Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2026.137

RESEARCH ARTICLE

Investigation of the Relationship Between TLR-3/9 Expressions and Apoptotic Markers in Bovine BOSCC Cases Using Immunohistochemical Methods

Hilmi Nuhoğlu[1*] Mizgin Kengiş[1] Ayfer Yıldız Uysal [1] and Serpil Dağ[1]

[1]Kafkas University, Faculty of Veterinary Medicine, Department of Pathology, Kars, Turkey
*Corresponding author: hilminuhoglu@gmail.com

ARTICLE HISTORY (26-194)

Received: February 26, 2026
Revised: May 12, 2026
Accepted: May 15, 2026
Published online: May 25, 2026

Key words:
Apoptosis
Cancer biology
Squamous cell carcinoma
Toll-like receptors

A B S T R A C T

Bovine ocular squamous cell carcinoma (BOSCC) constitutes a malignant epithelial neoplasm that has been linked to causative influences including ultraviolet light exposure and lack of pigment. Serving as key elements of the innate immune system, Toll-like receptors (TLRs) orchestrate inflammatory and immune reactions by sensing both pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Through the NF-κB pathway, TLR signal transduction exerts dualistic actions in tumor development thereby promoting inflammation while simultaneously inhibiting programmed cell death. The Bcl-2 protein family governs apoptotic processes, whereas matrix metalloproteinases (MMPs) hold crucial importance for tissue invasion and metastatic spread. In this study, TLR3 and TLR9 expressions in BOSCC were evaluated by immunohisto-chemistry and immunofluorescence and the relationships between Bax, Bcl-2, and MMP-9 were also investigated. TLR3 and TLR9 were highly expressed irrespective of tumor differentiation and did not show a strong direct correlation with apoptotic and invasion markers. However, in poorly differentiated cases, a significant increase in Bcl-2 and a relative decrease in Bax indicated a shift towards an anti-apoptotic phenotype. The high expression of TLR3/9 suggested that it may have the potential to be an immunotherapeutic target rather than an independent biomarker. The Bcl-2/Bax ratio increased from 0.26 in well-differentiated cases to 0.67 in poorly differentiated cases. An increased Bcl-2/Bax ratio suggests apoptosis resistance and this situation may explain resistance to conventional treatments and suggests that a combination of Bcl-2 inhibitors and immunotherapy may be beneficial.

To Cite This Article: Nuhoğlu H, Kengiş M, Uysal YA and Dağ S, 2026. Investigation of the Relationship Between TLR-3/9 Expressions and Apoptotic Markers in Bovine BOSCC Cases Using Immunohistochemical Methods. Pak Vet J, 46(6): 1429-1438. http://dx.doi.org/10.29261/pakvetj/2026.137

INTRODUCTION

Bovine ocular squamous cell carcinoma (BOSCC), also known as "cancerous eye" among veterinary pathologists, is a malignant epithelial tumor. These masses, notable for their cauliflower-like shapes and varying sizes, are malignant epithelial neoplasms that most often occur in the bulbar conjunctiva, cornea, and around the eyelid. This cancer, which has a higher incidence in breeds such as Hereford, Holstein, and Simmental, has been associated with environmental and hereditary factors such as conjunctival pigment deficiency and intense ultraviolet exposure (Tsujita and Plummer, 2010; Sharma *et al.*, 2020). It has also been noted that the disease may be linked to a number of viral agents, including papillomaviruses and herpesviruses. It has been found that the tumor commonly develops in cattle older than seven years, but is rarely seen in animals under three years of age. The most common location of the tumor in cattle is reported to be the limbus of the eye and the third eyelid (Tsujita and Plummer, 2010). Tumors formed by the transformation of plaque-like proliferations originating from the stratum spinosum layer of the epithelium into papillomas undergo various changes over time, progressing first to in situ and then to invasive carcinoma (Song *et al.*, 2012).

TLRs are members of the pattern recognition receptor (PRR) family, triggering the initiation of various defense mechanisms (Sakaniwa *et al.,* 2023; Alexopoulou and Irla, 2025). These same receptors also recognize damage associated molecular patterns (DAMPs) such as nucleic acids, proteins, lipids, and metabolites released from host cells, thereby triggering an inflammatory response (Kawai *et al.*, 2024). TLR signal transduction exhibits

bidirectional effects in the carcinogenesis process, both suppressing and supporting tumors (Salaun *et al.*, 2006; Salaun *et al.*, 2007; Sameer and Nisaa, 2021; Morana *et al.,* 2022). The NF-κB (Nuclear Factor-kappa B) pathway plays a significant role in both of these different effects. In the tumor microenvironment, NF-κB leads to the overexpression of numerous pro-inflammatory molecules, including the NF-κB-dependent TLR pathway, IL-1β, tumor necrosis factor-α (TNFα), and IL-6, while on the other hand, it causes the inhibition of proapoptotic pathways mediated by c-Jun kinase and also increases the expression of many anti-apoptotic genes. Thus, it has been observed to support carcinogenesis through pro-inflammatory, profibrogenic, proliferative, and anti-apoptotic signals in the tumor microenvironment or in the tumor cells themselves; on the other hand, it has been found to suppress the tumor by activating specific immune cells with antitumorigenic activity, such as dendritic cells (DCs), NKs, and cytotoxic T lymphocytes (Sameer and Nisaar, 2021).

The word 'apoptosis' was originally coined in 1972 by Kerr, Wyllie, and Currie as a descriptor for regulated cell death (Li and Yuan, 2008). Apoptosis is regulated by a group of proteins belonging to the Bcl-2 family, named after the Bcl-2 gene (B-cell lymphoma 2). The Bcl-2 family is broadly classified into two functional categories: proapoptotic members (Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim, Hrk) and antiapoptotic members (Bcl-2, Bcl-XL, Bcl-W, Bfl-1, Mel-1). Antiapoptotic proteins suppress apoptosis by inhibiting the release of cytochrome c from mitochondria, while proapoptotic proteins stimulate this release, initiating and supporting the apoptotic process (Wong, 2011). Matrix metalloproteinases (MMPs) are responsible for the remodeling and degradation of the extracellular matrix (ECM), including elastins, collagens, gelatin, matrix glycoproteins, and proteoglycans. MMPs play a significant role in programmed cell death (PCD), exhibiting both apoptotic and anti-apoptotic effects (Verma and Hansch, 2007). While these enzymes can support tumor growth by disrupting matrix barriers and promoting angiogenesis, they can also inhibit tumor development by limiting tumor neovascularization (Reunanen and Kähäri, 2000; Xu *et al.,* 2010).

This study investigated the intensity and distribution of TLR3 and TLR9 expression, and their associations with pro- and anti-apoptotic markers, in BOSCC cases in the veterinary field, using immunohistochemical staining methods. A literature review revealed a limited number of studies investigating TLR expression in BOSCC cases using immunohistochemical staining techniques, suggesting a significant role in veterinary oncology research. Therefore, the aim was to determine the expression of TLR3 and TLR9 in BOSCC cases, their association with apoptotic markers, and the role of TLR3 and TLR9 in tumor carcinogenesis.

MATERIALS AND METHODS

Ethical approval for the study was obtained from the Kafkas University Local Ethics Committee for Animal Experiments (Kafkas University HADYEK/2025-127). As the study material, a total of 12 BOSCC biopsy samples, including 6 well-differentiated and 6 poorly differentiated cases, obtained from the archive of the Department of Pathology, Faculty of Veterinary Medicine, Kafkas University, were used. In this study, all selected BOSCC cases were selected using a sequential selection method from cases retrospectively evaluated from archival records and diagnosed histopathologically as BOSCC. Cases with insufficient tissue integrity or unsuitable for immunohistochemical evaluation were excluded from this study. Table 1 lists the age, gender, race, and tumor location information for the cases included in the study.

Table 1: Information regarding age, race, gender, and tumor location of the cases

Case No.	Age	Gender	Race	Right/Left Eye
Case 1	5 age	Female	Simental	Left eye/third eyelid
Case 2	6 age	Female	Simental	Left eye/third eyelid
Case 3	4 age	Female	Simental	Left eye
Case 4	6 age	Female	Simental	Right eye
Case 5	5 age	Female	Simental	Left eye
Case 6	4 age	Male	Simental	Right eye
Case 7	6 age	Female	Simental	Left eye
Case 8	5 age	Female	Simental	Right eye
Case 9	5 age	Male	Simental	Left eye
Case 10	3 age	Female	Simental	Left eye
Case 11	4 age	Female	Simental	Right eye
Case 12	3 age	Female	Simental	Right eye

Histopathological method: Harvested samples underwent standard histological processing steps and were subsequently infiltrated with paraffin for block preparation. From each block, 4µm-thick sections were obtained and subjected to Hematoxylin-Eosin (H&E) staining. The sections were examined and photographed under a light microscope (Olympus BX53) (Dağ *et al.*, 2018). Tumors were evaluated according to their degree of differentiation. Based on keratinization, cellular atypia, and the prominence of desmosomes, tumors showing squamous differentiation in more than 50% of the cells were classified as grade 1–2 (well-differentiated), whereas tumors with less than 50% differentiation were classified as grade 3–4 (poorly differentiated) squamous cell carcinoma (Table 2) (Bălăşoiu *et al.*, 2008; Campoli *et al.*, 2014; Meier *et al.*, 2016; Hartmann *et al.*, 2018).

Table 2: BOSCC grading details

Grade	Differentiation Level	Histopathological Features	Squamous Differentiation Rate
Grade 1	Well-differentiated	Marked keratinization, large keratin pearls, prominent desmosomes/intercellular bridges, mild nuclear atypia	> 50%
Grade 2	Moderately-well differentiated	Keratinization present but reduced compared to Grade 1, moderate cellular atypia, intercellular bridges visible	> 50%
Grade 3	Poorly differentiated	Decreased keratinization, marked cellular and nuclear pleomorphism, indistinct desmosomes, increased mitotic activity	< 50%
Grade 4	Very poorly differentiated / Anaplastic	Anaplastic No or very little keratinization, severe atypia and pleomorphism, marked invasive structure	< 50%

Immunohistochemical method: The avidin-biotin immune-peroxidase method was used for immunohistochemical analysis. Sections of 3µm thickness were cut from paraffin blocks and mounted on Poly-L-

lysine–coated slides. Antigen retrieval was performed using citrate buffer (pH:6) in a microwave for 10 minutes. The sections were incubated overnight at 4°C with primary antibodies (TLR3, TLR9, Bax, Bcl-2, and MMP-9) diluted in PBS (Table 3). Subsequently, a biotinylated secondary antibody and peroxidase-conjugated streptavidin were applied to visualize the reaction. AEC was used as the chromogen, counterstaining was performed with Harris hematoxylin, and the slides were examined under a light microscope.

Table 3: Primary and secondary antibodies

Primary and secondary antibodies	Code	Dilution	Tissue
TLR -3 Polyclonal Antibody (in immunohistochemistry and immunofluorescence staining)	PA5-88251	1/100	Tumor tissue (BOSCC)
TLR-9 Polyclonal Antibody (in immunohistochemistry and immunofluorescence staining)	PA5-89960	1/100	Tumor tissue (BOSCC)
Bax (in immunohistochemistry and immunofluorescence staining))	STJ97729	1/100	Tumor tissue (BOSCC)
Bcl-2 (in immunohistochemistry and immunofluorescence staining))	Ab-BF9103	1/100	Tumor tissue (BOSCC)
MMP-9 Monoclonal Antibody ((in immunohistochemistry)	BT-MCA2606	1/100	Tumor tissue (BOSCC)
Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488	A-11008	1/500	Tumor tissue (BOSCC)
Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 555	A-21422	1/500	Tumor tissue (BOSCC)

Statistical analysis: In immunohistochemical staining, TLR3, TLR9, Bax, Bcl 2, and MMP 9 immuno-positive tumor cells were scored semi-quantitatively according to staining percentage and intensity. Cytoplasmic immunostaining percentage was categorized as follows: 0, ≤10% positive; 1, 11–25% positive; 2, 26–50% positive; 3, 51–75% positive; 4, ≥76% positive. Staining intensity was graded as light yellow: 1, yellow: 2, brown: 3. The total immunoreactivity score (IRS) was obtained by multiplying the staining percentage score by the intensity score for each case (range 0–12). For TLR3 and TLR9, total IRS values were classified according to the criteria of Hasimu *et al.* (2011): 0 → negative expression; 1–4 → low expression; 5–8 → moderate expression; 9–12 → high expression.

GraphPad 8.1 (San Diego, CA, USA) software was used for statistical analyses and calculations. Due to the small sample size and the failure of the data to meet the assumption of normal distribution, the non-parametric Mann–Whitney U test was applied to compare the total IRS values of the well-differentiated (n=6) and poorly differentiated (n=6) groups. All tests were performed two-way, and a $P<0.05$ value was considered statistically significant.

The Bcl 2/Bax ratio was calculated by dividing the total Bcl 2 IRS value by the total Bax IRS value for each case. A ratio <1 was interpreted as indicating the dominance of proapoptotic activation, =1 as indicating a balance between apoptosis and cell survival, and >1 as indicating the dominance of antiapoptotic mechanisms (Zhang *et al.*, 2009).

Double Immunofluorescence (IF): In immunofluorescence staining, the indirect Double IF staining technique was used. Deparaffinization and hydration were performed on sections taken from paraffin blocks. Subsequently, the sections were incubated in 3% hydrogen peroxide solution for 20 minutes to block endogenous peroxidase activity. After being kept in three different PBS buffer solutions for 5 minutes each, the sections were boiled in citrate buffer saline solution (pH:6.0) in a microwave oven for 10 minutes (600 W) to unmask antigenic sites, and then allowed to cool at room temperature for 20 minutes. After cooling, the sections were incubated in PBS solution 3 × 5 minutes, followed by drying the edges of the sections and drawing around them with a PAP pen. Then, to prevent background staining, the sections were incubated in blocking solution (1/100 goat serum) for 10 minutes at room temperature. Without PBS application, appropriately diluted primary antibodies were dropped onto the sections, which were then incubated overnight in a refrigerator at 4–6°C. On the following day, after washing the sections were incubated with goat anti-rabbit IgG (H+L) secondary antibody (Alexa Fluor 488) and goat anti-mouse IgG (H+L) secondary antibody (Alexa Fluor 555) at room temperature for 60 minutes in the dark. Following this incubation procedure, the sections were placed in distilled water. They were then kept in distilled water for 5 minutes, after which DAPI was applied and the sections were incubated in the dark for 5 minutes. Subsequently, the sections were coverslipped and examined under a fluorescence microscope.

RESULTS

Histopathological findings: Histopathological examination revealed that 6 cases were well-differentiated and 6 were poorly differentiated BOSCC. In well-differentiated cases, tumor cells invaded the dermis, forming tumor islands and cell cords. These cases showed prominent keratin pearls, intercellular bridges, and squamous keratinocyte differentiation (more than 50%). Additionally, atypia, dyskeratotic cells, mitotic figures, and in some cases mononuclear cell infiltrations were detected in malignant cells (Fig. 1A-1B).

In poorly differentiated BOSCC cases, the tumor surfaces were ulcerative and hemorrhagic, and tumor cell cords and island-like growth patterns were observed in the dermis. In these cases, keratin pearls were absent, while low squamous differentiation (less than 50%) and the presence of marked cellular atypia, pleomorphism, mitotic figures, and parakeratotic and dyskeratotic cells were noted (Fig. 1C-1D).

Immunohistochemical (IHC) staining results: TLR3 immunoreactivity was observed as intense and strong cytoplasmic positivity in all tumor cells of both well- and poorly differentiated BOSCC cases (Fig. 2A–2B). Similarly, TLR9 immunoexpression was detected as intense and strong intracytoplasmic positivity in all tumor cells of all well- and poorly differentiated BOSCC cases, in parallel with TLR3 expression (Fig. 2C–2D). However, although the IHC score for TLR9 was numerically higher in poorly differentiated cases, this increase was not statistically significant ($P>0.05$).

Bax expression was found to exhibit strong positive immunoreactivity with intracytoplasmic localization in all tumor cells in well-differentiated BOSCC cases, similar to TLR3 and TLR9. In contrast, in poorly differentiated BOSCC cases, Bax immunoreactivity was observed to be positive with an intensity varying between moderate and strong (Fig. 3A–3B). In well-differentiated BOSCC cases, Bcl-2 immunopositive staining was detected with intracytoplasmic localization in cells showing atypia located at the periphery of tumor nests and cords. In poorly differentiated BOSCC cases, similar to well-differentiated cases, Bcl-2 immunopositivity was determined to be concentrated in tumor cells located at the periphery of tumor foci and showing marked pleomorphism (Fig. 3C–3D). While the Bcl-2/Bax ratio was calculated as 0.26 in well-differentiated cases, this ratio was found to increase to 0.67 in poorly differentiated cases. MMP-9 immunoexpression was observed to be moderate and widespread in tumor cells in all well- and poorly differentiated BOSCC cases. In addition, positive reactions were noted in the tumor stroma and inflammatory cell infiltrations in the immunohisto-chemical staining (Fig. 3E–3F).

Statistical analysis results: The immunohistochemical staining results were statistically analyzed using the Mann–Whitney U test to evaluate the difference between the groups. Although a numerical increase was observed in TLR-3, TLR-9, Bax, and MMP-9 immunohisto-chemical staining between the well-differentiated and poorly differentiated groups, no statistically significant difference was detected ($P>0.05$). In contrast, the difference between the groups was statistically significant in Bcl-2 immunohistochemical staining ($P<0.01$) (Fig. 4).

The results of the TLR-3, TLR-9, Bax, Bcl-2, and MMP-9 immunohistochemical staining were analyzed using the Pearson correlation test. The test results showed no significant correlation between TLR3-TLR-9 ($P>0.05$/r: -0.28) and Bax-Bcl-2 ($P>0.05$/r:-0.33) immunohistochemical staining, while a positive correlation was observed between TLR-9-Bax ($P>0.05$/r: 0.24), and Bcl-2-MMP-9 ($P>0.05$/r: 0.41) (Fig. 5). (Blue tones indicate positive correlation, red tones indicate negative correlation, and darker colors indicate stronger correlation, while colors closer to white indicate weak correlation or no correlation).

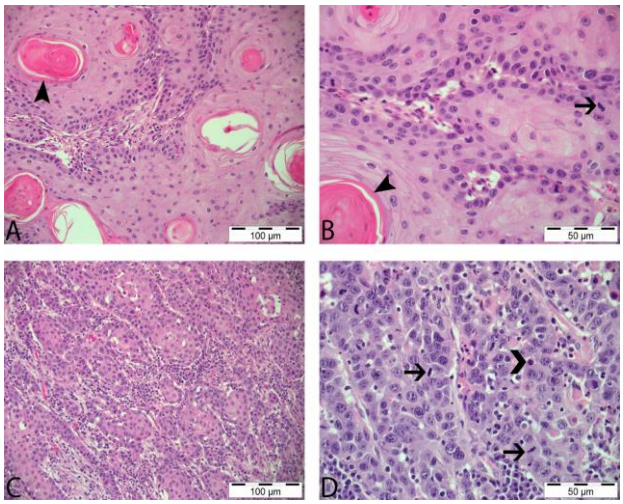

Fig. 1: Bovine ocular squamous cell carcinoma. A: Well-differentiated BOSCC; arrowhead: keratin pearl (H&E, 100µm). B: Well-differentiated BOSCC; arrowhead: keratin pearl and thin arrow: mitosis (H&E, 50µm). C: Poorly differentiated BOSCC (H&E, 100µm). D: Poorly differentiated BOSCC arrows: mitosis; band: dyskeratotic cell (H&E, 50µm).

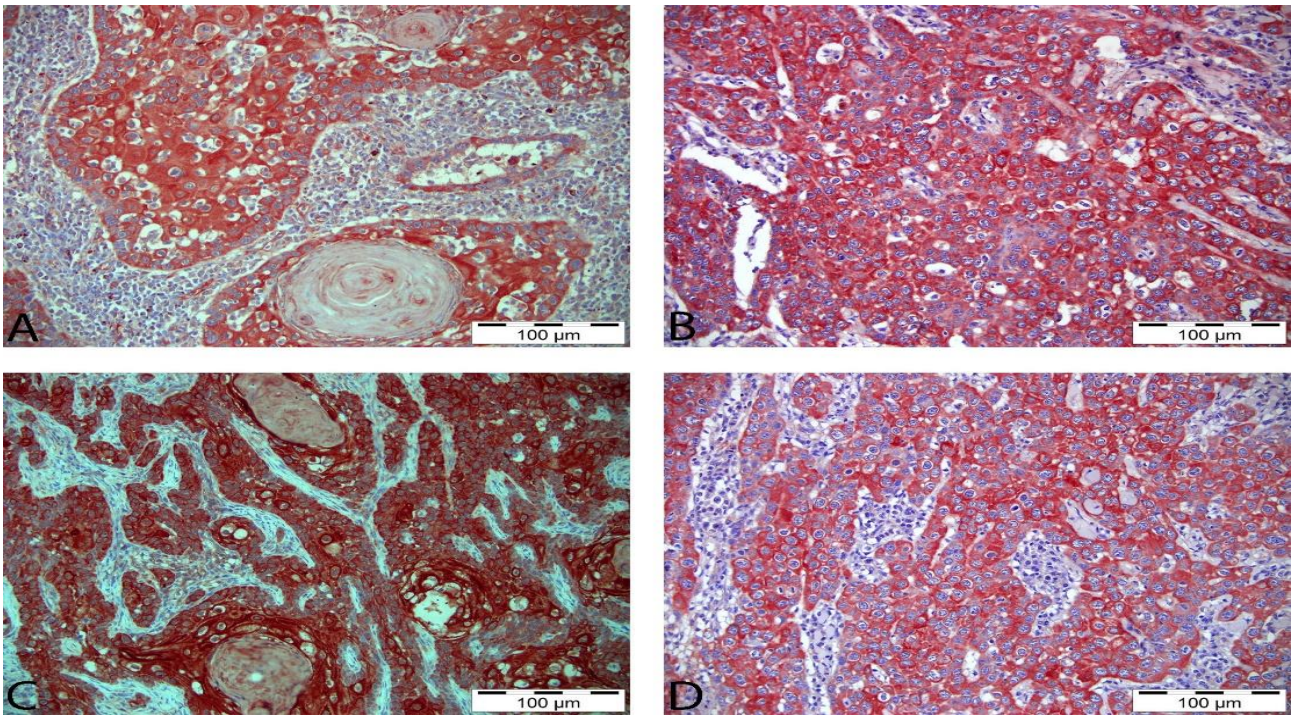

Fig. 2: Bovine ocular squamous cell carcinoma. A: TLR-3 immunohistochemical staining, well-differentiated BOSCC (IHC, 100µm), B: TLR-3 immunohistochemical staining, poorly differentiated BOSCC (IHC, 100µm), C: TLR-9 immunohistochemical staining, well-differentiated BOSCC (IHC, 100µm), D: TLR-9 immunohistochemical staining, poorly differentiated BOSCC (IHC, 100µm). In all figures red areas: indicate positive immunoreactive regions.

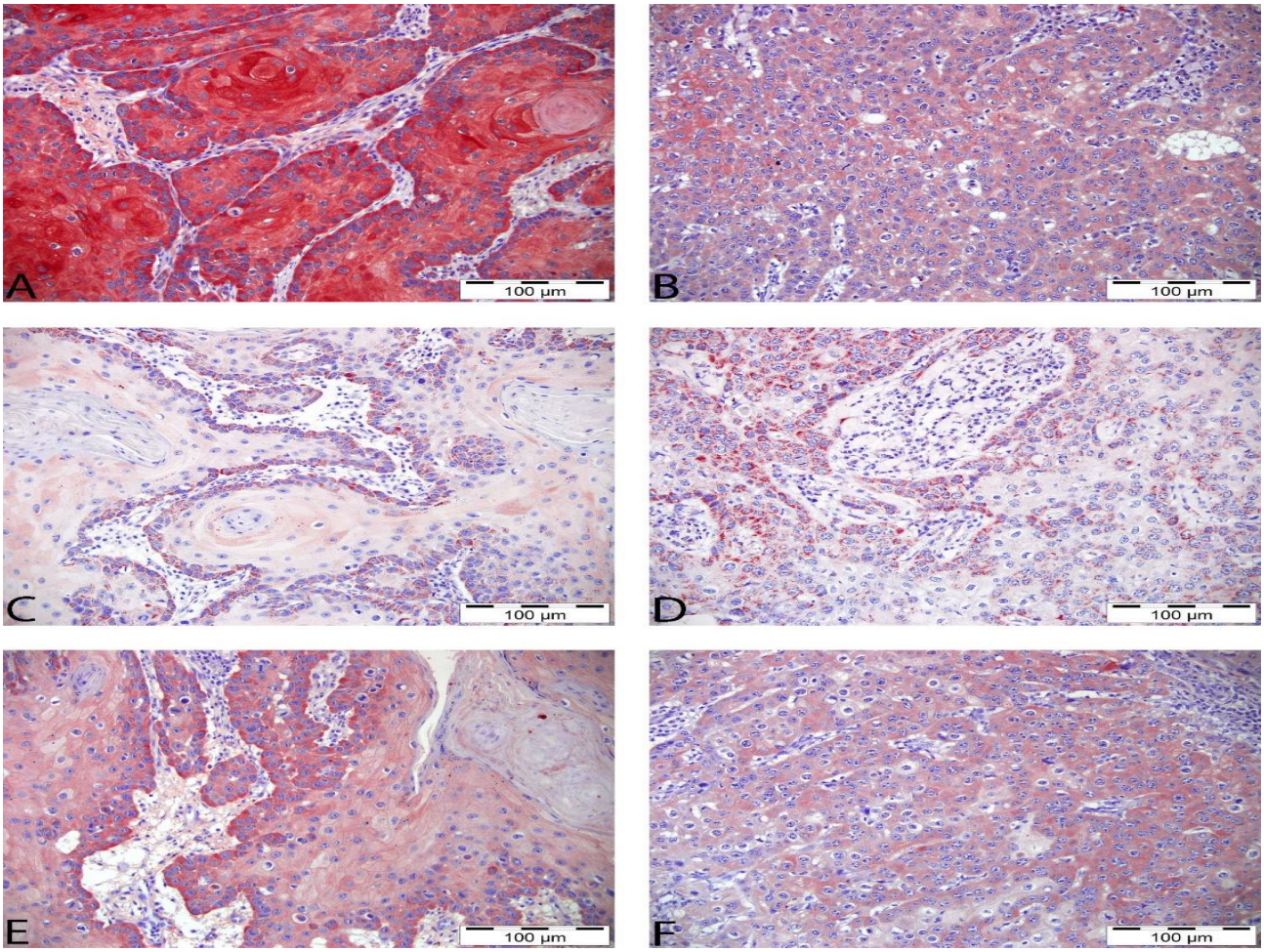

Fig. 3: Bovine ocular squamous cell carcinoma; A: Bax immunohistochemical staining, well-differentiated BOSCC; B: Bax immunohistochemical staining, poorly differentiated BOSCC, C: Bcl-2 immunohistochemical staining, well-differentiated BOSCC, D: Bcl-2 immunohistochemical staining, poorly differentiated, E: MMP-9 immunohistochemical staining, well-differentiated BOSCC; F: MMP-9 immunohistochemical staining, poorly differentiated BOSCC. In all figures red areas indicate positive immunoreaction regions.

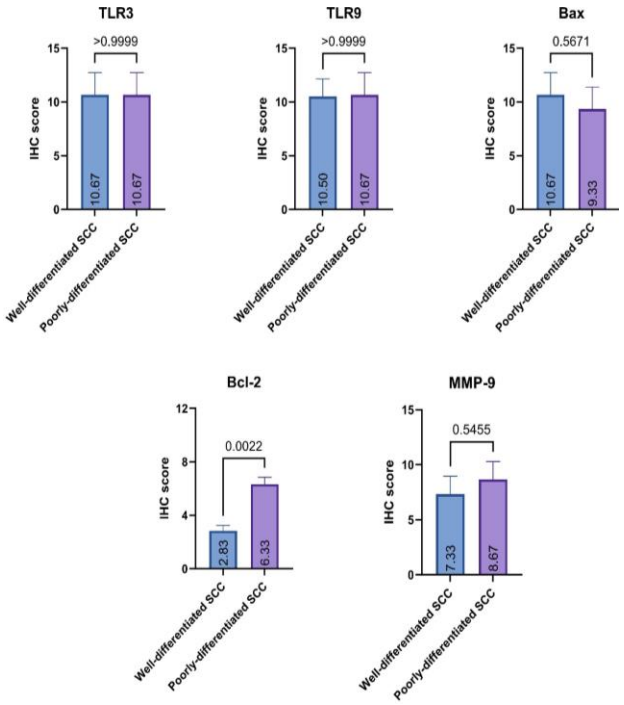

Fig. 4: Statistical evaluation of the immunohistochemical reactivities of TLR3, TLR9, Bax, Bcl-2, and MMP-9.

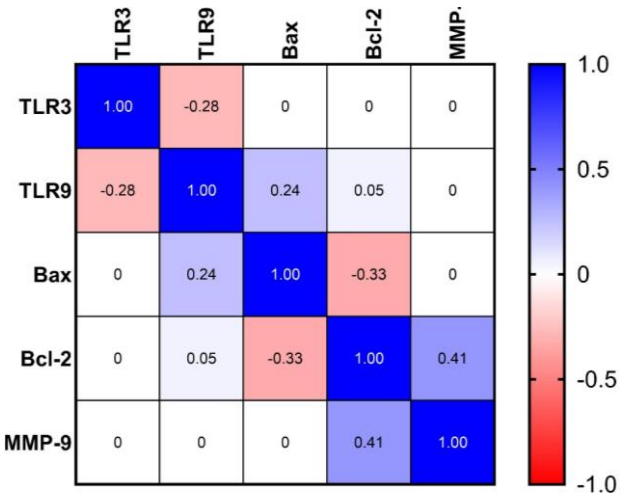

Fig. 5: Pearson correlation analysis of TLR3, TLR9, Bax, Bcl-2, and MMP-9.

The immunohistochemical and correlation findings obtained in this study revealed that Toll-like receptors (TLR3 and TLR9) are highly expressed in BOSCC regardless of tumor differentiation grade and their expression does not show a direct strong correlation with apoptotic and invasive markers. In contrast, the significant

increase in Bcl-2 expression and relative decrease in Bax expression in poorly differentiated BOSCC cases indicate a shift in the apoptotic balance toward the antiapoptotic direction.

Immunofluorescence findings: In TLR3-Bax dual immunofluorescence staining performed on well-differentiated and poorly differentiated BOSCC cases, positive reactions were observed in the cytoplasm of tumor cells. TLR3 and Bax expression were found to be similarly localized and expressed in all tumor cells (Fig. 6).

In well-differentiated BOSCC cases, dual immunofluorescence staining for TLR3-Bcl-2 showed that TLR3 expression was present in all cells of the tumor islands and cords, whereas Bcl-2 immunoreactivity was particularly observed in undifferentiated malignant cells at the periphery of the tumor islands and cords, with only a few Bcl-2 positive cells in the centers of the islands. In poorly differentiated BOSCC cases, Bcl-2 expression was intense in atypical tumor cells that did not exhibit squamous differentiation. Similar to well-differentiated cases, TLR3 expression was observed in all tumor cells of poorly differentiated BOSCC (Fig. 7).

According to the results of TLR9-Bax dual immunofluorescence staining in well-differentiated and poorly differentiated BOSCC cases, both were expressed in all tumor cells in both well-differentiated and poorly differentiated BOSCC tumors, and no significant difference was found between them (Fig. 8).

In well-differentiated BOSCC cases, TLR9 was found to be expressed at approximately the same level in all tumor cells, whereas Bcl-2 expression was high in cells located at the periphery of the tumor island and very low in a small number of cells at the center, according to the results of TLR9-Bcl-2 dual immunofluorescence staining. In poorly differentiated BOSCC cases, TLR9 expression was more intense than Bcl-2 expression. Bcl-2 expression was localized in undifferentiated cells at the periphery of the tumor foci, while expression was very low or absent in areas with increased differentiation (Fig. 9).

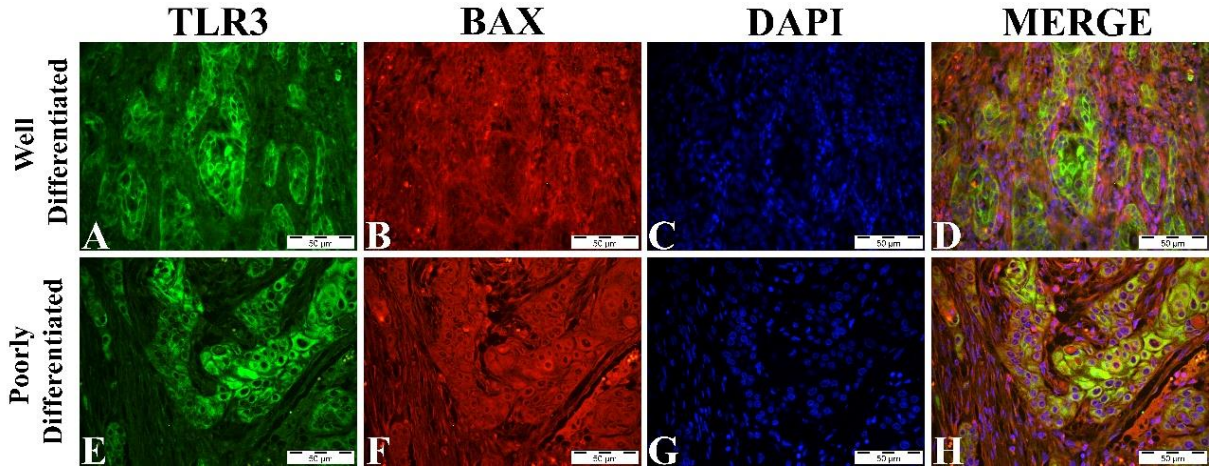

Fig. 6: BOSCC, TLR3-BAX immunofluorescence staining, A: TLR3 immunofluorescence staining, well-differentiated BOSCC (IF, 50µm), B: BAX immunofluorescence staining, well-differentiated BOSCC (IF, 50µm), C: DAPI immunofluorescence staining, well-differentiated BOSCC (IF, 50µm), D: TLR3, BAX and DAPI merging immunofluorescence staining, well-differentiated BOSCC (IF, 50µm), E: TLR3 immunofluorescence staining, Poorly-differentiated BOSCC (IF, 50µm), F: BAX immunofluorescence staining, Poorly-differentiated BOSCC (IF, 50µm), G: DAPI immunofluorescence staining, Poorly-differentiated BOSCC (IF, 50µm), H: TLR3, BAX and DAPI merging immunofluorescence staining, Poorly-differentiated BOSCC (IF, 50µm).

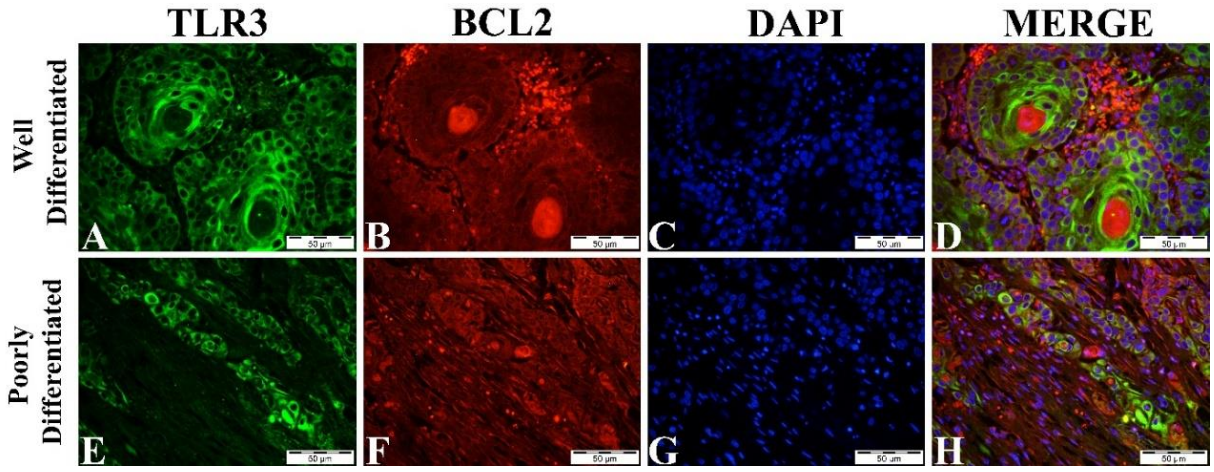

Fig. 7: BOSCC, TLR3-BCL2 immunofluorescence staining, A: TLR3 immunofluorescence staining, well-differentiated BOSCC (IF, 50µm), B: BCL2 immunofluorescence staining, well-differentiated BOSCC (IF, 50µm), C: DAPI immunofluorescence staining, well-differentiated BOSCC (IF, 50µm), D: TLR3, BCL2 and DAPI merging immunofluorescence staining, well-differentiated BOSCC (IF, 50µm), E: TLR3 immunofluorescence staining, Poorly-differentiated BOSCC (IF, 50µm), F: BCL2 immunofluorescence staining, Poorly-differentiated BOSCC (IF, 50µm), G: DAPI immunofluorescence staining, Poorly-differentiated BOSCC (IF, 50µm), H: TLR3, BCL2 and DAPI merging immunofluorescence staining, Poorly-differentiated BOSCC (IF, 50µm).

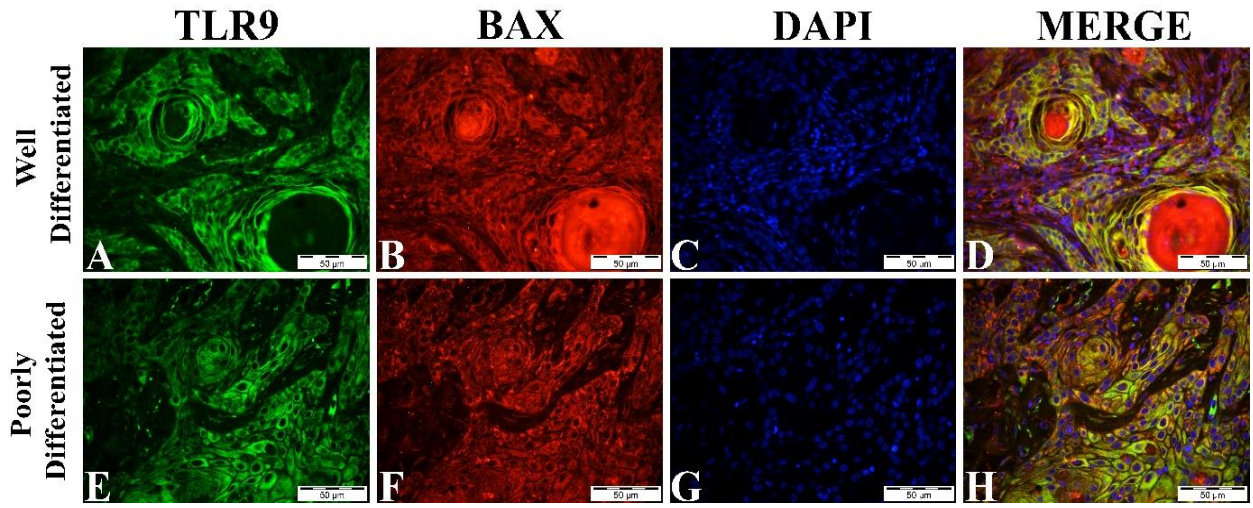

Fig. 8: BOSCC, TLR9-BAX immunofluorescence staining, A: TLR9 immunofluorescence staining, well-differentiated BOSCC (IF, 50µm), B: BAX immunofluorescence staining, well-differentiated BOSCC (IF, 50µm), C: DAPI immunofluorescence staining, well-differentiated BOSCC (IF, 50µm), D: TLR9, BAX and DAPI merging immunofluorescence staining, well-differentiated BOSCC (IF, 50µm), E: TLR9 immunofluorescence staining, Poorly-differentiated BOSCC (IF, 50µm), F: BAX immunofluorescence staining, Poorly-differentiated BOSCC (IF, 50µm), G: DAPI immunofluorescence staining, Poorly-differentiated BOSCC (IF, 50µm), H: TLR9, BAX and DAPI merging immunofluorescence staining, Poorly-differentiated BOSCC (IF, 50µm).

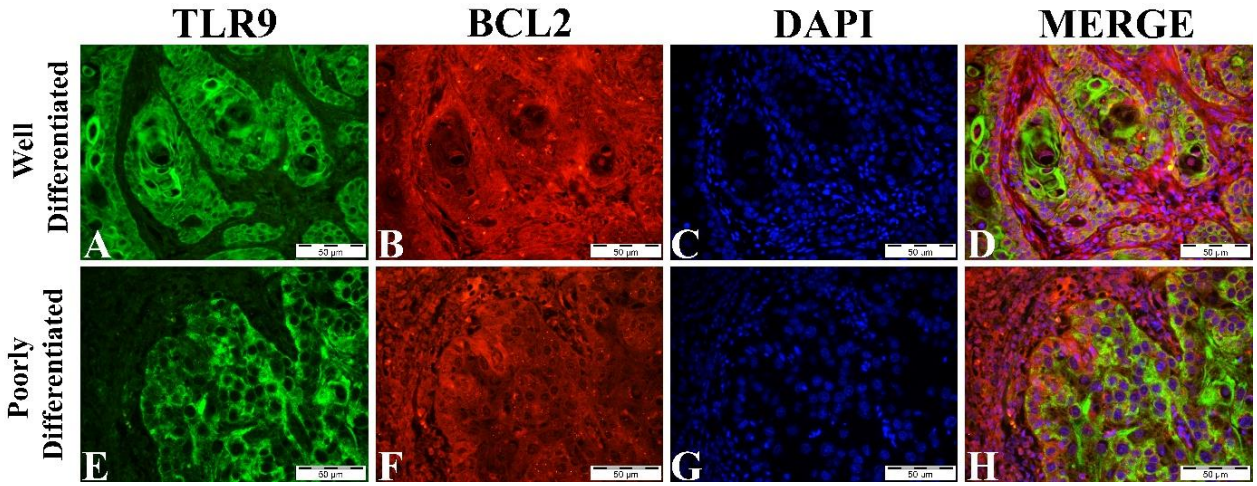

Fig. 9: BOSCC, TLR9-BCL2 immunofluorescence staining, A: TLR9 immunofluorescence staining, well-differentiated BOSCC (IF, 50µm), B: BCL2 immunofluorescence staining, well-differentiated BOSCC (IF, 50µm), C: DAPI immunofluorescence staining, well-differentiated BOSCC (IF, 50µm), D: TLR9, BCL2 and DAPI merging immunofluorescence staining, well-differentiated BOSCC (IF, 50µm), E: TLR9 immunofluorescence staining, Poorly-differentiated BOSCC (IF, 50µm), F: BCL2 immunofluorescence staining, Poorly-differentiated BOSCC (IF, 50µm), G: DAPI immunofluorescence staining, Poorly-differentiated BOSCC (IF, 50µm), H: TLR9, BCL2 and DAPI merging immunofluorescence staining, Poorly-differentiated BOSCC (IF, 50µm).

DISCUSSION

Bovine ocular squamous cell carcinoma (BOSCC) constitutes a common malignant ocular neoplasm of cattle that arises from squamous epithelial cells. The condition results in impaired vision, reduced productivity, and ultimately, notable economic detriment within affected herds (Dörtbudak and Yener, 2025). BOSCC cases are most frequently found in the conjunctiva, limbus, and third eyelid regions (Webb *et al.*, 2009). Ultraviolet light exposure, lack of periocular pigment, breed-linked susceptibility, advanced age, and persistent ocular irritation have all been implicated in the etiology of BOSCC (Winge *et al.*, 2023). Simmental and Holstein cattle breeds have been reported to be more susceptible to BOSCC development (Tsujita and Plummer, 2010). All 12 cases evaluated in this study were determined to be from the Simmental breed. In terms of tumor localization, left eye involvement was detected in 7 cases and right eye involvement in 5 cases. Furthermore, in 2 of the cases, the tumor formation was found to be located in the third eyelid. This distribution supports the idea that BOSCC tends to develop particularly in ocular regions exposed to UV radiation.

Histopathological examination of BOSCC revealed significant keratinization, keratin pearl formation, and the presence of intercellular bridges in well-differentiated cases. Additionally, the presence of tumor islands and cords, along with the observation of more atypical cells in the periphery, indicates a significant cellular heterogeneity even in well-differentiated tumors. In poorly differentiated BOSCC cases, a significant decrease in the number and size of keratin pearls was observed compared to the well-differentiated group, and in some cases, no keratin pearls were found at all. In this group, tumor cells showed a growth pattern in the form of islands and cords, and were characterized by significant pleomorphism and increased mitotic activity. The histomorphological features and

tumor grade assignments documented here align well with prior reports by Balasoiu *et al.* (2008), Campoli *et al.* (2014), Meier *et al.* (2016) and Hartmann *et al.* (2018).

Effective management of cancer depends largely on elucidating its pathogenesis. The limited number of studies on the pathogenesis of BOSCC makes elucidating the molecular mechanisms of this tumor even more important. In this study, the expression of different biomarkers was investigated to evaluate the apoptosis mechanism in order to contribute to understanding the pathogenesis of BOSCC.

Apoptosis is one of the organism's fundamental cell-autonomic tumor surveillance mechanisms against neoplastic development and plays a critical role in maintaining cellular homeostasis. Conversely, apoptosis evasion is considered one of the most distinctive features of tumor cells. Among the genes regulating apoptosis, the proapoptotic group (Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim, and Hrk) suppress tumor development by promoting cell death, while antiapoptotic genes (Bcl-2, Bcl-XL, Bcl-W, Bfl-1, and Mcl-1) contribute to tumor progression by increasing cell survival (Bose *et al.*, 2012).

Several studies in the literature have highlighted the importance of Bax and Bcl-2 expression in tumor biology. Madewell *et al.* (2001) immunohistochemically investigated Bax and bcl-2 expression in normal (healthy) cat skin and in 24 benign feline cutaneous basal cell tumors. They reported that in feline cutaneous basal cell tumors, compared to normal tissue, an increase in Bcl-2 and a decrease in Bax altered the apoptotic balance in favor of the tumor, and emphasized that this balance may have prognostic value (Madewell *et al.*, 2001). Wootipoom *et al.* (2004) investigated the expression of Bax, Bcl-2, and p53 in cervical squamous cell carcinoma treated with radiotherapy using immunohistochemical methods and reported that high Bax expression was associated with good prognosis, while high Bcl-2 expression was associated with poor prognosis, and that the evaluation of Bax and Bcl-2 expression could provide independent prognostic information for the clinical course of the disease. Zhang *et al.* (2009) reported that high Bcl-2 expression was associated with poor prognosis and metastasis in biopsy samples of human oral squamous cell carcinoma (OSCC), and that Bax expression was higher in well-differentiated tumors compared to poorly differentiated ones. Furthermore, decreased Bax expression has been reported to play a significant role in aggressive tumor development. The same study emphasized that the Bcl-2/Bax ratio reflects the activation level of the mitochondrial apoptosis pathway, and that an increase in this ratio supports cell survival, while a decrease increases apoptotic tendency. Immunohistochemical evaluations in this study revealed that Bax expression was located intra-cytoplasmically in tumor cells and showed moderate to severe positive immunoreactivity. Bcl-2 expression was determined to be localized intra-cytoplasmically, especially in cells located at the periphery of tumor islands and cords. It was observed that Bax expression was strong and widespread in well differentiated BOSCC cases, while it decreased to moderate to severe levels in poorly differentiated cases.

Statistical analysis revealed a significant increase in Bcl-2 expression in poorly differentiated BOSCC cases ($P<0.01$). Similarly, the Bcl-2/Bax ratio increased from 0.26 in well differentiated cases to 0.67 in poorly differentiated cases. These findings demonstrate that apoptotic suppression plays a critical role in BOSCC progression, and that anti-apoptotic mechanisms become dominant as the tumor progresses.

These results are consistent with findings reported by Madewell *et al.* (2001), Wootipoom *et al.* (2004), and Zhang *et al.* (2009), supporting the association between increased Bcl-2 and decreased Bax with tumor aggressiveness. Our study suggests that anti-apoptotic mechanisms are activated, particularly in poorly differentiated BOSCC cases, but this response is insufficient to completely suppress tumor progression. This indicates that disruption of apoptotic balance in BOSCC is one of the key determining factors in tumor biology.

TLRs are essential components of the innate immune response, and have been reported to have both tumor suppressive and tumor promoting effects on cancer development, progression, and treatment response in different tumor types (Salaun *et al.*, 2006; Sato *et al.*, 2009; Chuang *et al.*, 2018). Various studies have reported that Toll like receptor (TLR) activation in tumor cells affects processes associated with tumor progression, such as cell proliferation, escape from apoptosis, invasion, and metastasis. Conversely, different studies have shown that the activation of TLRs on immune cells or TLR ligands directly stimulates the antitumor immune response by inducing apoptosis in tumor cells (Sameer and Nisaar, 2021). These findings suggest that TLRs exert a bidirectional effect in cancer, and that balanced and targeted modulation of TLR signaling pathways could make these receptors candidate receptors for cancer immunotherapy. TLRs additionally participate meaningfully in tumor formation and apoptotic regulation. Published data show that, in various epithelial cell types, TLR engagement activates NF-κB signaling, leading to up-regulation of anti-apoptotic mediators such as Bcl-x, c-IAP-1, and c-IAP-2; this mechanism fosters carcinogenesis through dampening of the cellular apoptotic response.

A breast tumor cell-based in vitro study described how TLRs can engage FADD (Fas-associated death domain) and caspase-8 platforms either through MyD88-coupled cascades (used by TLR2, TLR7, TLR8, TLR9), the TRIF-dependent axis (employed by TLR3), or, in the case of TLR4, both routes; this interaction subsequently triggers apoptotic cell death (Kaiser and Offermann, 2005; Salaun *et al.*, 2006; Salaun *et al.*, 2007). Bonnin *et al.* (2019) reported that Toll-like receptor 3 (TLR3) exhibited a proapoptotic effect in both human hepatocellular carcinoma cases and experimental hepatocellular carcinoma models. In contrast, Muresan *et al.* (2022) demonstrated that TLR3 exhibited both proapoptotic and antiapoptotic properties in both experimental prostate tumor models in mice and human prostate biopsy samples. In contrast, investigations performed on human head and neck squamous cell carcinoma (HNSCC) tissues and corresponding laboratory models have revealed an antiapoptotic function for TLR3 (Nomi *et al.,* 2010; Chuang *et al.*, 2018). Sheyhidin *et al.* (2011) demonstrated that elevated TLR3 expression in

human oesophageal squamous cell carcinoma (ESCC) biopsies correlate with more aggressive tumor behavior. However, some studies have reported that TLR3 activation increases the apoptotic response and reduces cell migration in tumor cells. *In vivo* and in vitro studies conducted on oral squamous cell carcinoma models have shown that TLR3 agonists induce apoptotic effects and suppress tumor growth (Luo *et al.*, 2012; He *et al.*, 2014).

Employing immunohistochemistry, the current work assessed how TLR3 expression relates to proapoptotic (Bax) and antiapoptotic (Bcl-2) indicators across well- and poorly differentiated BOSCC samples. In well-differentiated cases, TLR3 and Bax expression showed intracytoplasmic positivity in all malignant cells, while Bcl-2 expression was detected more in cells located at the periphery of tumor islands that did not show squamous differentiation. In poorly differentiated cases, significant Bcl-2 expression was observed in peripherally located undifferentiated cells, while TLR3 and Bax expression was found to persist widely in all malignant cells. These results suggest that TLR3 may be associated with proapoptotic pathways in BOSCC and that, when evaluated together with Bax expression, it activates the apoptotic response. The findings are consistent with the studies of Luo *et al.* (2012) and He *et al.* (2014), who reported that TLR3 agonists induce apoptotic effects and can suppress tumor progression, suggesting that TLR3 may play an important role in tumor biology and apoptosis regulation in BOSCC.

Ma *et al.* (2024) found that TLR9 promotes tumor progression through antiapoptotic pathways, as evidenced in human oral squamous cell carcinoma (OSCC) biopsies as well as in both cellular and animal OSCC models. Similarly, Lee *et al.* (2007) documented markedly higher TLR9 levels in human cervical intraepithelial neoplasia (CIN) and invasive squamous cell carcinoma (ISCC) relative to normal cervical squamous epithelium. In line with these findings, it has been suggested that TLR9 may be an important marker associated with tumor progression in cervical neoplasia and may be used as a potential biomarker in the evaluation of malignant transformation.

In our study, TLR9 expression was found to exhibit intense and strong positive staining in the intracytoplasmic region of all tumor cells in both well-differentiated and poorly differentiated BOSCC cases. The similar immunoreactivity pattern of TLR9 expression to Bax and TLR3, and their co-expression in the same cells, suggests that TLR9 may exhibit not only anti-apoptotic effects but also different biological effects depending on the context in BOSCC.

Immunofluorescence analyses supported the immunohistochemical findings and contributed to obtaining important morphological information. The homogeneous distribution of TLR3 and TLR9 in all tumor cells confirms that these receptors are active throughout the entire tumor mass. In contrast, the concentration of Bcl-2, particularly in peripheral and undifferentiated cells, indicates that anti-apoptotic mechanisms are more active in regions that can be described as the "invasive front" of the tumor.

However, it has been observed that similar expression patterns of TLR3 and TLR9 in well-differentiated and poorly differentiated BOSCC cases alone are insufficient as prognostic markers reflecting tumor differentiation. Therefore, it is concluded that when TLR3 and TLR9 are used in conjunction with histopathological evaluation and apoptotic markers, they can contribute to a more accurate understanding of tumor biology and support diagnostic processes.

Metastasis initiation requires tumor cells to detach from the primary mass and gain invasive competence. This step is made possible through extracellular matrix breakdown, executed chiefly by MMP-9—a central matrix metalloproteinase (MMP) family member—alongside additional proteolytic enzymes. MMP-9 not only degrades the extracellular matrix but also facilitates the intravasation of tumor cells into blood and lymphatic vessels, contributing to the development of distant organ metastasis (Chakraborty *et al.*, 2023). Furthermore, MMP-9 has been reported to be associated with tumor progression and poor prognosis in several malignancies, including gastric, breast, colorectal, and non-small cell lung tumors (Zeng *et al.*, 2013). In cattle, BOSCC tumors have been reported to be generally slow-growing, locally invasive, and rarely metastasize (Feng *et al.*, 2024). Tsukamoto *et al.* (2023) reported that high MMP-9 expression was associated with poor prognosis in esophageal squamous cell carcinoma (ESCC) cases in cell culture studies.

In our study, it was determined that MMP-9 expression was at a moderate level in terms of intensity and severity in both well-differentiated and poorly differentiated BOSCC cases, and that all tumor cells showed intracytoplasmic immunoreactivity. Although a slight increase in MMP-9 expression was observed in poorly differentiated BOSCC cases compared to the well-differentiated group, this difference was not statistically significant ($P>0.05$). This suggests that, compared with the findings of Tsukamoto *et al.* (2023), who associated high MMP-9 expression with metastatic potential and poor prognosis, MMP-9 expression in BOSCC may indicate a biological behavior more limited to local invasion.

Therefore, the moderate MMP-9 expression observed in bovine BOSCC cases appears consistent with the tumor's low metastatic potential and its biological character characterized by more local invasion.

Conclusions: In this study, the presence and distribution of TLR3 and TLR9 expression, as well as their relationship with pro- and anti-apoptotic markers, were investigated using immunostaining methods in BOSCC cases encountered in veterinary oncology. We believe that TLR3 and TLR9 play a role in tumor biology, but are potential candidates as immunotherapeutic targets rather than independent markers. The increased Bcl-2/Bax ratio in poorly differentiated BOSCC cases indicated that these tumors are resistant to apoptosis. This situation may explain the resistance of tumors to conventional treatments and suggests that agents targeting apoptosis, such as Bcl-2 inhibitors, may theoretically be beneficial when used in combination with immunotherapy. Our study is one of the first to investigate TLR expression in BOSCC cases and the relationship between this expression and pro- and anti-apoptotic markers using immunostaining methods. It is important in terms of

laying the groundwork for future studies on TLRs in veterinary oncology and contributing to the literature.

Funding: This study did not receive any financial support from any institution or organization.

Conflicts of Interest: The authors declare that there are no conflicts of interest regarding this study.

Authors Contribution: Hilmi NUHOĞLU, Ayfer Yıldız UYSAL, Mizgin KENGİŞ: Conducted the laboratory analyses. Hilmi NUHOĞLU and Serpil DAĞ: Prepared the original draft of the manuscript. Hilmi NUHOĞLU, Ayfer Yıldız UYSAL, and Serpil DAĞ: Reviewed and edited the manuscript. Hilmi NUHOĞLU and Serpil DAĞ: Supervision, consultancy, and overall oversight of the study.

REFERENCES

Alexopoulou L and Irla M, 2025. Toll-like receptors (TLRs) in the trained immunity era. eLife 14: e106443.

Bose P, Klimowicz AC, Kornaga E, *et al.,* 2012. Bax expression measured by AQU Analysis is an independent prognostic marker in oral squamous cell carcinoma. BMC Cancer 12: 332.

Campoli M, Brodland DG and Zitelli J, 2014. A prospective evaluation of the clinical, histologic, and therapeutic variables associated with incidental perineural invasion in cutaneous squamous cell carcinoma. Journal of the American Academy of Dermatology 70(4):630–636.

Chakraborty S, Suresh TNR and Mohiyuddin AS, 2023. Role of Matrix Metalloproteinase 9 in Predicting Lymph Node Metastases in Oral Squamous Cell Carcinoma. Cureus 15(1): e33495.

Dörtbudak MB and Yener K, 2025. Investigation of DNA damage and apoptosis in bovine ocular squamous cell carcinoma. Veterinary Medicine and Science 11(4):e70448.

Feng J, Lardé H, King A, *et al*., 2024. Ocular and perineal squamous cell carcinomas in a Holstein Friesian cow. Open Veterinary Journal 14(11):3113–3119.

Hartmann D, Krammer S, Bachmann MR, *et al.,* 2018. Ex vivo confocal microscopy features of cutaneous squamous cell carcinoma. Journal of Biophotonics 11(4):e201700318.

Hasimu A, Ge L, Li QZ, *et al.,* 2011. Expressions of Toll-like receptors 3, 4, 7, and 9 in cervical lesions and their correlation with HPV16 infection in Uighur women. Chinese Journal of Cancer 30(5):344-50.

He Z, Huang X, Ni Y, *et al.,* 2014. Functional toll-like receptor 3 expressed by oral squamous cell carcinoma induced cell apoptosis and decreased migration. Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology 118(1):92–100. i e33495.

Kawai T, Ikegawa M, Ori D, *et al.,* 2024. Decoding Toll-like receptors: Recent insights and perspectives in innate immunity. Immunity 57(4):649–673.

Lee JW, Choi JJ, Seo ES, *et al.,* 2007. Increased toll-like receptor 9 expression in cervical neoplasia. Molecular Carcinogenesis: Published in cooperation with the University of Texas MD Anderson Cancer Center 46(11):941–947.

Li J and Yuan J, 2008. Caspases in apoptosis and beyond. Oncogene 27(48):6194–6206.

Luo Q, Hu S, Yan M, *et al.,* 2012. Activation of Toll-like receptor 3 induces apoptosis of oral squamous carcinoma cells in vitro and in vivo. The International Journal of Biochemistry & Cell Biology 44(8):1266–1275.

Ma L, Qin N, Wan W, *et al.,* 2024. TLR9 activation induces immunosuppression and tumorigenesis via PARP1/PD-L1 signaling pathway in oral squamous cell carcinoma. American Journal of Physiology-Cell Physiology 326(2):C362–C381.

Madewell BR, Gandour-Edwards R, Edwards BF, *et al.,* 2001. Bax/bcl-2: cellular modulator of apoptosis in feline skin and basal cell tumours. Journal of Comparative Pathology 124(2-3):115-121.

Meier K, Drexler SK, Eberle FC, *et al.,* 2016. Silencing of ASC in cutaneous squamous cell carcinoma. PLoS One 11(10):e0164742.

Morana O, Wood W and Gregory CD, 2022. The Apoptosis Paradox in Cancer. International Journal of Molecular Sciences 23(3):1328.

Nomi N, Kodama S and Suzuki M, 2010. Toll-like receptor 3 signaling induces apoptosis in human head and neck cancer via survivin associated pathway. Oncology Reports 24(1): 225–231.

Reunanen N and Kähäri VM, 2013. Matrix Metalloproteinases in Cancer Cell Invasion. In: Madame Curie Bioscience Database [Internet]. Austin (TX): Landes Bioscience; 2000-2013.

Sakaniwa K, Fujimura A, Shibat, T, *et al.,* 2023. TLR3 forms a laterally aligned multimeric complex along double-stranded RNA for efficient signal transduction. Nature Communications 14(1):164.

Salaun, B, Romero P and Lebecque S, 2007. Toll-like receptors' two-edged sword: when immunity meets apoptosis. European Journal of Immunology 37(12):3311–3318.

Sameer AS and Nissar S, 2021. Toll-Like Receptors (TLRs): Structure, functions, signaling, and role of their polymorphisms in colorectal cancer susceptibility. BioMed Research International 2021:1157023.

Sato Y, Goto Y, Narita N, *et al.,* 2009. Cancer cells expressing Toll-like receptors and the tumor microenvironment. Cancer Microenvironment 2 Suppl 1(Suppl 1):205–214.

Sharma S, Gupta RP, Jangir BL, *et al.,* 2020. Pathomorphological studies and immunohistochemical expression of p53 and Pan-Cytokeratin in bovine epithelial tumors. Indian Journal of Veterinary Pathology 44(1):1–6.

Sheyhidin I, Nabi G, Hasim A, *et al.,* 2011. Overexpression of TLR3, TLR4, TLR7 and TLR9 in esophageal squamous cell carcinoma. World Journal of Gastroenterology 17(32): 3745–3751.

Song CH, Ku SK, Jang HS, *et al.,* 2012. Eyelid Squamous Cell Carcinoma in a Dog. Pakistan Veterinary Journal 32(3):474–476.

Tsujita H, Plummer CE, 2010. Bovine ocular squamous cell carcinoma. Veterinary Clinics: Food Animal Practice 26(3):511-29.

Tsukamoto S, Koma YI, Kitamura Y, *et al.,* 2023. Matrix Metalloproteinase 9 induced in esophageal squamous cell carcinoma cells via close contact with tumor-associated macrophages contributes to cancer progression and poor prognosis. Cancers 15(11):2987.

Verma RP and Hansch C, 2007. Matrix metalloproteinases (MMPs): chemical-biological functions and (Q)SARs. Bioorganic & Medicinal Chemistry 15(6):2223–2268.

Webb JL, Burns RE, Brown HM, *et al.,* 2009. Squamous cell carcinoma. Compendium (Yardley, PA), 31(3):E9.

Winge MCG, Kellman LN, Guo K, *et al.,* 2023. Advances in cutaneous squamous cell carcinoma. Nature Reviews Cancer 23(7):430–449.

Wootipoom V, Lekhyananda N, Phungrassami T, *et al.,* 2004. Prognostic significance of Bax, Bcl-2, and p53 expressions in cervical squamous cell carcinoma treated by radiotherapy. Gynecologic Oncology 94(3):636-642.

Wong RS, 2011. Apoptosis in cancer: from pathogenesis to treatment. Journal of Experimental & Clinical Cancer Research 30(1):87.

Xu L, Wang C, Wen Z, *et al.,* 2010. Selective up-regulation of CDK2 is critical for TLR9 signaling stimulated proliferation of human lung cancer cell. Immunology Letters 127(2):93–99.

Zeng R, Duan L, Kong Y, *et al.,* 2013. Clinicopathological and prognostic role of MMP-9 in esophageal squamous cell carcinoma: a meta-analysis. Chinese Journal of Cancer Research 25(6):637–645.

Zhang M, Zhang P, Zhang C, *et al.,* 2009. Prognostic significance of Bcl-2 and Bax protein expression in the patients with oral squamous cell carcinoma. Journal of Oral Pathology & Medicine 38(3):307-313.