Original Article

An investigation of bovine papillomaviruses from ocular squamous cell carcinomas in cattle

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Abstract

Background: Ocular squamous cell carcinomas (OSCCs) in cattle has been studied for many years, but no definite etiology has been established. Squamous cell carcinomas (SCCs) may occur in different body parts of cattle. Depending on the location, it can cause an economic loss of varying degrees. **Aims:** The aim of this study was to investigate the causes of OSCCs in the eye region of cattle. **Methods:** Sixty tumoral masses taken form 60 cattle with proliferation in the eye region that were collected between the years 2012-2022 were used. These cases were admitted to our department for routine diagnosis. The tissues were diagnosed as OSCC using histopathological methods. The presence of bovine papillomavirus (BPV), one of the causative factors, was investigated using immunohistochemical and polymerase chain reaction (PCR). **Results:** Macroscopically masses were nodular or cauliflower-like and fragile and had hemorrhagic surfaces. Considering the keratin pearls, tumoral islands, and squamous differentiation, 20 out of 60 cases were classified as well, 20 as moderately, and 20 as poorly-differentiated OSCCs. 47 of the 60 cases were BPV positive using immunohistochemical methods. However, BPV nucleic acid was detected in only two cases with PCR. Only one of the cases could be sequenced. After phylogenetic analysis, virus strain was identified as BPV-1. **Conclusion:** Our results indicated that papillomaviruses can contribute to the development of OSCCs, in both precursor lesions and also advanced stage OSCCs. We found that BPV-1 has a possible causative role; however, more studies are needed to investigate the role of other viral agents and their interaction with secondary factors.

Key words: Bovine papillomavirus, Immunohistochemistry, Ocular squamous cell carcinoma, PCR

Introduction

Ocular squamous cell carcinomas (OSCCs), known as cancer eye, happen worldwide with high incidence (Podarala *et al*., 2020; Vala *et al*., 2020; Karakurt *et al*., 2021). OSCCs have malign characteristics with an epithelial origin. Lesions can be located in different ocular and periocular areas like the corneal area, sclera, eyelids, palpebra tertia, limbus, the epithelial surface of the cornea, and conjunctiva (Fornazari *et al*., 2017; Sözmen *et al*., 2019). In addition, OSCCs are most common in non-pigmented areas since the photo-protective effect of melanin is not present (Carvalho *et al*., 2005). Because of this reason, OSCCs are mostly seen in Hereford and Holstein or hybrids of these breeds that have non pigmented eye areas (Vala *et al*., 2020). OSCCs have four development stages, plaque (stage 1), keratoma or keratoacanthoma (stage 2), papillomas (stage 3), and eventually carcinomas (stage 4). Plaques, keratomas, and papillomas are benign precursor lesions that can transform into carcinomas. OSSCs can metastasize to lymph nodes or lungs when not treated (Tsujita and Plummer, 2010).

OSCCs cause production losses and cost of treatments and may lead to early culling and carcass defects, all of which have a significant economic burden (Gharagozlou *et al*., 2007; Pugliese *et al*., 2014).

The etiology of the disease consists of environmental factors (latitude, high altitude, and exposure to sunlight), hereditary factors (breed and lack of pigmentation in eyelids), and viral infection (papillomaviruses and herpesviruses 1-5) (Sözmen *et al*., 2019; Podarala *et al*., 2020). Herpesviruses and papillomaviruses have a debatable role in the development of precursor lesions and progression of these lesions to SCCs (Tsujita and Plummer, 2010).

SCCs have been studied extensively in different species like cats (Yamashita-Kawanishi *et al*., 2018; Altamura *et al*., 2020; Chu *et al*., 2020; Yamashita-Kawanishi *et al*., 2021), horses (Hibi *et al*., 2019; Ramsauer *et al*., 2019; Alloway *et al*., 2020; Greenwood *et al*., 2020; Armando *et al*., 2021), and dogs (Munday *et al*., 2015; Luff *et al*., 2016; Munday *et al*., 2016; Sabattini *et al*., 2016; Chang *et al*., 2020). There are some studies investigating SCCs in cattle; however, there are not many studies that investigate SCCs in the ocular region. In fact, only a few OSCC studies on the etiology was found in the literature (Ford *et al*., 1982; Rutten *et al*., 1992; Fornazari *et al*., 2017).

This study aimed to investigate the causes of OSCCs from the eye region of cattle.

Materials and Methods

Tissue samples

The material of the study consists of 60 OSCC cases collected between the years 2012-2022. All of the tumoral lesions were excised from the orbital/periorbital area. For negative control, ten tissues obtained from healthy cattle were used. Paraffin-embedded tissues were used for older cases (older than the last three years) in molecular studies; fresh tissue samples were used for newer cases when available. The study was approved by the local Ethics Committee of Kafkas University with the decision number (KAU-HADYEK-2021-08).

Histopathology

Excised masses were fixed in 10% buffered formaldehyde solution (Merck). Tissues were embedded in paraffin after following routine procedures. Sections of 5 µm for Hematoxylin and Eosin (H&E) and sections of 4 µm for immunohistochemical staining were taken.

H&E staining was used for observing histo-pathological changes. Slides were examined thoroughly under a compound light microscope (Olympus Bx53) by at least two pathologists. Photographs were taken using Cell ^P software (Olympus Soft Imaging Solutions GmbH, 3,4). Detailed analyses of the taken photographs were made with Image J software (1.51j8).

Differentiation criteria for grading of tumors were based on Carvalho *et al*. (2005) and Sözmen *et al*. (2019). Tumors were graded in three categories: well, moderate, and poorly differentiated.

Immunohistochemistry

Streptavidin-Biotin Peroxidase kit (Thermo Scientific Histostain IHC Kit, HRP, broad-spectrum, REF: TP-125-HL) was used based on the manufacturer's directions for staining. Primary antibody (BPV antibody, mouse monoclonal, MyBioSource, MBS320197, Dilution: 1/100, at 4°C, overnight protocol) was applied after antigen retrieval and prevention of nonspecific staining. Amino ethyl carbazole (AEC, Thermo Scientific, REF: TA-125-HA) was applied as chromogenic substrate and incubated for 15 min. Slides were rinsed for 5 min with distilled water and stained with Mayer Hematoxylin then covered with AEC mount. A different protocol of increasing the percentage of alcohol and xylol baths was used for slides for which 3,3'-Diaminobenzidine (DAB) was used as a chromogenic substrate, and mounting was done with Entellan (Merck).

The analysis of BPV immunopositivity was evaluated using a grading system based on positively stained cell count in the most intensively stained areas. Three different areas were examined for each tumor. Grading was determined as following: (-) no immunoreactivity, (+) low with 1-10% positivity in the cell of the area, (++) moderate with 11-59% positivity in the cell of the area, and (+++) intense with higher than 60% positivity in the cell of the area (Beytut, 2017).

Nucleic acid extraction

Fresh tissue samples were homogenized by adding PBS at a ratio of 1:10 and vortexing. homogenized tissues were centrifuged at 3000 g for 15 min and supernatants were collected after centrifugation. Nucleic acid extraction procedure defined by Sambrook *et al*. (2001) was used.

DNA extractions from paraffin-embedded blocks were carried out based on Pikor *et al*. (2011). All extracts were stored at -20°C for further processing.

Molecular analysis

Papillomavirus nucleic acid was investigated by PCR method. L1 ORF (open reading frame) was chosen because of its being conservative. For detection of BPV genome as described by FAP59/FAP64 (Forslund *et al*., 1999) and MY09/MY11 (Ogawa *et al*., 2004), primer pairs were used. The primer pairs produced 478 and 450 bp, respectively. PCR products were analyzed using 1% agarose gel containing ethidium bromide. The gels were visualized for specific-sized bands by UV transillu-mination.

Phylogenetic analysis

Amplicon having sufficient DNA yield and suitable for sequence analysis was sent to a commercial company (BM Yazilim Danis. ve Lab. Sis. Ltd. Sti, Ankara) for Sanger sequencing. Sequence assembly and editing were done with Bioedit (version 7.0.5.3) and Clustral W (Hall, 1999). Sequence similarities were compared with the GenBank nucleotide sequence database using the basic length alignment search tool (BLAST) software of the National Center for Biotechnology Information (NCBI) (Altschul *et al*., 1997). Phylogenetic analysis of gene sequences was performed using MEGA7 software (Tamura *et al*., 2011). Neighbor-joining (NJ) method of the software was selected and sequence differences were calculated with the Kimura two-parameter model. Confidence level was assessed by bootstrapping using 1000 replicates.

Statistical analysis

Kruskal-Wallis-H analysis was used for multiple comparison of differential grades of tumors. Mann-Whitney U test was used for binary comparison of

significant parameters. Bonferroni correction was applied to obtain the p-value and a significant adjustment was noted. Statistical analysis of the results was performed using SPSS (SPSS 26.0, Chicago, IL, USA) software. The results were given as ± standard error (SE). $P<0.05$ was considered statistically important.

Results

Macroscopical results

Thirty-five of the masses were originated from the left eye and 25 were originated from the right eye. Five of the tumor masses were located in the third eyelid, nine in the lower eyelid, six in the upper eyelid, eight in the lateral cantus, and 32 of the tumors were spread in all eye regions. Masses were different in size ranging from 0.2 cm to 10 cm. Tumors were seen as either solitary or multiple form. The masses were mostly pink in color, and tumors had nodular, papillomatous, or cauliflower-like proliferations. The consistency of the masses differed from fragile to firm. The surface of the lesions was hemorrhagic and ulcerative. In addition, some masses were covered in purulent exudate (Figs. 1A and B).

Histopathological and immunohistochemical results

20 well-differentiated OSCC cases had numerous and large-sized keratin pearls. Additionally, tumor islands were extensive and had distinct squamous differentiation. Moderately differentiated cases (n=20) had small or average-sized keratin pearls; squamous differentiation and a smaller number of undifferentiated cells were compared to well-differentiated cases. Poorly differentiated cases (n=20) had almost no keratinization; only a couple of dyskeratotic cells were observed. Tumor island size was smaller compared to moderately and well-differentiated cases. Pleomorphic areas were greater in number and also numerous mitotic figures were detected (Figs. 2A, B, D, E).

BPV immunoreactivity scores of all differentiation groups are shown in Table 1. The control group of apparent healthy tissues had no BPV staining. Well, moderately, and poorly differentiated OSCC groups had gradually increased the number of stained cells respectively when compared to the negative control group. This change in well, moderately, and poorly differentiated OSCC groups was also statistically

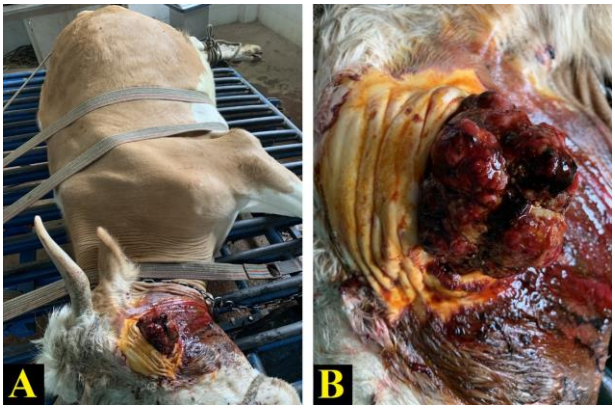

Fig. 1: Solitary tumoral mass covering entire left eye. The lesion has cauliflower-like appearance, firm consistency, 8-9 cm diameter with hemorrhagic surface. Photographed from different angles (**A-B**)

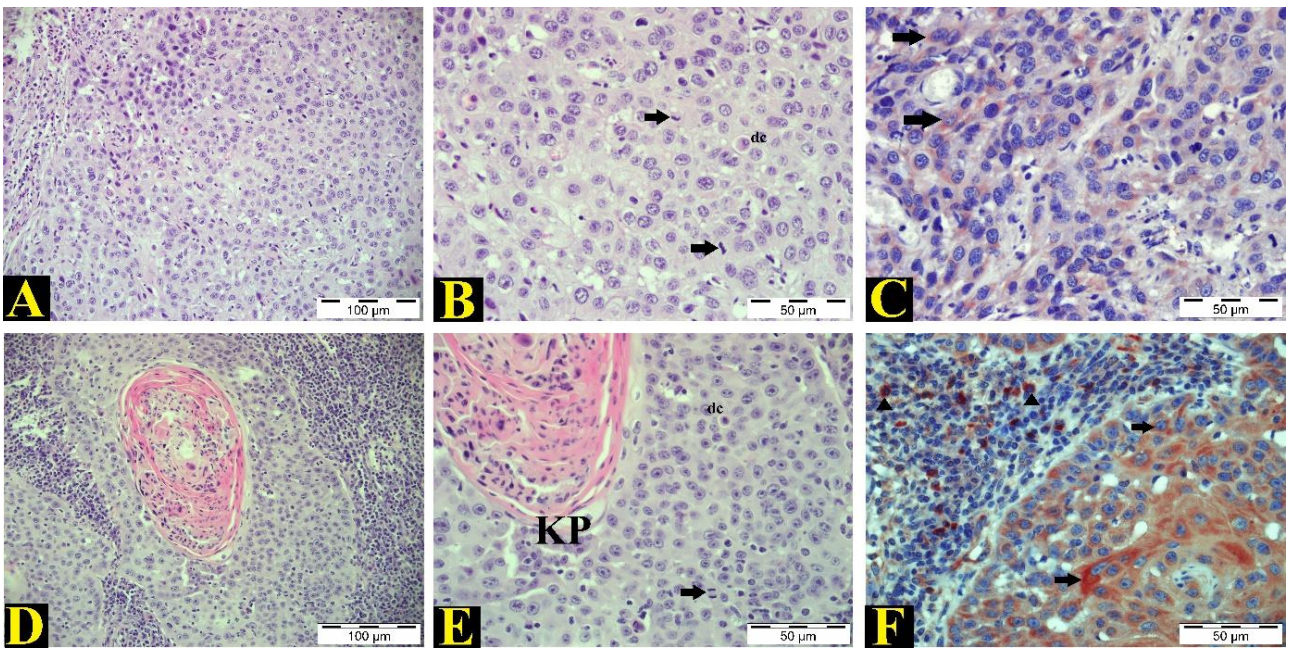

Fig. 2: Images of the cases which are tested positive with PCR for BPV. (**A-B**) Poorly differentiated OSCC, mitotic figures (arrows) and dyskeratotic cells (dc) (H&E staining), (**C**) Poorly differentiated OSCC, BPV positive reactions in the cytoplasm of tumoral cells (arrows) in pleomorphic areas (IHC method), (**D-E**) Well differentiated OSCC, keratin pearl (KP), dyskeratotic cell (dc), and mitotic figure (arrow) (H&E staining), and (**F**) Severe intracytoplasmic BPV immunoreactivity in the cytoplasm of cells in tumoral islands (arrows) and inflammatory cell infiltration (arrowheads) around these islands

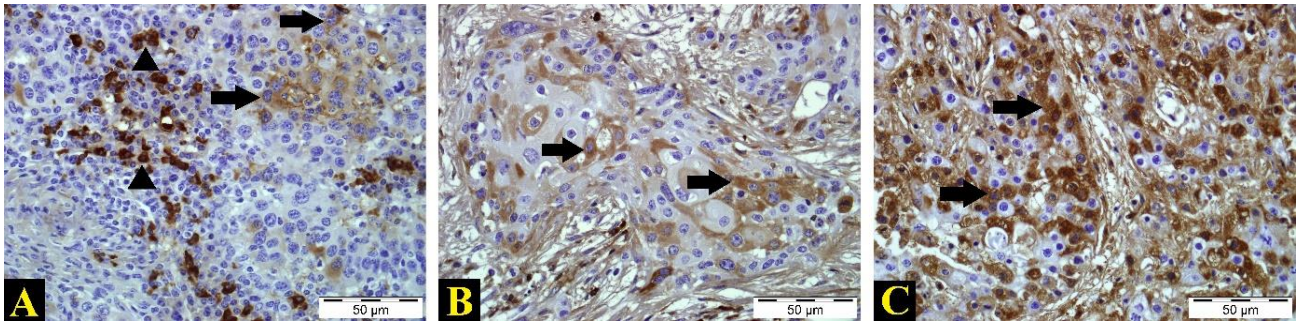

Fig. 3: Images of the cases which tested negative for BPV nucleic acid with PCR. BPV antibody is used for immunohistochemistry with chromogen substrate DAB (Hematoxylin staining). (**A**) Well-differentiated OSCC, BPV positive reactions in tumoral cells (arrows) and inflammatory cells (arrowheads), (**B**) Moderately differentiated OSCC, in the cytoplasm of neoplastic cells forming tumor islands (arrows), BPV immune-positive expressions, and (**C**) Poorly differentiated OSCC, severe intracytoplasmic (arrows) BPV, immunoreactivity in tumoral cells, especially in the periphery of tumoral islands

significant. The poorly differentiated OSCC group had the highest values of BPV immunopositivity among other groups. In well-differentiated OSCC group, BPV immunopositive reactions were localized in the cytoplasm of tumor cells in tumor islands. Intracytoplasmic BPV staining was also present in cells consisting of mostly mononuclear cells, in inflammation infiltration areas surrounding the tumor islands. In moderately differentiated cases, BPV immunopositivity was intense in less differentiated cells. The staining of inflammatory cells was weaker compared to that of well-differentiated cases. BPV positivity of poorly differentiated cases was localized in the cytoplasm of cells with evident pleomorphism. Staining of inflammatory cells was also nonexistent in this group (Figs. 2C, F and Figs. 3A-C).

Table 1: BPV immunoreactivity scores of the differentiated groups

Groups*	BPV
Control (n=10)	0.00 ± 0.00^{a}
Well differentiated OSCC (n=20)	1.22 ± 0.15^{b}
Moderately differentiated OSCC (n=20)	1.30 ± 0.22^{b}
Poorly differentiated OSCC (n=20)	2.35 ± 0.17^{c}
P-value	<0.001

* Grading values, analyzed statistically, were determined as following: (- = 0) no immunoreactivity; (+ = 1) low immunoreactivity, 1-10% cell positivity; (++ = 2) moderate immunoreactivity, 11-59% cell positivity; and (+++ = 3) intensive immunoreactivity, higher than 60% cell positivity. [a-c] Statistical differences between the differentiated groups according to the Kruskal Wallis-H test (P<0.001). The degrees of differentiation are given as mean±SE

Molecular results

Two cases were found to be positive with PCR. One case was detected as positive with the primer pair MY09/MY11, while the other was detected with the primer pair FAP59/FAP64.

One of the cases (detected with the MY09/MY11 primer pair) was classified as BPV-1 after sequence analysis (Fig. 4), while the amplicon of the other case could not be sequenced because of the suboptimal yield. Agarose gel image of FAP59/FAP64 positive case is shown in Fig. 5. The sequence obtained from this study was deposited to GenBank (accession No. OK157506).

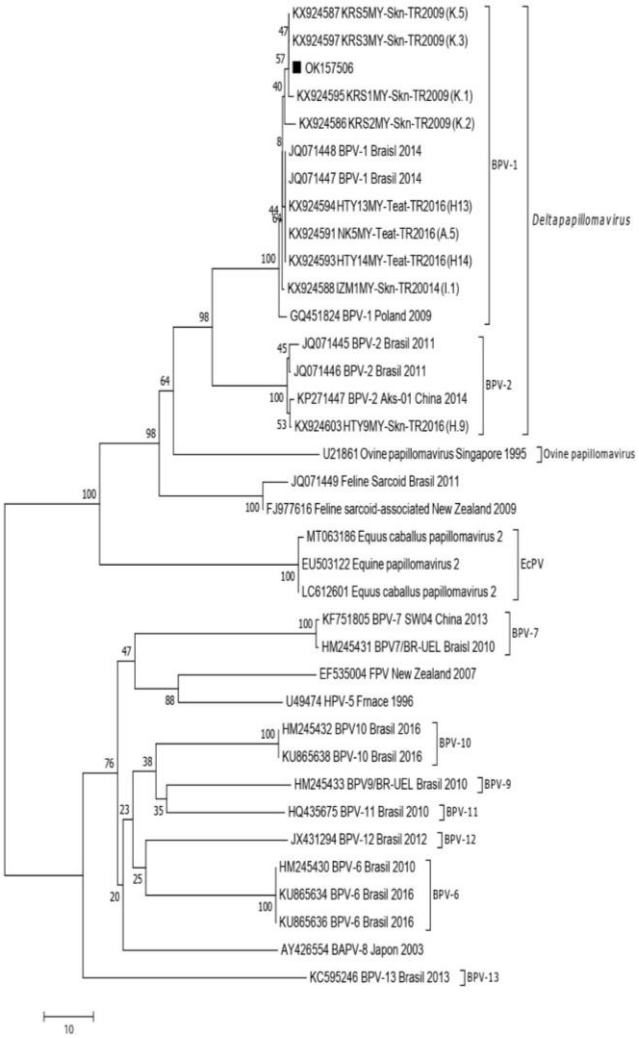

Fig. 4: Phylogenetic tree constructed using neighbor-joining (NJ) method using Kimura-Two parameter and 1000 bootstraps (MEGA 7.0) comparing our sequence with other reference papillomaviruses. Our sequence is indicated with black square (OK157506)

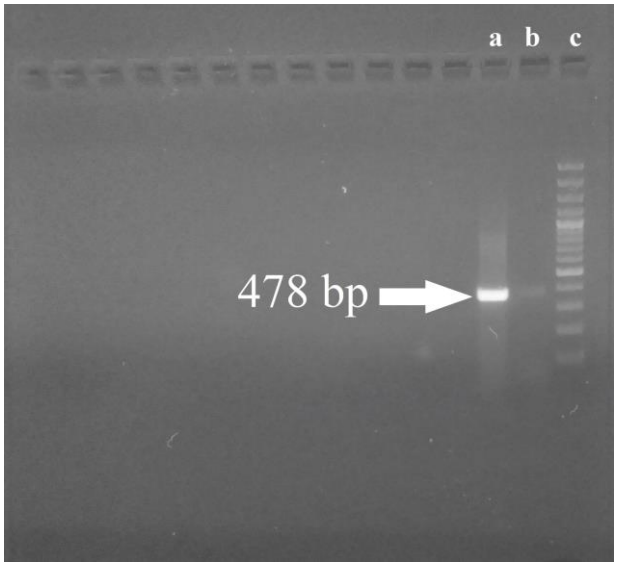

Fig. 5: Agarose gel image of PCR amplification with FAP59/FAP64 primer pair. Lane a: Positive control, Lane b: Sample, and Lane c: 100 bp (Thermo Scientific) DNA marker

Discussion

OSCCs develop depending on interaction of many different factors. To this day, a definite mechanism of progression has not been determined. The etiology of OSCCs in cattle is associated with several viral agents such as bovine papillomavirus and bovine herpes virus type 1 and type 5 (Pugliese *et al*., 2014). These viral agents can induce preneoplastic lesions as well as leading to neoplastic transformation (Fornazari *et al*., 2017). In veterinary medicine, it is stated that BPV types 1, 2, 4 and 13 and feline papillomavirus (FcaPV) types 2 and 3 are highly associated with malignant neoplasms such as squamous cell carcinoma, bowenoid carcinoma *in situ*, and transitional cell carcinoma (Chang *et al*., 2020).

BPVs are suggested to have a role in the OSCC of cattle, especially in precursor lesions (Tsujita and Plummer, 2010). There are not many studies investigating the role of BPVs in OSCCs in the literature. Ford *et al*. (1982) investigated BVPs etiology in 25 different cases of bovine OSCCs and found papillomavirus-like particles using electron microscopy in eight of the cases. Rutten *et al*. (1992) investigated 14 bovine OSCCs and 9 cell lines derived from bovine OSCCs using different hybridization assays and electron microscopy. They were unable to find any BPV DNA. However, as they only investigated BPV types 1-6, they thought that BPVs can cause precursor lesions and that they lead to carcinogenic transformation without being relevant to tumor development. Fornazari *et al*. (2017) investigated BPV etiology in OSCC of cattle using *in situ* hybridization and PCR and they could not find BPVs in any of the cases. For the most part, these studies were the main studies to investigate papillomavirus etiology in OSCCs. In this study, 47 of the 60 cases (78.33%) were BPV positive using immunohistochemical methods. In addition, BPV DNA was detected using PCR in two of the cases. In this regard, this is the first study to reveal BPV in OSCCs with immunohistochemistry and PCR. Our data suggested that PCR has a relatively low positivity rate when compared to immunohistochemistry. Nucleic acid extraction process from paraffin-embedded tissue blocks is known to cause loss of nucleic acids. For this reason, some positive cases may have been falsely evaluated as negative with PCR. Furthermore, one of the positive cases could not be sequenced even if it was submitted to sequencing. A phylogenetic tree was constructed based on the sequence obtained from this study, comparing it with other reference papillomavirus sequences. The sequence was designated as BPV-1. BPV-1 is classified in subgenera *Deltapapillomavirus* with BPV-2, BPV-13, and BPV-14. BPV-1 and BPV-2 are known as highly pathogenic papillomaviruses and cause bladder cancer in cattle fed with bracken fern. In fact, they are considered to have malign transformation properties (Yamashita-Kawanishi *et al*., 2019; Cutarelli *et al*., 2021; Emin *et al*., 2022). The case that was designated as BPV-1 was poorly-differentiated, and the other case that was positive with PCR was well-differentiated. This may indicate that BPVs can be of importance in etiology not only in precursor lesions but also in advanced stage SCCs.

Rutten *et al*. (1992) previously reported no viral antigens could be detected using BPV specific antibodies in OSCCs. Contrary to this data, our study had a high rate of positivity (78.33%) with immunohistochemistry. This could be due to using of monoclonal antibody in this study, which has higher affinity. Also, we could not find any recent study investigating BPVs using immunohistochemical methods. Additionally, sample sizes of the previous studies were not large, and OSCC tumoral differentiation variation in the samples were not taken into account. Our study had three differential stages of OSCCs (well, moderate, and poorly-differentiated cases) and the sample size was larger than that of previous studies. These could explain the high rate of positivity in our results. According to our immunohistochemical results, the number of BPV positive cells was highest in poorly-differentiated cases compared to moderate and well-differentiated cases. This may indicate a correlation between BPV presence and tumoral progression.

Kars region, where the study was performed, has a high altitude and there is a high risk of UV light exposure. Some studies report BPV and retroviruses are present in the region (Coşkun, 2022; Karakurt *et al*., 2023). OSCC seemed to be the most common malign tumor in cattle population when cases were submitted to our department. Ford *et al*. (1982) also suggested that papillomavirus infection and exposure to sunlight in excess can cause OSCCs. We believe BPVs have a contribution in cancer progression with the interaction of secondary environmental factors like UV exposure and high altitudes.

In conclusion, we investigated 60 cases of OSCCs, 47 of which were found positive for BPV immunoreactivity

and two of which were positive with PCR. This is the first study to have BPV immunopositivity with IHC and to find molecular evidence of BPV presence in OSCC cases. Our data suggest BPVs have a role in the development of OSCCs and that there may be a possible promoting effect of the virus in the progression of this tumor. Based on our molecular results, we could only find BPV-1 association, but more studies are needed to further elaborate on the association of other viral agents such as bovine herpesviruses and other BPV types with OSCCs.

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Conflict of interest

The authors declare no competing interests.

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