Case Report

Vulvar squamous cell carcinoma *in situ* within viral papillomas in an aged Quarter Horse mare

M. A. Smith*, D. G. Levine, L. M. Getman, E. J. Parente and J. B. Engiles

University of Pennsylvania, New Bolton Center, George D. Widener Hospital for Large Animals, 382 West Street Road, Kennett Square, Pennsylvania 19348, USA.

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Summary

This report describes the diagnosis and treatment of vulvar papillomatosis with carcinoma *in situ* in a 25-year-old mare. Papillomavirus was demonstrated via immunohistochemical staining of biopsies of the lesion. The papillomas were removed via laser excision and the surrounding vulvar tissue injected with 5-fluorouracil. Additional more extensive laser excisions were required due to recurrence.

Introduction

Papillomas are among the most common equine cutaneous neoplasms (Baker and Leyland 1975; Sundberg *et al.* 1977). Acquired cutaneous papillomas in horses are viral-induced neoplasms which occur in 2 distinct forms: viral papillomatosis and ear papilloma (aural plaques) (Scott and Miller 2003). Viral papillomatosis most often occurs in horses aged <3 years, and spontaneous remission usually occurs within 3–4 months (Scott and Miller 2003). Lesions occur most commonly on the muzzle and lips, and less commonly on the eyelid, external genitalia and distal limbs.

Papillomaviruses, the causative agents of acquired cutaneous papillomas in horses, have been shown to have malignant potential in several species of animals with neoplastic progression to squamous cell carcinoma *in situ* (Bowen's disease) and invasive squamous cell carcinoma (SCC). Human cervical and vaginal papillomas and squamous neoplasms are highly associated with human papillomavirus (HPV). These are characterised by low to high grade cervical/vaginal squamous intraepithelial neoplasms (CIN and VAIN, respectively) and invasive squamous cell carcinoma. However, only a few subsets of HPV (HPV-16, HPV-18, HPV-6/11) have been identified in invasive squamous cell carcinomas (Daling *et al.* 2002). In dogs, a novel canine papillomavirus (CFPV-2) with unique tropism has been cloned in persistent cutaneous papillomas of the footpad and interdigital tissue (Yuan *et al.* 2007). When this virus is transplanted into immunocompromised dogs with X-linked severe combined immunodeficiency (X-linked SCID), the resultant papillomas can progress to malignant invasive and metastatic squamous cell carcinoma (Goldschmidt *et al.* 2006). Cynomolgus monkeys have also demonstrated papillomavirus-associated cervical and vaginal papillomas, CIN/VAIN, and invasive cervical SCC (Wood *et al.* 2004). Although papillomaviruses have been implicated in the aetiology of SCC in the aforementioned species, papillomavirus antigen has not been previously detected by immunoperoxidase staining in equine SCC, despite the similarity of distribution to that of equine squamous papillomas (Junge *et al.* 1984).

This report describes the clinical and histopathological findings and treatment of an aged mare with persistent vulvar papillomatosis and carcinoma *in situ*. To the authors' knowledge this is the first report of vulvar papillomatosis undergoing malignant transformation to carcinoma in a horse.

Case history and clinical findings

A 25-year-old chestnut Quarter Horse was initially examined for a complaint of 'lumps' on the vulva. The mare was found to be in good general health. Examination of the vulva and vaginal area revealed several exophytic and verrucous growths within the ventral commissure of the vulva, the largest of which was 5 x 5 cm. There were also multiple smaller frond-like papillomatous lesions along the vestibular walls (**Fig 1**). The lesions did not appear to extend into the vagina. Incisional biopsies of the largest lesion revealed squamous cell carcinoma *in situ* arising within squamous papillomas with viral cytopathic effect and moderate submucosal inflammation. Due to

*Author to whom correspondence should be addressed.

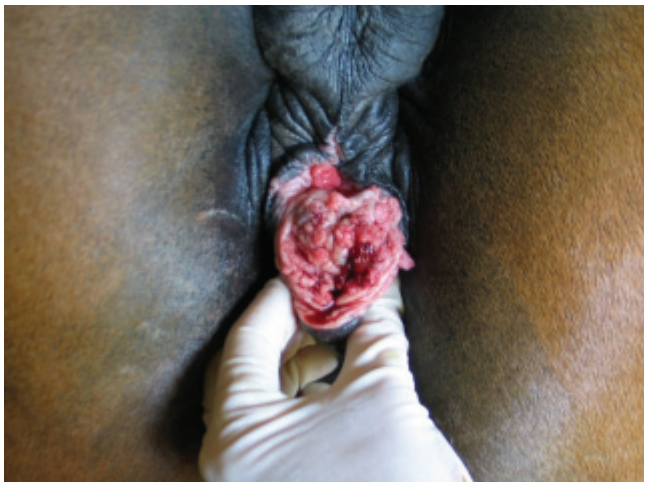

Fig 1: Macroscopic view of the diseased vulvar mucosa (the bloody area on the affected tissue is a clot which formed at the site of the incisional biopsy).

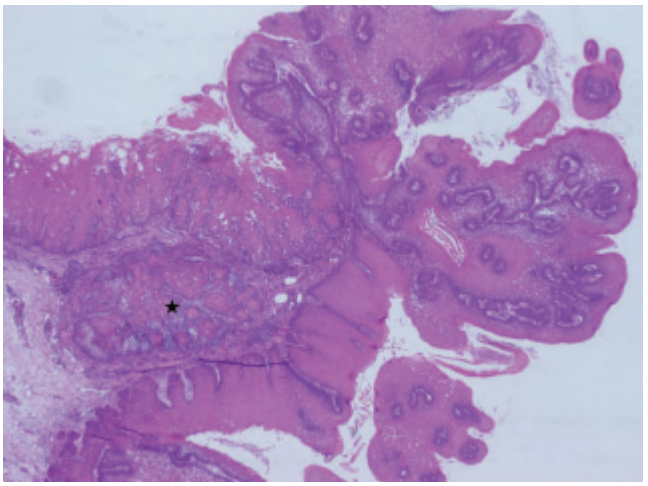

Fig 2: Papilloma. H&E stain of one lesion surgically excised from the vulva that demonstrates the formation of papillary projections and plaque-like thickenings by a hyperplastic and acanthotic epithelium, x2. Focal extension into the submucosa is observed (star).

evidence of neoplastic transformation, the mare was referred for complete surgical excision to the George D. Widener Hospital for Large Animals at the University of Pennsylvania's New Bolton Center. At the time of referral, there were no clinical signs associated with the growths.

Surgical procedure

The mare was sedated with detomidine (0.01 mg/kg bwt), butorphanol (0.01 mg/kg bwt), and acepromazine (0.02 mg/kg bwt) i.v. Regional anaesthesia was achieved with a caudal epidural using a combination of xylazine (0.17 mg/kg bwt) and lidocaine (0.22 mg/kg bwt) as previously described. The largest mass encompassed the clitoris and was removed *en bloc* using a diode laser[1] at 18 watts with a bare 600 µm fibre in direct contact. Several lesions measuring less than 3 mm in diameter were removed in similar fashion. Additional abnormal tissue within the clitoral fossa and along the vestibular walls was photoablated using the same diode laser with an air-cooled large round contact probe[1]. The surrounding tissue was then injected with a total of 10 ml of 5-fluorouracil (5-FU; injectable USP, 50 mg/ml). The mare received flunixin meglumine (1.1 mg/kg bwt i.v.), dexamethasone (0.04 mg/kg bwt i.v.), and procaine penicillin G (22,000 iu/kg bwt i.m.) intraoperatively. She was discharged the following day with instructions for treatment with flunixin meglumine (1.1 mg/kg bwt *per os* b.i.d.) for 4 days, and 2 repeat treatments of 5-FU starting in 3–4 weeks, at 3–4 week intervals. The clitoral mass was submitted for histological evaluation.

Histology

Routine 5 µm thick haematoxylin and eosin (H&E) stains of formalin-fixed, paraffin-embedded (FFPE) biopsy sections were performed. Histopathology revealed acanthotic plaque-like-thickenings to papilliferous projections of a hyperplastic epithelium supported by a fine fibrovascular stroma (**Fig 2**). Papilloma viral cytological effect was present throughout the proliferations characterised by blue-grey dyskeratosis, round to oval vesicular nuclei with peripheralised chromatin, hyaline intranuclear inclusions, and distinct perinuclear clearing (koilocytes) (**Fig 3a**). Some areas demonstrated features consistent with malignant transformation characterised by mild to marked epithelial dysplasia with the formation of epithelial nests and keratin pearls, expansion of the basal epithelium, loss of nuclear polarity, nuclear atypia, frequent individual cell necrosis/apoptosis and mitotic figures present within the spinous layer (0–1 per high power field) (**Figs 3a**,**b**). Proliferations were associated with moderate to severe lymphoplasmacytic to neutrophilic dermal inflammation, but none showed invasion through the basement membrane. Immunohistochemical (IHC) stains of FFPE 5 µm sections were performed. Slides were routinely deparaffinised and rehydrated through a series of ethanol solutions, and subsequently treated with hydrogen peroxide to eliminate endogenous peroxidase. A 1:500 dilution of the primary antibody (rabbit polyclonal papillomaviral antibody DAKO B0850) was applied and incubated for 30 min, followed by application of the detection system kit (DAKO LSAB2), which utilises a biotinylated secondary antibody and a streptavidin-horseradish peroxidase label, followed by incubation with a DAB (3,3'-diaminobenzidine) chromagen. Slides were then counterstained with haematoxylin. IHC stained sections revealed focal to confluent papilloma viral antigen expression within occasional nuclei of koilocytes (**Fig 3c**). More frequently, dense perinuclear and punctate cytoplasmic staining was observed within both atypical cells of the *stratus spinosum* and koilocytes (**Fig 3d**). Positive and negative controls for the immunohistochemical staining were utilised, and worked appropriately (data not shown).

Progression and outcome

Three weeks after the initial resection, the mare was re-examined for the second 5-FU treatment. Multiple papillomatous masses, ranging in size from 0.5–1 cm in diameter, were present within the clitoral fossa. The masses and surrounding tissue were injected with 5-FU. Subsequent examinations at 6 and 10 weeks post operatively revealed that the masses were growing in size and number. Almost 5 months after the initial laser resection of the vulvar papillomas, the mare was admitted for evaluation and excision of the recurrent masses. Examination of the vulva revealed multifocal papillomatous lesions around the clitoral fossa, with one

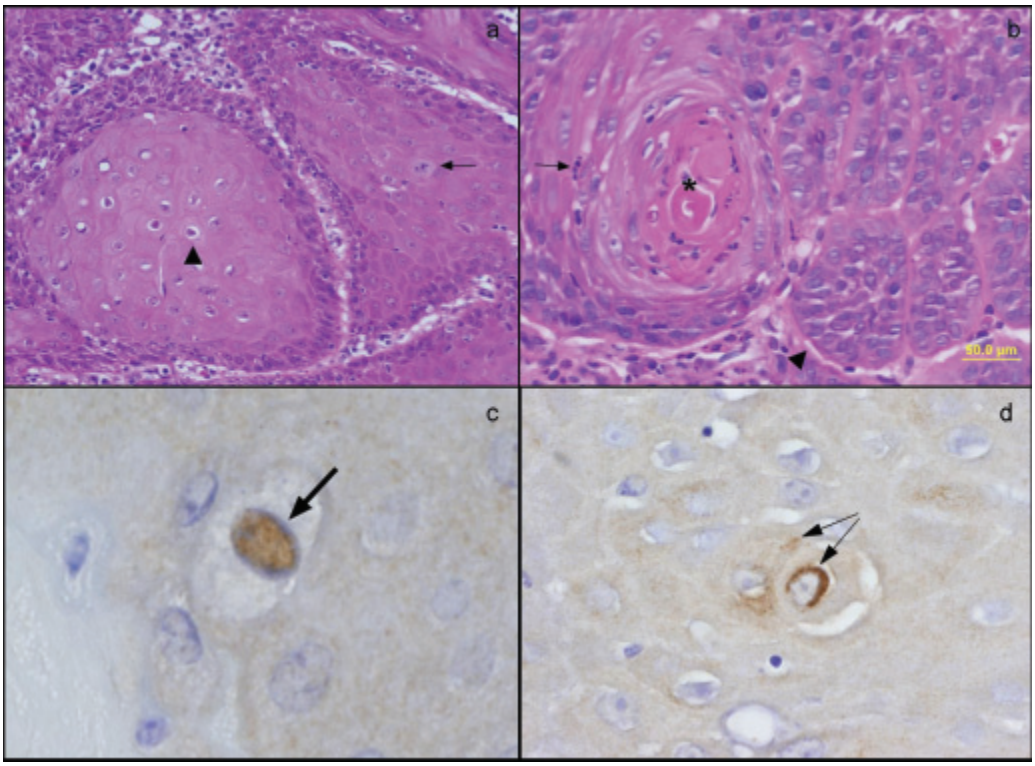

Fig 3: Viral papilloma with carcinoma* in situ.** ***a) H&E stain of an island of squamous epithelium that demonstrates viral cytopathic effect, characterised by koilocytes with intranuclear inclusions (arrowhead), as well as malignant transformation characterised by atypical mitoses within the spinous layer (thin arrow), x20. b) H&E stain showing formation of epithelial nests (arrowhead) and keratin pearls (asterisk), and individual cell necrosis (thin arrow), x40. c) Intranuclear papilloma viral antigen expression (arrow) detected within an epithelial cell by indirect immunohistochemistry, x60. d) Dense perinuclear and punctate cytoplasmic antigen (double arrows) detected by indirect immunohistochemistry, x60.

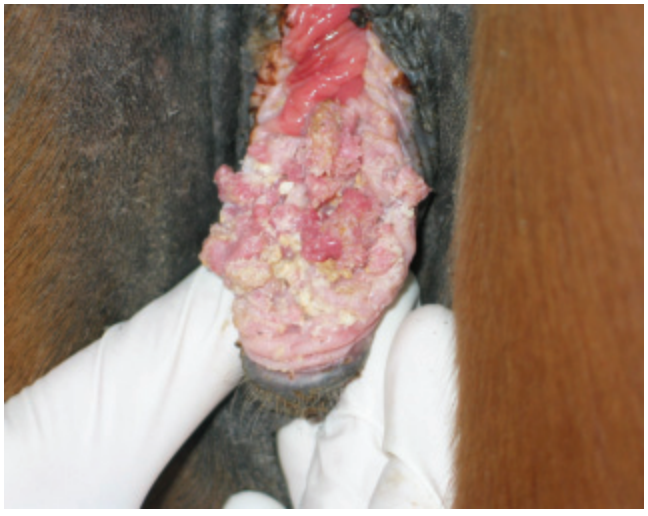

Fig 4: Recurrence of the papillomas with carcinoma* in situ *(image taken 5 months after initial surgical resection).

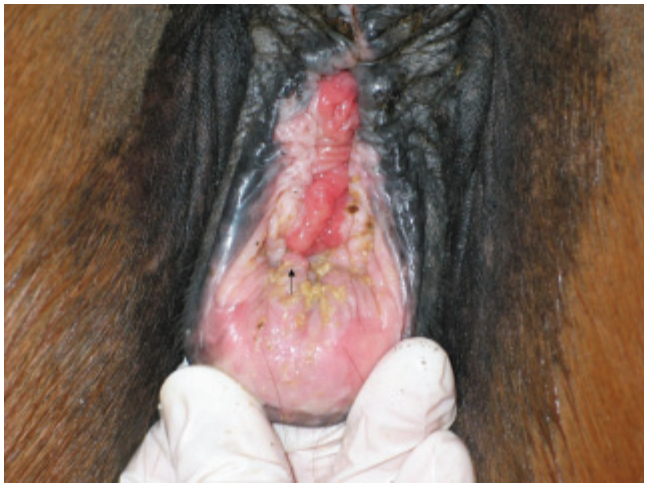

Fig 5: Recurrence of papillomas 2 months after second surgery (arrow). Histopathology of this lesion indicated viral papilloma with no signs of carcinoma.

large central mass (**Fig 4**). The mare was treated preoperatively with phenylbutazone (4.4 mg/kg bwt i.v.) and procaine penicillin G (22,000 iu/kg bwt i.m.). The second surgical procedure was performed in a similar fashion to the previously described surgical excision. Due to the post surgical recurrence, greater efforts were made to photoablate deeper into the submucosa and achieve wider margins. Tissue submitted for histological evaluation revealed similar exophytic papillomatous proliferations with foci of dysplasia and squamous cell carcinoma *in situ*. Demonstration of viral cytopathic effect and immunohistochemically-detected papilloma viral antigen expression was also present within the keratinocytes of this biopsy. The mare was discharged the following day with recommendations to return in 30 days for a follow-up examination. No further medical or adjunctive treatment was recommended.

Two months later the mare returned for re-evaluation. Examination of the vulva revealed grossly normal tissue except for 2 small (<2 mm diameter) papillomatous masses in close proximity to the clitoral fossa. Laser resection was performed using standing sedation and local anaesthetic. Histopathology of these lesions revealed papillomas with viral cytopathic effect and foci of dysplastic epithelium; however, carcinomatous lesions were not observed. Papillomaviral antigen expression within keratinocytes was detected by immunohistochemistry. Recommendations were made for a follow-up examination in 4–6 months. Examination of the vulva 2 months after the last surgery showed no evidence of recurrence of the lesions.

Discussion

Papillomaviruses are double-stranded DNA viruses that affect the stratified squamous epithelium. These viruses are highly species and site specific pathogens (Jackson 2003). In horses, the lesions resulting from papillomaviral infection (viral papillomatosis and aural plaques) are characterised as benign, proliferative, epithelial, cutaneous neoplasms (Scott and Miller 2003). The lesions of equine viral papillomatosis occur in young horses on haired skin as well as mucocutaneous junctions. They can vary from solitary to multiple, and typically undergo spontaneous regression (Jackson 2003). Cutaneous papillomas are occasionally observed in equine fetuses and neonates; however, neither papilloma viral cytopathic effect nor viral antigen expression has been detected within these proliferations, thus they are considered incidental congenital lesions (White *et al.* 2004).

Viral-induced papillomas are contagious, and transmission can occur directly by intradermal injection or indirectly via fomites (Scott and Miller 2003). Natural transmission most likely requires damaged skin, such as that which occurs from environmental trauma, ultraviolet light and ectoparasites. Lesions on the penis or vulva can result in transmission of the equine papillomavirus through breeding (McCue 1998).

Although papillomaviruses are generally host-specific, multiple strains of papillomavirus can affect one given host species. Over 100 antigenically distinct papillomaviruses affect people, and 6 are known to cause infection in cattle (Carter *et al.* 2005). At this time, only one type of equine papillomavirus (EPV) has been sequenced; the nucleotide sequence of *Equus caballus papillomavirus* type 1 (EcPV-1) was determined using DNA obtained from an equine cutaneous papilloma (Ghim *et al.* 2004). However, 2 different studies failed to find the presence of this EcPV-1 DNA in 8 different equine genital papillomas, suggesting the existence of a novel EPV type (O'Banion *et al.* 1986; Postey 2007). The possibility should be considered that a different strain of EPV can specifically infect the external genitalia of horses, much like the papillomaviral infection that causes canine footpad and interdigital papillomas.

There has been much research into the role of papillomaviruses in oncogenesis. The papillomaviral genome encodes 3 oncoproteins (E5, E6 and E7) which interfere with the function of cellular proteins involved in cell-cycle control, such as p53 (Campo 2003). In addition to the subtypes of HPV that have been implicated as causes of human cervical cancer, bovine papillomavirus (BPV), the only papillomavirus known to cause cross-species infection, has been associated with the pathogenesis of sarcoids, the most common equine cutaneous neoplasm (Nasir and Reed 1999). BPV infection not only causes papillomatosis of the skin, teats, udder and penis of cattle, but infection also causes papillomatosis and neoplasia of the upper gastrointestinal tract and urinary bladder in cattle. Malignant transformation of papillomas to SCC *in situ* and invasive SCC and other malignant neoplasms, such as haemangiosarcoma, can develop in cattle in some cases, and is thought to result from interactions between the virus, chemical carcinogens and immunosuppressants present in bracken fern (Campo 1997). The development of urinary neoplasia in cattle infected with specific strains of BPV illustrates the multifactorial origin of some papillomaviral-induced neoplasms.

Differences in immunocompetency and host genetic constitution can also influence disease persistence and progression. It is thought that host immunosurveillence is required for regression of papillomatous lesions, thus immunosuppression is suspected in horses chronically affected by viral papillomatosis (Williams 1997). In human X-linked severe-combined immunodeficiency disease (X-linked SCID), and in the canine model of this disease, cutaneous and mucosal (oesophageal) papillomas frequently occur and persist; a proportion of these lesions undergo malignant transformation to SCC *in situ* (Bowen's disease) and invasive SCC (Goldschmidt *et al.* 2006). The impact of the host's genetic constitution on the course of disease is exemplified by cottontail rabbit papillomaviral (CRPV) infection. In cottontail rabbits, CRPV infection results in the proliferation of papillomas,

the vast majority of which persist in a benign state. This is in contrast to CRPV infection of domestic rabbits, which results in the majority of papillomas progressing to a more aggressive neoplasm, although among domestic rabbits the rate of neoplastic progression differs (Kreider and Bartlett 1981). Genetic studies have shown that rabbits with a certain MHC class II haplotype are more susceptible to tumour progression than rabbits with a different haplotype (Han *et al.* 1992). This association between different MHC class II haplotypes and increased risk of neoplastic progression has also been observed in man (Wank and Thomssen 1991).

This report describes the diagnosis and treatment of vulvar papillomatosis in a mare that was unusually old (25 years) for the development of this disease. The duration of the disease prior to her diagnosis is unknown. A review of the literature on viral papillomatosis in horses revealed numerous reports of genital papillomas in both male and female horses. In the one report that specifically noted the age of the affected horses, the mean age of horses with viral papillomas (4 years) was significantly less than that of horses with genital papillomas (17 years) (Valentine 2006). The difference in the mean age of these 2 groups of horses also suggests that a different strain of EPV is involved in the aetiology of genital papillomas.

It is possible that the existence and/or persistence of infection with papillomavirus in this case were due to altered immune function. Pituitary *pars intermedia* dysfunction (PPID) is common in horses >15 years of age, and immunosuppression induced by elevated levels of circulating endogenous glucocorticoids increases susceptibility of affected horses to secondary infections (Dybdal 1997; Beech 1999). Although this mare did not exhibit any of the phenotypic or clinical signs of PPID, additional recurrence of the papillomas would warrant investigation into concurrent disease as a possible cause for immunosuppression. In this mare, we could speculate that age-related immunosuppression, or that her particular genetic constitution, contributed to her susceptibility to infection, the persistence of the lesions, and/or the malignant progression.

Papillomas and SCCs have a similar site distribution on the horse, with overlapping age distributions (Junge *et al.* 1984). SCC is the most common neoplasm of the equine external genitalia (Scott and Miller 2003). The average age of mares affected with SCC is 11.9 years, but all ages can be affected. Suggested causes of SCC in mares include decreased hair and skin pigmentation, ultraviolet light, chronic irritation, trauma and papillomaviruses (McCue 1998). Although papillomaviruses have been implicated in the aetiology of SCCs in man, dogs and cats, papillomaviral structural antigens were not detected in 90 equine SCCs tested in one retrospective study, including 4 SCCs of the female genitalia (Junge *et al.* 1984). In a different retrospective study examining SCC of the equine penis, squamous papilloma (SP) in addition to SCC was identified in 12/41 (29%) cases, and 5 of the 12 SPs showed premalignant features on histological examination (Howarth *et al.* 1991). As a result, Howarth *et al.* (1991) concluded that any papillomatous lesion present on the equine penis should be considered a potential premalignant proliferation.

Diagnosis of equine viral papillomatosis is usually based on distribution and clinical appearance of the lesions, but immunohistochemical detection of viral antigen within biopsy tissue can confirm the disease (Scott and Miller 2003). In this case distinct antigen expression was detected in many cells within these lesions; however, the distribution of antigen staining was not always typical for papilloma (described as diffuse nuclear staining of koilocytes). In a study performed on Cynomolgus monkeys with VAIN and CIN lesions, immunohistochemical staining for papillomavirus using both polyclonal and monoclonal antibodies directed against BPV antigens and HPV antigens, respectively, demonstrated epithelial-specific, selective nuclear and/or cytoplasmic staining patterns (Wood *et al.* 2004). Although structural proteins of papillomavirus are transcribed and assembled in the host cell nucleus, these proteins are preassembled in the cytoplasm and translocated to the nucleus via complex interactions between host cell molecular chaparones, or heat shock proteins, such as Hsc70 (Florin *et al.* 2004). In addition, HPV protein expression is reported to change with malignant transformation of papilloma lesions and transformation to invasive carcinoma (Davy *et al.* 2005). Given these 2 studies, one could speculate that the variable expression patterns seen in our case may reflect either a novel or aberrant form of an EPV, altered expression pattern related to duration of infection or malignant progression, or specific host genetic make-up or immunological status.

Treatment of equine viral papillomatosis is rarely indicated since lesions typically spontaneously resolve in 3–4 months. If treatment is opted for aesthetic or health reasons, surgical excision or cryosurgery is effective, and anecdotal evidence exists for successful treatment using a number of topical agents, autogenous tumour cell vaccines, as well as i.v or intralesional immunostimulant treatments (Scott and Miller 2003). In the case described in this report, surgical therapy was pursued due to neoplastic transformation of virally-infected cells. Carcinomas of the vulva and perineum are locally aggressive, invading surrounding tissue and metastasising to local lymph nodes and occasionally the lungs in later stages of the disease (McCue 1998).

Initial therapy for this malignant lesion consisted of removal of all grossly abnormal tissue and injections of 5-FU into the surrounding and underlying tissue. 5-Fluorouracil is a fluorinated pyrimidine antimetabolite that interferes with nuclear DNA biosynthesis, leading to cell death or increased susceptibility to the immune system (Théon 1998). Topical 5% 5-FU cream has been used successfully in

the treatment of SCCs of the external genitalia of horses with and without prior cytoreductive surgery (Fortier and Mac Harg 1994). Treatments in this case were performed using the 5-FU injectable solution at 3 week intervals, instead of daily application of the cream formulation. Because gross papillomatous lesions recurred within 3 weeks of the initial treatment, chemotherapeutic treatment was discontinued in lieu of more extensive laser excision and photoablation, which to date has shown more favourable results.

This Case Report demonstrates that viral-induced genital papillomas in mares should be considered potential precarcinomatous lesions. The findings in this mare support the assertion that biopsies of all genital papillomas should be performed. Treatment of this case also suggests that aggressive resection with wide margins is needed for resolution of the lesions as tumour resection and adjunct chemotherapy did not resolve the condition. This case also demonstrates the need for further evaluation of equine papillomas, including papillomaviral genotype analyses and characterisation of lesions and/or lesion progression.

Manufacturer's address

[1]PhotoMedex Surgical, Montgomeryville, Pennsylvania, USA.

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