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Equine ocular squamous cell carcinoma: a case report

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ABSTRACT: An eight-year-old gelding, Wielkopolski Horse was presented with a pink tumour, which filled the entire palpebral fissure of the left eye. Ultrasonography revealed it to be well-demarcated from the cornea mass, which covered its entire surface. Due to the extensive size of the lesion and the lack of owner's consent to post-operative treatment, it was decided to perform enucleation. A histopathological examination revealed corneal squamous cell carcinoma (CSCC). The primary cause of the tumour has not been established. Immunohistochemistry was negative for p16 immunoreactivity which is typical for the E7 oncoprotein in PV infection, and is suspected of involvement in the aetiopathogenesis of ocular squamous cell carcinoma (OSCC).

Keywords: eyeball neoplasm; SCC; p16 protein; horse

Ocular squamous cell carcinoma (OSCC) is a malignant tumour of the eye and ocular adnexa (Dubielzig 2002). It is reported in both small animals such as dogs (Takiyama et al. 2010), cats (Scurell et al. 2013) and in large animals such as cows (Carvalho et al. 2005), reindeer (Gonzalez-Alonso-Alegre et al. 2013) and horses (Kaps et al. 2005; Lassaline et al. 2014).

In horses, SCC is the most common ocular tumour (Lavach and Severin 1977). A study by Kafarnik et al. (2009) showed that it constitutes the majority of all ocular tumours. Lymphoma (Schnoke et al. 2013), angiosarcoma (Hacker et al. 1986), melanoma (Ramadan 1975) and other neoplasms are also observed, although they are diagnosed less frequently (Bosh and Klein 2005; Gilger 2013). OSCC may affect the cornea (Kafranik et al. 2009), limbus (Bosch and Klein 2005; Lassaline et al. 2014), eyelid conjunctiva and globe conjunctiva (Chahory et al. 2002; Bosch and Klein 2005), third eyelid (Mair et al. 2012) as well as the eyelids (Giuliano et al. 2008).

SCCs of the cornea may be primary when only the corneal cells are involved (Kafarnik et al. 2009) or secondary such as a corneolimbal form, when the limbus is primarily infiltrated followed by the cornea (Michau et al. 2012; Scurrell et al. 2013). Corneal SCC (CSCC) may affect the corneal epithelium (Rebhun 1990), epithelium and stroma (van der Woerdt et al. 1996) or the stroma alone as a corneal stromal invasive SCC (Kafranik et al. 2009). Despite numerous publications on deep neoplasia of the cornea, no tumour penetrating the Descemet's membrane has yet been described (Dubielzig 2002; Kaps et al. 2005; Kafarnik et al. 2009). It seems that this membrane is resistant to the invasion of neoplastic cells, which spread around its borders (Monlux et al. 1957).

To date, the pathogenesis of OSCC has not been fully elucidated (Dugan et al. 1991a; Sironi et al. 1999; Mosunic et al. 2004; Lassaline et al. 2014). It is thought that prolonged sunlight exposure plays a significant role in the occurrence of SCCs (Dugan et al. 1991a). Ultraviolet radiation causes mutations in the p53 protein encoding gene which is the major regulator of the cell cycle and genome integrity (Lane 1992). Mutation of this protein interferes with proper arrest of the cell cycle, compromises DNA repair, causes abnormalities in apoptosis and stimulates the formation of neoplastic cells (Lane 1992; Kamb 1994). p53 over-expression has been detected in SCCs in humans (Brash et al. 1991; Hall et al. 1993) and animals (Teifke and Lohr 1996; Sironi et al. 1999; Albaric et al. 2001). It is suspected that viral factors such as papillomavirus (Kim et

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al. 2009; Nasir and Brandt 2013) are also involved in SCC aetiology and pathogenesis in horses. An increase in immunoreactivity for the p16 protein is one of the markers of papillomavirus infection, because an increase in its expression is elicited by the E7 oncoprotein (Konig et al. 2007; Munday et al. 2011a).

Changes in oestrogen and androgen levels in the blood are additional elements of SCC aetiology in horses (Lavach and Severin 1977; Dugan et al. 1991a; Mosunic et al. 2004). The disease mainly affects horses eight to 13 years old (Gelatt et al. 1974; Schwink 1987; Mosunic et al. 2004; Michau et al. 2012; Lassaline et al. 2014). A more frequent incidence of the neoplasm has been reported in equine breeds with a grey hair coat component than in dark-pigmented horses (Gelatt et al. 1974; Lavach and Severin 1977; Dugan et al. 1991a). A high probability of OSCC incidence has also been described in Haflinger horses (Lassaline et al. 2014).

OSCC has features of malignancy, such as local invasiveness, metastatic potential and recurrences. Local OSCC invasiveness involves the iris (Monlux et al. 1957), sclera (Malalana et al. 2010) as well as the extraocular structures: the orbit, nasal cavity and sinuses (Mair et al. 2012; Albanese et al. 2014). SCC may also cause bony destruction (Gelatt et al. 1974; Eversole and Lavach 1978; Albanese et al. 2014).

OSCC has a low metastatic rate of 18% (Schwink 1987; King et al. 1991). The recurrence rate ranges from 11.1% to 66.7% and is associated with factors such as the type of applied treatment (King et al. 1991; Mosunic et al. 2004; Michau et al. 2012). In addition, recurrences are more common in OSCC affecting the eyelids than in the corneal form of the tumour (Dugan et al. 1991b; King et al. 1991).

Case description

An eight-year-old gelding, Wielkopolski Horse, was referred to the ophthalmologic division of our clinic with a pink tumour, which filled the entire palpebral fissure of the left eye. It was learned that the lesion had been first noticed by the owners over seven months earlier and had initially only involved the temporal area of the cornea.

A physical examination revealed a dense pink, non-movable lesion covering the entire corneal surface of the left eye (Figure 1). The lesion was densely vascularised. As the cornea could not be accessed, it was impossible to perform an ophthalmologic examination. The ophthalmologic examination of the right eye with a slit lamp biomicroscopy and both indirect and direct ophthalmoscopy and tonometry did not reveal any abnormalities.

Ultrasonography of the orbital and ocular structures was performed after auriculopalpebral and frontal nerve blocks. Two ml of 2.0% lidocaine (Lignocainum hydrochloricum, Polfa, Warszawa, Poland) were injected subcutaneously along the

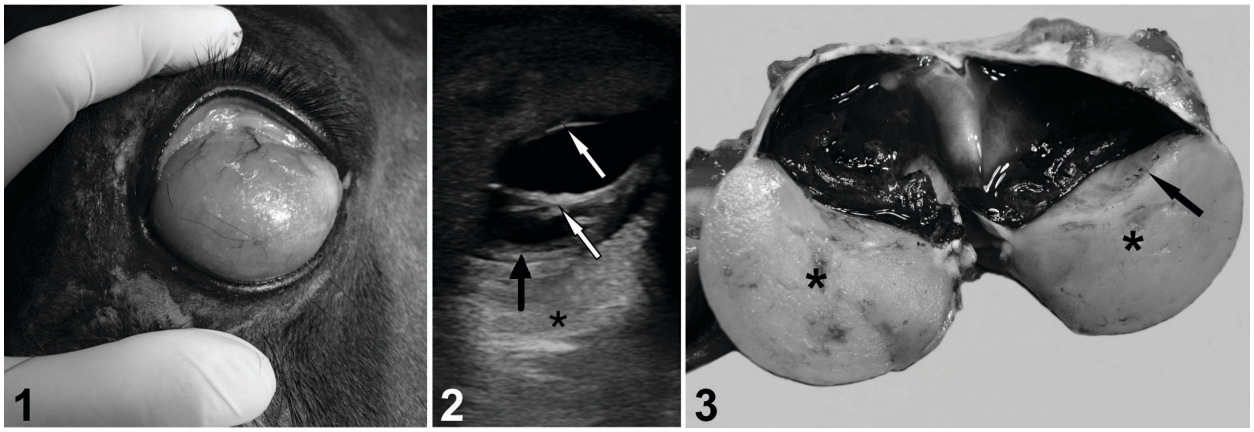

Figure 1. Appearance of the well-vascularised mass covering the entire corneal surface of the left eye at initial clinical examination

Figure 2. Ultrasonographic image of the left eye. Well-demarcated from the cornea (black arrow) dense hyperechogenic mass (asterisks), anterior and posterior lens capsule (white arrows)

Figure 3. Cross section of the left eyeball. Mass of the tumour (asterisks) covers the entire surface of the cornea (black arrow)

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dorsal zygomatic arch to block the auriculopalpebral nerve and 1 ml was injected over the opening to the supraorbital foramen to block the frontal nerve.

B-mode USG with a 10 MHz transducer demonstrated a hyperechogenic mass, up to 25 mm thick, situated in the central part of the cornea, which covered its entire surface (Figure 2). The anterior and posterior segments of the left eye were normal. Ultrasonography of the orbital and ocular structures of the right eye was normal.

Differential diagnosis included neoplasia such as an OSCC (with a special emphasis on CSCC), parasitic infestation, and inflammatory lesions such as an abscess, an inflammatory granuloma or a foreign body reaction (Lavach 1990).

The patient was qualified for a surgical treatment. Due to the extensive size of the lesion and the lack of owner's consent to post-operative treatment, it was decided to perform enucleation. The clinical general examination revealed no abnormality. The total blood count and serum biochemical profile were within normal limits. The horse was premedicated with medetomidine (Domitor Pfizer GmbH, Karlsruhe, Germany) at a dose of 0.006 mg/kg intravenously (*i.v.*). The catheter was placed into the jugular vein. Anaesthesia was induced by administration of ketamine (Vetaketam, Vet-Agro, Lublin, Poland) 2 mg/kg and diazepam (Relanium, Polfa, Warsaw, Poland) 0.025 mg/kg *i.v.* After intubation, anaesthesia was maintained with isoflurane (Aerrane, Baxter Polska, Warsaw, Poland) at a concentration of 1.5–2.0%. Intravenous fluids – NaCl 0.9% (Natrium Chloratum 0.9%, Baxter Polska, Warsaw, Poland), were administered throughout the surgical procedure at a rate of 10 ml/kg/h. Retrobulbar nerve block was obtained with the use of 10–12 ml of 2.0% lidocaine (Lignocainum hydrochloricum, Polfa, Warsaw, Poland) injected into the retrobulbar space (Gilger and Davidson 2002). Enucleation was performed according to the well-known transpalpebral technique (Pierce and Townsend 2012).

The removed globe was fixed in 10% neutral-buffered formalin (Figure 3). Transverse paraffin sections of the globe were stained with haematoxylin-eosin and examined under a microscope.

In the microscopic pattern of the tumour, neoplastic cells were clearly predominant, with connective tissue forming small bands that surrounded tumour cells (Figure 4a). The cells, with their polygonal shape, although being similar to each other, were quite often of different sizes (Figure 4b). Clusters of small hyperchromatic cells were also observed; they lay adjacent to each other and were highly anaplastic (Figure 4b). The neoplastic cells were always orderly arranged to each other. They sometimes formed structures that mimicked the stratified squamous epithelium and were occasionally grouped in packs resembling the glandular architecture (Figure 4c). They also had similar, roundish nuclei that were usually pycnotic and sometimes hyperchromatic, most often with a

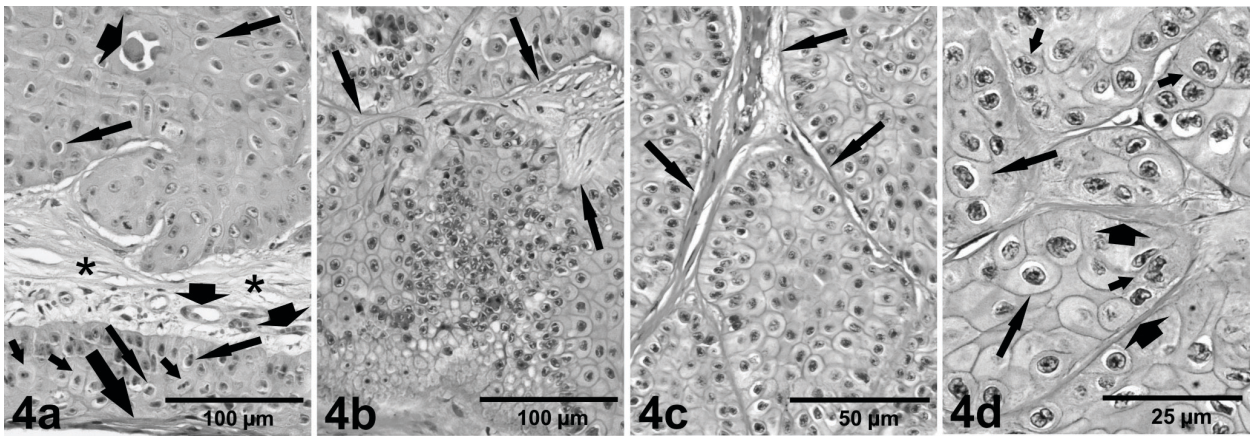

Figure 4. Microscopic pattern of CSCC (**a**). Neoplastic infiltration, connective tissue (asterisks), blood vessels (head arrows), neoplastic cells in stratified squamous epithelium (short arrows), koilocytes (long arrows), Bowman's membrane (long thick arrow) (**b**). Differentiated size of neoplastic cells, highly anaplastic small cells (in the middle), connective tissue interstitium (arrows) (**c**). Tumour parenchyma structure resembling glandular architecture, clusters of cells surrounded with connective tissue (arrows) (**d**). Neoplastic cells with differentiated nuclear chromatin, numerous koilocytes (long arrows), direct division of nuclei (short arrows), connective tissue (head arrows)

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single nucleolus. The chromatin in the nuclei was arranged in polymorphic papules with different sizes and varied locations. The cytoplasm of these cells was markedly eosinophilic. Occasionally, it contained differently-sized vacuoles and koilocytes were quite often found. They had a wrinkled (i.e. "raisinoid") nucleus surrounded by a clear halo (Figure 4d).

The mitotic index was 20 (20 mitotic figures per 10, 40 × high power fields). Multipolar cell divisions were sometimes detected and direct cell divisions were also seen. The corneal stroma and the anterior and posterior segment of the globe were normal. A histopathological examination confirmed CSCC.

Complementary investigations

Protocol for p16 detection. The antibody used in the CINtec Histology Kit (Ventana Medical Systems, San Jose, CA, USA) – (an immunohistochemistry assay for the qualitative detection of the p16INK4a antigen on formalin-fixed, paraffin-embedded tissue sections prepared from human cervical biopsies) is a murine monoclonal anti-human clone E6H4, supplied in 50 mmol/l Tris buffer pH 7.2 containing 15 mmol of sodium azide and stabilising protein. The system revelation- chromogen is DAB. A negative control (aspecific) reagent provided in the kit and a positive tissue which serves as the positive control were also used.

Protocol for CAM 5.2 detection. After deparaffinization, the endogenous peroxidase was inactivated, and the non-specific background was blocked with 5.0% goat serum (Vector Laboratories, Peterborough, UK). Sections were incubated overnight at 4 °C with monoclonal CAM 5.2 (ready to use; BD Biosciences, San Jose, CA, USA). After a second incubation for 30 min with secondary biotin-conjugated goat anti-mouse antibody at a dilution of 1 : 200 (Vector Laboratories, Peterborough, UK), slides were incubated with avidin-biotin-peroxidase reagent (ABC Kit Vectastain Elite, Vector Laboratories, Peterborough, UK). Peroxidase activity was detected using 0.1% hydrogen peroxide in diaminobenzidine solution (Sigma, Saint Louis, MO, USA). Finally, sections were counterstained with Mayer's haematoxylin.

The immunohistochemical reactions for p16 protein expression were completely negative. In some parts the tumour resembled a Meibomian-type tumour with foamy cytoplasm, which might have originated from the third eyelid, but it was completely negative for immunohistochemical staining for CAM 5.2., in contrast to the positive control (normal skin) in which the expression of the molecule was clearly evident.

DISCUSSION AND CONCLUSIONS

In horses, CSCC constitutes from 19.1% (Mosunic et al. 2004) to approximately 21% of all OSCCs (Dugan et al. 1991b). There are a number of OSCC treatment options. The selection of the proper technique depends on the features of a given tumour: its location, size, depth of invasion and factors such as treatment costs and availability of equipment. Surgical excision is the most often treatment option for OSCC (Gelatt 1975; Koch and Cowles 1971; Payne et al. 2009). It is effective when the lesion is excised with an appropriately wide safety margin or, if not feasible, an adjunctive therapy may be applied to eliminate other tumour cells (Schwink 1987; Dugan et al. 1991b). It has been demonstrated that a combination of surgical treatment and supportive therapy results in a lower rate of recurrences (King et al. 1991; Theon and Pascoe 1995; Theon et al. 1999; Mosunic et al. 2004). A study by Mosunic et al. (2004) showed that the recurrence rate after surgical excision was 44.1%, whereas this index was reduced to 11.9% when surgical treatment was combined with an adjunctive therapy.

Adjunctive treatment may involve cryotherapy (Schoster 1992; Bosh and Klein 2005), radiofrequency hyperthermia (Wilkie and Burt 1990), photodynamic therapy (English et al. 1990; Guiliano et al. 2008; Michau et al. 2012), immunotherapy (McCalla et al. 1992), chemotherapy with cisplatin (Theon et al. 1993), 5-fluorouracyl (Pucket and Gilmour 2014) or mitomycin C (Clode et al. 2012), radiotherapy such as brachytherapy (King et al. 1991), interstitial therapy (Chachory et al. 2002), and beta radiation (Walker et al. 1986; Rebhun 1990; Plummer et al. 2007).

Enucleation (Kafranik et al. 2009), exenteration (Albanese et al. 2014) or zygomatic arch excision (Beard and Wilkie 2002) are performed in very severe cases and when the disease recurs after surgical excision and adjunctive treatment. In numerous publications, recurrences have not been reported

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in surrounding tissues after enucleation (Beard and Wilkie 2002; Mosunic et al. 2004; Bosch and Klein 2005; Kafranik et al. 2009; Michau et al. 2012; Albanese et al. 2014).

The recurrence rate of CSCC treated with an adjunctive therapy is up to 44.1% (Mosunic et al. 2004). Despite the good results of combined therapy, some animals were surgically treated with enucleation (Mosunic et al. 2004; Bosch and Klein 2005; Kafranik et al. 2009; Michau et al. 2012). It has been shown that CSCCs which are less than 10 mm in diameter treated with keratectomy and beta radiation have a better prognosis than lesions with diameters over 10 mm (Rebhun 1990). Michau et al. (2012) found the recurrence rate of extensive (over 10 mm) CSCCs treated with keratectomy and laser photoablation to be 17.8%. 100% of tumours larger than 2 cm^2 did not respond to keratectomy combined with cryotherapy and were treated using enucleation (Bosch and Klein 2005).

In this case report, due to the size of the tumour and the lack of data on a totally effective combined treatment, a radical approach (enucleation) was selected.

The histopathological findings in our case were indicative of a well-differentiated corneal squamous cell carcinoma, keratinising, infiltrating, with epithelial changes (koilocytes) that could indicate a viral aetiology. The presence of areas with adenomatous pattern suggested a glandular origin of the tumour but the CAM 5.2 immunohistochemical staining was negative, excluding this hypothesis. The presence of koilocytes in a typical squamous cell tumour and the possible role of Papillomavirus in the pathogenesis of the tumour, prompted us to analyze the expression of the p16 protein because it has proved to be an important biomarker in human papillomavirus (HPV) cervical intraepithelial neoplasia and head and neck SCC (Konig et al. 2007).

The p16 protein (INK4a) is an inhibitor of the cyclin-dependent kinases CDK4 and CDK6 and plays an important role in regulating the cell cycle of eukaryotic cells. It participates in the control mediated by retinoblastoma protein (pRB) and mediated the cell cycle arrest in the processes of cell differentiation. Its distinguishing feature is that it is overexpressed in cells in which the RB1 gene is inactivated by the oncoprotein E7 of papillomaviruses; this means that p16 is not only a marker of viral infection, but also of activation of expression of viral oncogenes (E7) and alterations induced by the virus on the cell cycle (Munday et al. 2011a). Therefore, it indicates not only the presence of papillomavirus or activation of the E6-E7 oncogenes but goes well beyond oncogenesis, demonstrating the presence of serious virus-induced molecular damage on the cell, mediated by E7. Abnormal RB expression results in an accumulation of p16CDKN2A protein (p16), which can be detected using immunohistochemistry. While the presence of p16 has not previously been investigated in horse SCC lesions, recent studies of skin and oral samples revealed that p16 immunoreactivity is rarely present within feline-negative PV SCCs (Munday et al. 2011b), while there was an intense cytoplasmic and nuclear immunoreactivity in oral Papillomas associated with Felis catus Papillomavirus Type 1 (Munday et al. 2015).

In the present study, p16 immunoreactivity was not visible within the OSCCs. This could suggest that the neoplasm was not due to PV infection, in agreement with the literature; however, it is also possible that the absence of p16 immunoreactivity was due to infection with a PV that does not express E7 or a tumour in which this protein plays only a minor role. Moreover, no putative pRB binding sites (LXCXE) have been identified in equine papilloma viruss (EcPV-2) and 3 E7 amino acid sequences, so E7 is probably not able to modulate pRB and consequently the p16 protein. In human pathology the prevalence of HPV in ocular surface disease varies dramatically, according to variations in the assays used to detect the virus, as well as geography and genetic susceptibility (Woods et al. 2013), and the utility of p16INK4a immunoexpression for predicting HPV in ocular surface squamous neoplasia was evaluated but, unlike cervical cancers, it seems that there is no correlation. In fact, the overexpression of p16INK4a in OSSN was significantly associated with HPV (Chauhan et al. 2012), while Auw-Haedrich et al. (2008) have reported HPV and p16INK4a positivity in conjunctival intraepithelial neoplasias (CIN) but they did not observe any association between p16INK4a and HPV.

The negative stain for p16 may be due to the lack of specificity of the monoclonal antibody used (anti-human) against the horse p16 but previously similar antibodies were shown to be useful in cats, where the specificity of monoclonal anti-human p16 antibodies for feline p16 has been documented (Munday et al. 2015). Unfortunately, all these considerations are speculative because Papillomaviral

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DNA PCR assays were not performed. In conclusion, even if a major limitation of our work is the lack of DNA detection and a control of the specificity of the p16 antibodies, our results support the hypothesis that OSCCs may not be associated with EcPV in contrast to penile SCCs and/or may indicate that p16 is not a useful biomarker for ocular EcPV lesions.

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