



NOTE

Clinical Pathology

Detection of bovine papillomavirus type 2 DNA in calf conjunctival myofibroblastoma

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ABSTRACT. An 8-month-old male Japanese Black calf was referred for the evaluation of a slow-growing conjunctival mass in the right eye. A superficial keratectomy was performed followed by recurrence on two occasions. No metastases were found in surrounding tissues. Histological, immunohistochemical, and ultrastructural investigation revealed that both the primary and the recurrent lesions were benign, conjunctival, myofibroblastomas. Interestingly, bovine papillomavirus type 2 (BPV-2) DNA was detected in both myofibroblastoma lesions. Archival bovine myofibroblastomas from the vulva and neck were also analyzed for papillomaviral genomes. BPV-2 DNA was also amplified from these lesions. To the best of our knowledge, this is the first report describing a potential causal relationship between BPV-2 infection and conjunctival myofibroblastoma.

KEY WORDS: bovine papillomavirus type 2, calf, conjunctival myofibroblastoma

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Myofibroblastoma is an uncommon, benign, mesenchymal tumor, predominantly occurring in the breast tissue of menopausal women and older men [19]. Sarcomas of myofibroblastic origin have also been described in the leg and the mammary glands of two cats [5, 11] and in the mesentery and abdomen of two horses [12, 20]. In cattle, three cases of myofibroblastoma of the nose, neck, and abdomen [12, 26], and two cases of the vulva [1, 26] have been described. Myofibroblastoma of the eye has not yet been described.

Myofibroblasts are seen in both normal and damaged tissue; in the intestine, lung, testis, ovary, and adrenal gland and in granulation and scar tissue, where they perform both a contractile and a secretory function [8]. Myofibroblasts are also found in numerous fibroblastic tumors, e.g., fibroma, fibrosarcoma, and liposarcoma [8]. Some histologically-defined cases of myofibroblastoma have been composed almost entirely of myofibroblasts [8] and could have originated from different types of precursor cells, e.g., fibroblasts, pericytes, smooth muscle cells, bone marrow-derived stem cells, epithelium, and endothelium [14]. Neoplastic transformation of myofibroblasts occasionally occurs in humans and various other animal species but their etiology is not yet clearly defined. In humans, genetic evidence for an association with human herpesvirus 8 and Epstein-Barr virus has been described [2, 9].

Tumorigenesis caused by bovine papillomavirus (BPV) infection in cattle is well studied and the causal virus is believed to be highly epitheliotropic. However, new BPV type 2 (BPV-2) tissue tropisms are increasing and a recent study revealed that BPV-2 can infect not only bovine epithelial, but also bovine mesenchymal cells, e.g., glomus tumors [21], hemangioma [4], bovine peripheral blood mononuclear cells [23], and bovine trophoblast cells, such as the placenta [22]. The goal of this study was to describe the clinical and histological features of a calf conjunctival myofibroblastoma and to investigate its potential relationship with BPV infection.

An 8-month-old male Japanese Black calf with a history of a slow-growing, isolated, conjunctival mass in the right eye was referred to the Livestock Hygiene Service Center, Hokkaido, Japan for diagnosis. No systemic abnormalities were noted on routine physical examination. Ophthalmic examination revealed a soft, milky-white, pedunculated nodule with red areas of hemorrhage, 1.2 × 1.0 cm in size and localized in the dorsolateral aspect of the cornea (Fig. 1). The limbus was not involved. The tissue on the cut surface was traversed by bands of fibrous tissue. Two months after the first superficial keratectomy, recurrence was detected, and the lesion was surgically removed. A further recurrence occurred 2 months later, and a third keratectomy was performed. After

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these treatments, no further recurrence was observed during a 24-month follow-up. The primary and the first recurrent lesions, which had similar macroscopic features, were processed for histological and immunohistochemical examination. The second recurrent lesion was not available. Surgical treatments were performed in the lying position (the first keratectomy) or the standing position (the second and the third keratectomy) under sedative and analgesic conditions via intravenous injection of 0.15 mg/kg xylazine, and under nerve block condition via injection of 10 ml of 2% lidocaine. Animal housing, treatment, and sampling conformed to the institutional guidelines approved by the ethics committee of National Institute of Animal Health.

The lesions were fixed in 10% buffered formalin and embedded in paraffin. Four μm sections were prepared for hematoxylin-eosin (H&E) and immunohistochemistry. Immunohistochemical staining was carried out using a streptavidin-biotin-peroxidase complex method (Nichirei, Tokyo, Japan). The primary antibodies used were mouse monoclonal antibodies to alpha smooth muscle actin (SMA) (DAKO A/S, Glostrup, Denmark), vimentin (Dako Corporation, Carpinteria, CA, U.S.A.), desmin (Bio-Science Products, Emmenbrücke, Switzerland), proliferating cell nuclear antigen (PCNA) (BioGenex Laboratories, San Ramon, CA, U.S.A.), rabbit polyclonal antibody to BPV-1 L1 protein (Quartett, Berlin, Germany), and mouse monoclonal antibody to BPV-1 E2 protein (1E2, Thermo Scientific, Rockford, IL, U.S.A.).

Small blocks of formalin-fixed neoplastic tissue from the primary mass were post-fixed with 1% osmium tetroxide and embedded in epoxy resin. Ultra-thin sections were cut, stained with uranyl acetate and lead citrate, and examined with an electron microscope (EM) [1].

Total DNA was extracted from paraffin sections using the phenol-chloroform-isoamyl alcohol method [15] and it was used as a template for polymerase chain reaction (PCR). PCR amplification of the BPV L1 gene with primers subAup/subAdw followed by nucleotide sequencing and genotyping was performed as previously described [18]. These primers were designed to target highly conserved L1 regions of *Delta* and *Epsilon papillomavirus* and could amplify all the known BPVs in both genera [10, 17].

For comparison, bovine herpesviruses-specific PCR, which targeted infectious bovine rhinotracheitis virus, bovine herpes mammillitis virus, Movar-type herpesvirus, bovine herpesvirus type 5, and alcelaphine herpesvirus 1, was performed as described by Kálmán *et al.* [16]. Similar PCR for detection of BPVs and bovine herpesviruses and nucleotide sequencing analyses was also performed on the previously diagnosed neck and vulva myofibroblastomas [1, 13, 26].

To prepare the gene probe for *in situ* hybridization (ISH), 1 μg of cloned BPV-2 DNA (~8 kb) was labeled with digoxigenin-11-dUTP (DIG) using a DIG-High Prime kit (Roche Applied Science, Basel, Switzerland) and stored at -20°C until used. ISH was performed according to the manufacturer's protocol (Roche Applied Science). Tissue sections were deparaffinized with xylene, rehydrated, and digested with proteinase K. Hybridization was carried out at 42°C for 3 hr with 2 ng/ μl of DIG-labeled DNA probe in the hybridization solution. After washing twice in $2 \times \text{SSC}$ and once in $0.1 \times \text{SSC}$, the slides were soaked in antibody dilution (AB) buffer and then in blocking solution. Antibody incubation was performed for 1 hr at room temperature (RT) with alkaline phosphatase (AP)-labeled goat-anti-DIG antibody (1:1,000 dilution; Roche Applied Science) in blocking solution. Sections were then washed thoroughly in AB buffer and equilibrated in AP buffer. AP activity was detected by the addition of the chromogen/substrate 4-nitrobluetetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP, Roche) in AP buffer. The sections were briefly washed with PBS, counterstained with neutral red, and examined by light microscopy. Sections of fibropapilloma with BPV-2 infection and normal bovine conjunctiva for positive and negative controls were also included.

Histologically, the primary tumor mass was composed of tumor cells and collagen fibers and had relatively unclear boundaries with the adjacent normal tissue. The predominant tumor cells were large and fusiform in shape, having plump cytoplasm (Fig. 2). The nuclei were oval to spindle-shaped, with finely dispersed chromatin and small to medium-sized nucleoli. Mitoses were occasionally seen. In less cellular and more fibrous areas; however, smaller and slender tumor cells with oval to elongated nuclei were observed, and mitotic structures were rare. Immunohistochemistry revealed positive staining for SMA and vimentin in most neoplastic cells, but desmin was absent (Figs. 3 and 4). Most tumor cells stained intensely or moderately for PCNA (data not shown), although faintly positive or negative cells predominated in less cellular areas. The BPV-1 L1 antigen, the major capsid protein, was not detected in the tumor cells or stratified squamous epithelium (data not shown). However, the BPV-1 E2 antigen, the transcription and replication regulatory protein E2, was detected in most of the tumor cells (Fig. 5) and intact, overlying squamous cells. Similar histological and immunohistochemical data were collected from the recurrent lesions. Ultrastructurally, the tumor cells were characterized by moderately developed rough endoplasmic reticulum (RER) and thin filaments with or without focal densities (data not shown). Accumulations of intermediate filaments were also observed in some cells.

Partial L1 genes in the BPV genome, which encode major capsid proteins, were amplified from both the primary and the recurrent tumor samples using *Delta papillomavirus* and *Epsilon papillomavirus*-specific subAup/subAdw primer sets (Fig. 6). Both amplified DNAs were 437 bp in length and were 100% homologous with 99.5% identity to previously reported BPV-2 sequences (accession number AB823004). On the other hand, bovine herpesvirus-specific DNA could not be detected in any of the myofibroblastoma lesions. For further verification of the presence of BPV-2 in myofibroblastomas, similar analyses were performed on two previously diagnosed myofibroblastoma samples from the neck and vulva [1, 13]. DNA with 99.7% homology to previously reported BPV-2 sequences was detected in each myofibroblastoma sample (accession number AB823005 and AB823006) (Fig. 6).

The ISH results confirmed that BPV-2 specific DNA was present in the nuclei of most tumor cells in the primary lesion (data not shown). Intense ISH-positive cells predominated in more cellular areas, in which most cells stained positive for PCNA. Similar results were obtained from the recurrent lesions.

Myofibroblasts display several biological traits that are intermediate between fibroblasts and smooth muscle; they are spindle-shaped cells that secrete collagen and have well-defined contractile properties. In conventional histological sections, myofibroblasts cannot be readily distinguished from fibroblasts, but immunohistochemical detection of SMA (not seen in fibroblasts) and

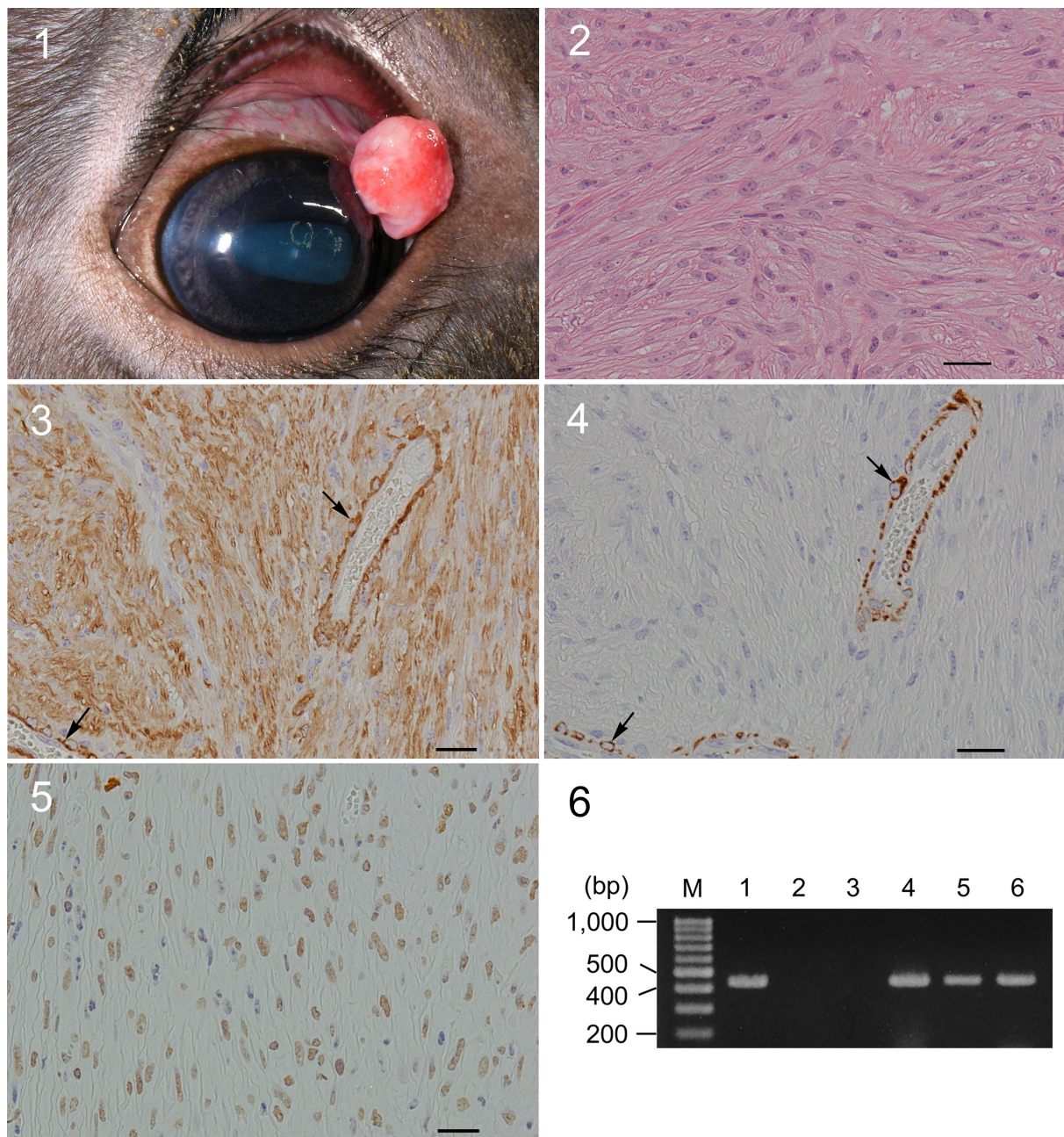


Fig. 1. Conjunctival myofibroblastoma in an 8-month-old male Japanese Black calf. A pedunculated solid nodule is observed in the dorsolateral aspect of the cornea.

Fig. 2. Fascicles of spindle-shaped tumor cells with interstitial collagen are visible; H&E; scale bar=10 μ m.

Fig. 3. SMA immunolabeling is observed not only in vascular smooth muscle cells (arrows) but also in surrounding tumor cells; immunohistochemistry; scale bar=20 μ m.

Fig. 4. In contrast to Fig. 3, smooth muscle cells alone are positively stained for desmin (arrows); immunohistochemistry; scale bar=20 μ m.

Fig. 5. Nuclei of most tumor cells are immunoreactive for BPV-1 E2; immunohistochemistry; scale bar=20 μ m.

Fig. 6. PCR detection of BPV DNA using *Delta* and *Epsilon papillomavirus*-specific primers (subAup: CCAGAYTAYYTMAAAATGGC and subAdw: ATAAMKG CTAGCTTATATTC). Amplicons of 443 bp were detected in all myofibroblastoma samples. M: 100 bp ladder; lane 1: positive control (pBPV-2); lanes 2 and 3: negative control (pBPV-9 and normal bovine skin); lane 4: conjunctival myofibroblastoma; lanes 5 and 6: bovine myofibroblastoma of the neck and vulva.

ultrastructural detection of contractile proteins show that they are distinct [25]. Although myofibroblasts and smooth muscle cells in bovine tissues are both positive for SMA, only myofibroblasts show minimal or absent staining with the anti-desmin antibody [28]. The present neoplasm, displaying SMA positivity in almost all cells and no detectable staining for desmin, was clearly different from a fibroma, of fibroblast cell origin, and a leiomyoma, of smooth muscle cell origin. Based on these findings, a myofibroblast progenitor is confirmed. The neoplasm recurred after inadequate surgical excision, but not after complete excision. There appeared to be regressive changes in the eye and no metastases were found. Therefore, the present case was diagnosed as a benign conjunctival myofibroblastoma. Normally myofibroblastoma in cattle arises in adult animals [1, 13, 26], but was present in an immature young animal in the present case.

Recently, the presence of the papillomavirus (PV) genome in various non-epithelial cells has been described [7, 23]. Schwann cells, fibroblast cells, and peripheral blood mononuclear cells of mesenchymal origin are possible reservoirs of PV [7, 23]. Glomus cells of mesenchymal origin and placenta of cytotrophoblast origin are also possible reservoirs of BPV-2 [21, 22]. The detection of BPV-2 DNA in the conjunctival myofibroblastoma, as well as in the neck and vulval myofibroblastoma, reported here, suggests that BPV-2 can cause neoplasia derived from myofibroblasts in different locations of the body [1, 13]. A pairwise comparison of the nucleotide sequences of amplified DNA from each lesion showed that each DNA was different by one or two bases. We suggest that three different BPV-2 variants are responsible for these lesions.

BPV E2 proteins have been identified to activate viral transcription and replication by binding with E2-specific recognition sequences located within the promoter region of the viral genome [3]. Expression of BPV E6 and E7 transforming genes are under the control of the E2 proteins [3]. E2 proteins also tether BPV genomes to the mitotic chromosomes via cell cycle regulator proteins, such like bromodomain-containing protein 4; thus, they advance the distribution and retention of the viral genomes in the tumor tissue [3]. We report here the expression of the E2 proteins in most of the tumor cells, suggesting that the harboring BPV-2s are transcriptionally active in the myofibroblastoma and are likely to be associated with tumorigenesis through the multifunctional E2 proteins.

Ford *et al.* [6] isolated and identified PV-like particles by electron microscopy in approximately 33% of putative bovine ocular squamous cell carcinoma precursor lesions. Although they did not study myofibroblastoma cases, these results combined with our results suggest the presence of BPV in various ocular lesions in cattle. BPV structural antigens were not detected by immunohistochemistry using a cross-reactive, BPV-1-induced antibody, and PV-like particles were not ultrastructurally observed in the conjunctival myofibroblastoma. However, it should be noted that this finding is not unusual because similar results were obtained from previous observations of BPV-associated feline and equine sarcoids [27, 29]. Also, BPV replication and virion production are confined to epithelial regions of the lesion, whilst infection of mesenchymal cells is generally non-productive [24]. Thus, we consider that the BPV infection is non-productive and that productive BPV replication, with synthesis of complete virions, does not occur in myofibroblastoma. Although further investigation of the molecular mechanism of myofibroblastoma development is required, we report here the first firm evidence for BPV infection in myofibroblastoma in cattle.

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