisms is maintained through a balance between cell proliferation and cell death (Thompson 1995, Bentz et al 2002, Sankari et al 2012). Cell death, especially apoptosis, is one of the most studied topics regarding cells (Wong 2011).

When the cell is deprived of growth factors and its proteins are damaged beyond repair, the cell kills itself in a way called apoptosis, characterized by the disintegration of the nucleus by maintaining membrane continuity. Briefly, programmed cell death is called apoptosis (Thompson 1995, Bentz et al 2002, Sankari et al 2012). Disruption of apoptotic regulation may lead to various pathological processes characterized by excessive cell accumulation or loss (Bentz et al 2002, Wong 2011, Sankari et al 2012). While some diseases inhibit apoptosis (cancer, autoimmune diseases, viral infections), some diseases trigger apoptosis (acquired immunodeficiency syndrome (AIDS), neurodegenerative diseases, ischemic injury, toxic liver diseases) (Thompson 1995). Understanding the mechanism of apoptosis in diseases is very important as it not only gives insights into the the pathogenesis of diseases but may also leaves clues on how to treat diseases (Thompson 1995, Wong 2011, Sankari et al 2012).

This study aims to examine the apoptosis levels in BOSCC by using the TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) method by grading according to anaplastic features of BOSCC in bovines.

Material and Methods

A total of 30 buffered formalin-fixed tumor tissues consisted of 10 poorly-differentiated, 10 moderately-differentiated, and 10 well-differentiated tissues obtained from the Department of Pathology, Faculty of Veterinary Medicine, Kafkas University were used

to examine apoptosis. Clinical findings observed in animals and macroscopic findings related to tumors were evaluated retrospectively from the surgery and pathology department records.

Histopathologic examination

Tissues fixed in 10% buffered formalin were washed overnight to remove formalin under tap water. Routine tissue process was followed. Tissues were dehydrated in ascending grades of ethanol (70, 80, 90, 96, and 100%), cleared in xylene, embedded in paraffin, and sectioned at 4µm thickness from each block and deparaffinized in xylol then passed through a series of 100%, 96%, 80%, and 70% alcohol, respectively. After the tissues were stained with Hematoxylin and Eosin (H&E), microphotographs (Olympus DP12) of the tissues were obtained under a light microscope (Olympus CX31).

In order to examine apoptosis, 3 groups were formed as well, moderately and poorly differentiated BOSCC based on anaplastic features. Histopathological classifications and grading of 10 tumors in each group were made as presented in Table 1. (Carvalho et al 2005, Sözmen et al 2019).

Determination of apoptotic cells by TUNEL method

TUNEL method was applied in order to identify apoptotic cells in tissues. For this, In Situ Cell Death Detection and POD (Roche, Germany, Cat. No. 11684817910) apoptosis kit was used and the standard prescribed procedure was applied for formalin-fixed paraffin-embedded tissues. Endogenous peroxidase activity was removed in 3% H₂O₂ methanol. Proteinase K (Abcam, ab64220) was used as an antigen retrieval and DAB (ScyTek Laboratories, Logan, UT) was used as a chromogen. The samples were counterstained with hematoxylin. TUNEL stained sections were evaluated semi-quantitatively and graded according to the following scale: negative, 0, <33% of keratinocytes positive, +1; 33–66% of keratinocytes positive, +2; >66% of keratinocytes positive, +3 (Schoelch et al 1999).

Statistical analysis

Statistical analysis was performed with SPSS (SPSS 26.0, Chicago, IL, USA). Kruskal Wallis-H Test and Mann-Whitney U Tests were used to compare the mean and median values between groups. Obtained results were given as mean ± standard error (SE). A value of p<0.05 was considered as statistically significant.



Table 1. Histopathological classifications and grading BOSCC (Carvalho et al 2005, Sözmen et al 2019)

Grade 1: Well differentiated	Large keratin pearls Large tumor islands Prominent intercellular bridges Obvious squamous differentiation
Grade 2: Moderately differentiated	Small-to medium sized keratin pearls Smaller islands, Moderate degree of keratinization and differentiation Squamous differentiation Increased number of poorly differentiated cells
Grade 3: Poorly differentiated	No sign of keratinization Individual cell keratinization, Few small tumor islands, Poor cellular differentiation

Results

According to the macroscopic localization of the 30 tumors, they covered the lower and upper eyelids, the third eyelid, and the entire eye. It was determined that the lesions were macroscopically nodular, showed cauliflower or papillomatous growth patterns, and were bleeding. It was determined that the tumor tissues caused an increase in lacrimation and vision loss as a result of the pressure on the eye.

Histopathological classifications of tumors presented in Table 1 above were made according to the method suggested by Carvalho et al (2005) and Sozmen et al (2019). The well-differentiated tumor types had squamous differentiation with large keratin pearls and large tumor islands (Figure 1a). The moderately differentiated types, had less prominent, small, and medium-sized keratin pearls, squamous differentiation, and an increasing number of poorly differentiated cells (Figure 1b). Cell-based keratinization, poorly

differentiated cells and mitotic figures were observed in the poorly differentiated types. Tumor islands were smaller in poorly differentiated type than those in well and moderately differentiated BOSCC cases. Similarly, pleomorphic areas were found to be more common in the poorly differentiated group. Keratinized single cells were large and round. The nuclei of these cells were pycnotic, and the cytoplasm was dark eosinophilic (Figure 2).

TUNEL method was applied in order to identify apoptotic cells in 3 groups (30 tumor tissues). Apoptosis levels in TUNEL staining are presented in Table 2. In TUNEL staining performed for the detection of apoptosis, intense positivity of keratinocytes, especially nuclei, was observed in well-differentiated BOSCC cases (Figure 3a, 3b) ($p<0.001$). However, similar staining in the moderately differentiated type showed mild intensity (Figure 3c, 3d) ($p<0.001$). Apoptosis was observed in the poorly-differentiated type and mild TUNEL positivity was observed in neoplastic cells (Figure 3e, 3f) ($p<0.001$).

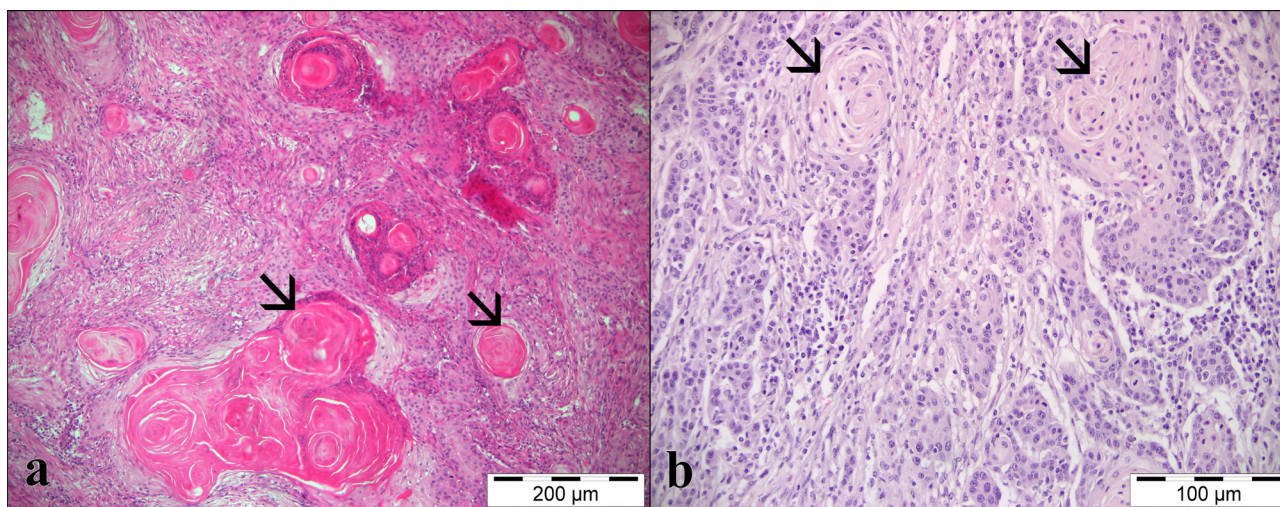


Figure1: Histopathologic appearance of BOSCC a) Well-differentiated tumor type with large keratin pearls (arrows), H&E. b) Moderately differentiated tumor type, small, and medium-sized keratin pearls (arrows), H&E.

Table 2. Apoptosis levels in TUNEL staining of BOSCC

BOSCC	Apoptosis
Well differentiated	3,0±0,0 ^a
Moderatly differentiated	1,0±0,0 ^b
Poorly differentiated	1,0±0,0 ^b
p value	<0.001

a-b: Means that there was a statistically significant difference between the defined groups

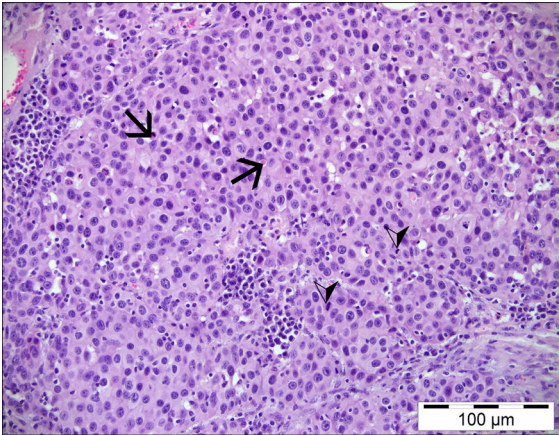


Figure 2: Histopathologic appearance of poorly differentiated BOSCC tumor type, cell-based keratinization (arrows) , mitotic figures (arrowheads) , H&E.

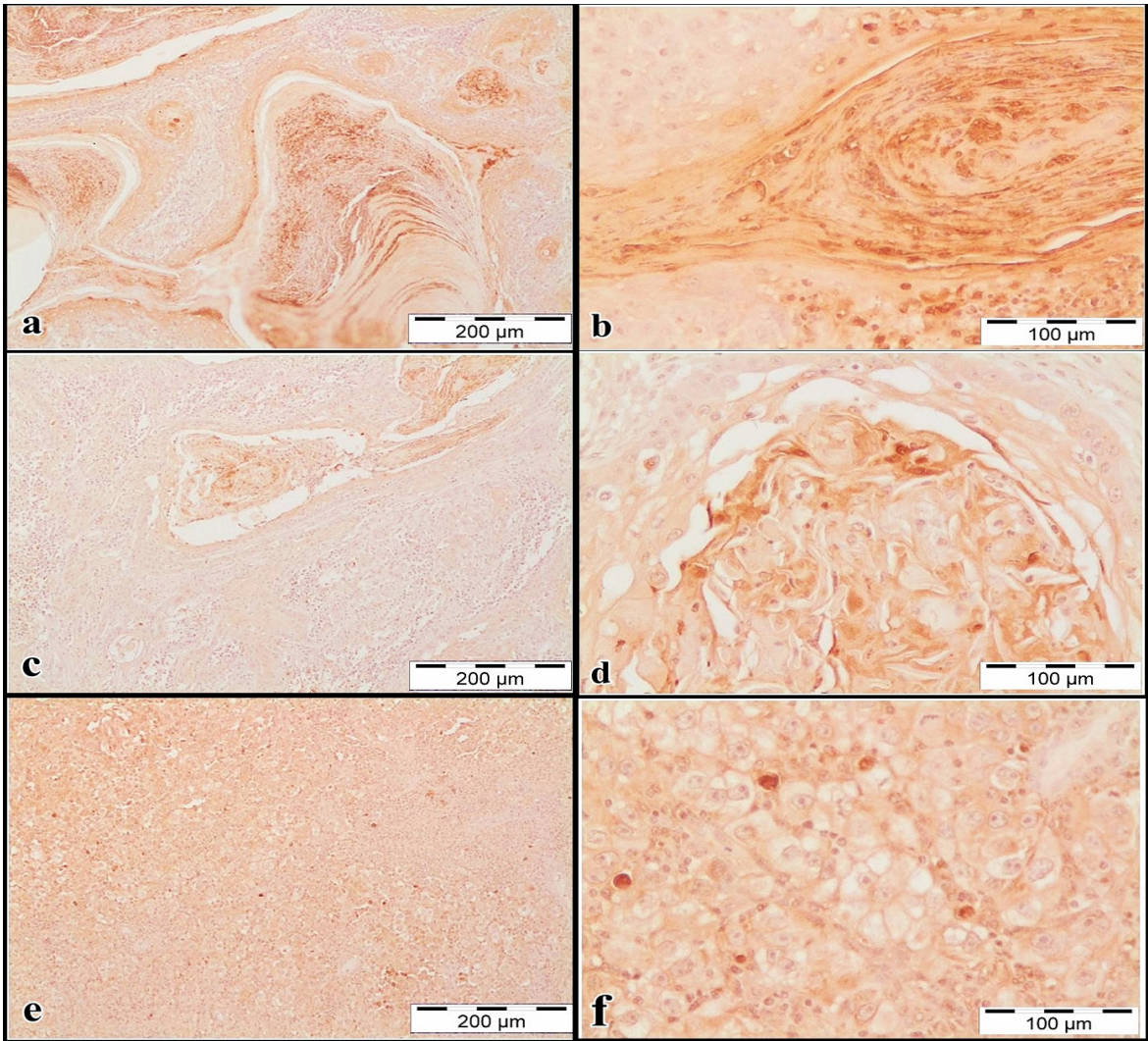


Figure 3: TUNEL staining for demonstrating apoptotic cells of BOSCC. a) Intense positivity of keratinocytes in well-differentiated BOSCC. b) Intense positivity of keratinocytes in well-differentiated BOSCC. c) Mild positivity of keratinocytes in moderately differentiated BOSCC. d) Mild positivity of keratinocytes in moderately differentiated BOSCC. e) Mild positivity in neoplastic cells in poorly differentiated BOSCC. f) Mild positivity in sporadic cells in poorly differentiated BOSCC.



Discussion

Squamous cell carcinoma cases have an important place among the tumors encountered in domestic animals, and ocular squamous cell carcinoma is mostly seen in bovines and horses, and rarely in cats and dogs (Aksoy et al 2006, Mara et al 2005, Yüksel et al 2005, Ferreira et al 2008, Wilcock 1993, Tsujita et al 2010, Taş et al 2009, Yavuz and Yumuşak 2017). BOSCC is typically ulcerative, solid, lobular, and cauliflower-like (Taş et al 2009, Yakan et al 2017, Vala et al 2020). In this study, it was determined that BOSCCs similarly had nodular in structure, a cauliflower-like or papillomatous growth pattern, and were ulcerative.

Histologically, BOSCC can be observed in many forms ranging from well-differentiated to undifferentiated and is divided into three categories as poorly-, moderately-, and well-differentiated based on the characteristics of tumors. (Carvalho et al 2005, Fornazari et al 2017, Sözmen et al 2019, Vala et al 2020). In the present study, it was seen that the presence and size of keratin pearls and tumoral islands were significant in the classification of BOSCC as in previous studies (Carvalho et al 2005, Sözmen et al 2019). In addition, in this study mitotic figure and pleomorphism also contributed to the classification. Vala et al (2020) reported that 26.3% of these tumors were poorly-differentiated, 26.3% were moderately-differentiated, and 47.4% were well- differentiated.

Cell death, especially apoptosis, is one of the most studied topics regarding cells (Townson et al 2003, Wong 2011, Sankari et al 2012). The controlled cell death process is shaped under the influence of several signalling pathways and triggered by many factors including cellular stress, DNA damage, and the immune system (Sankari et al 2012, Carneiro et al 2020). As a result of the loss of balance in cell division and cell death in cancer, cells cannot receive the necessary signals to cell death (Thompson 1995, Townson et al 2003, Wong 2011, Sankari et al 2012). The loss of apoptotic regulation has been hypothesized to result in excessive cellular or loss, leading to various pathological processes (Bentz et al 2002). Indeed, reduction of apoptosis or resistance to apoptosis plays a vital role in carcinogenesis (Thompson 1995, Townson et al 2003, Wong 2011, Sankari et al 2012). For example, if the DNA damage caused by prolonged exposure to ultraviolet-light (UV) radiation is not repaired, or if these damaged cells are not eliminated by apoptosis, these cells undergo a transformation with uncontrolled proliferation which leads to the formation of cancers such as melanoma, basal cell carcinoma, and squamous cell carcinoma of the skin (Erb et al 2005). In this study, it was determined that apoptosis decreased as the malignancy increased and damaged cells are not eliminated by apoptosis especially

in moderately and poorly differentiated BOSCCs. With this study, it was concluded that apoptosis plays a role in carcinogenesis of BOSCCs. These tumors were generally examined clinically and histopathologically in Turkey (Aksoy et al 2006, Yüksel et al 2005, Taş et al 2009, Ceylan et al 2012, Yavuz and Yumuşak 2017). In this study, the role of apoptosis in tumor progression in BOSCC's was studied.

Most cancer cells exhibit defective apoptotic mechanisms or form an uncontrolled structure as an escape mechanism from apoptosis, allowing them to develop in an uncontrolled way (Friesen et al 1999, Townson et al 2003, Erb et al 2005, Wong 2011, Carneiro et al 2020). As early as the 1970's, Kerr et al (1972) had linked apoptosis to the elimination of potentially malignant cells, hyperplasia and tumour progression. Therefore, restoring apoptosis is considered a method in the treatment of cancer. One of the main goals of clinical oncology for more than 30 years has been the development of a treatment method that ensures the elimination of cancer cells through apoptosis (Carneiro et al 2020, Wong 2011, Sankari et al 2012). Many recent and important discoveries have opened new doors into potential new classes of anticancer drugs. (Wong 2011). It was evaluated that drugs which act on apoptosis-related proteins can be studied on BOSCC's, which are commonly seen in bovine.

Conclusion

Consequently, in this study, it was determined that apoptosis decreased as anaplasia increased and differentiation decreased in bovine ocular squamous cell cancers. In addition, with this study, it was concluded that apoptosis-focused treatment options can be tried in ocular squamous cell cancer treatments of cattle.

Conflict of Interest

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

Funding

During this study, any pharmaceutical company which has a direct connection with the research subject, a company that provides and / or manufactures medical instruments, equipment and materials or any commercial company may have a negative impact on the decision to be made during the evaluation process of the study or no moral support.

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Ethical Approval

Kafkas University Experimental Research and Application Center, Animal Experiments Ethics Committee KAU-HADYEK/2021-141 Number Ethics Committee Decision

CITE THIS ARTICLE: Kutlu T, Karakurt E, Aydın U, Beytut E, Özaydın İ, Dağ S, Aksoy Ö, Nuhoglu H, 2022. Apoptosis in bovine ocular squamous cell carcinomas. *Eurasian J Vet Sci*, 38, 2, 131-136