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Invited review

Papillomavirus infection and squamous cell carcinoma in horses

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ABSTRACT

Squamous cell carcinoma (SCC) is a common disease that seriously impairs the health and welfare of affected horses and other equids. In humans, almost all cervical carcinomas, a high percentage of anogenital SCCs and a subset of SCCs of the head and neck are caused by high-risk human papillomavirus (hrHPV) infection. Since hrHPV-induced human cancers and equine SCC have similar cytological and histopathological features, it has been hypothesised that equine SCCs could also be induced by papillomaviruses. This review provides an overview of the current evidence for an aetiological association between papillomavirus infections and equine SCCs and SCC precursor lesions. SCC of apparently papillomavirus-unrelated aetiology are also discussed, as are recent advances in equine SCC prophylaxis.

Introduction

Squamous cell carcinoma (SCC) is frequently diagnosed in horses and other equids. It is the most common malignant skin neoplasm in horses, with an incidence of about 7–37% of equine skin tumours. Equine SCCs arise from cutaneous or mucosal keratinocytes. In the initial stages, lesions can present as whitish plaques or papillomas that can progress to carcinoma in situ (CIS) and ultimately, to invasive SCC (Scott and Miller, 2003; Knottenbelt, 2009). SCC and related precursor lesions can affect the skin and mucosa at any site, but preferentially develop on non-pigmented skin and at mucocutaneous junctions, such as the ocular region and the external genitalia (Scott and Miller, 2003; Van den Top et al., 2008a; Knottenbelt, 2009). Treatment mainly consists of wide surgical excision of the affected tissue; however, this is not always indicated or feasible, e.g. in case of large, metastasising lesions or inaccessible tumours, such as SCCs of the sinus or the parapharyngeal region (Mair et al., 2000; Scott and Miller, 2003; Van den Top et al., 2008a; Knottenbelt, 2009). Although equine SCC is a common tumour that seriously impairs the health and welfare of affected animals, the biological mechanisms underlying tumour development and progression are still unclear. The identification of aetiological agents and factors involved in equine SCC carcinogenesis is complex because lesions are not uniform e.g. equine sarcoids, but have variable, sometimes unpredictable causes, depending on the tumour site (Knottenbelt, 2009; Nasir and Brandt, 2013).

Papillomaviruses and cancer

Papillomaviruses (PVs) are relatively small, non-enveloped viruses that consist of an icosahedral capsid that harbours a circular, double-stranded DNA genome. The capsid is composed of 72 major capsid protein L1 pentamers termed capsomeres, and a minimum of 12 minor capsid protein L2 monomers. The viral genome contains open reading frames (ORF) for early (E) regulatory (E1, E2 and E4) and up to three transforming proteins (E6, E7 and frequently E5), and for the late (L) capsid proteins L1 and L2. A non-coding long control region (LCR) is located between the L1 and E6 ORFs and is responsible for viral replication and transcription (Campo, 2006b). The productive life cycle of PVs is tightly linked to the differentiation of keratinocytes. De novo infection occurs via micro-abrasions which allow virions to gain access to basal keratinocytes presenting the necessary surface molecules for virion attachment and endocytosis (Giroglou et al., 2001; Day et al., 2003; Spoden et al., 2008). Following virion disassembly, the virus expresses its early regulatory and transforming genes in the basal and suprabasal epidermal layers (Doorbar, 2005). In the differentiated cells of the spinous and granular layer, the viral genome replicates by 10–100 times. The major L1 and the minor L2 capsid proteins are expressed in the granular and final squamous layers. The genomes are then packaged by directed assembly of L1 and L2 into virions which are shed via desquamation (Graham, 2006). PV infection is not always productive. The viral genome can be maintained in infected

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Table 1
Equus caballus papillomavirus type 2 (EcPV2) detection rates in precancerous lesions and SCC using PCR (2010-2014).

Study by	PCR type	FF	Material type	Sample information	EcPV2+
Scase et al. (2010)	EcPV2 L1-specific PCR	Yes	SCC-related	13 penile papillomas	11/13
				12 penile SCC	6/12
				4 vulval papillomas	4/4
				1 vulval papilloma	1/1
				1 vulval CIS	1/1
				4 vulval SCC	3/4
			Other lesions	1 sarcoid	0/1
			Normal	1 intact skin	0/1
	EcPV2 E6- and E1-specific PCR	No	SCC-related	1 penile CIS	0/1
				4 penile SCC	4/4
				10 ocular SCC	0/10
			Normal	27 smegma samples	0/27
				13 scrotal tissue samples	0/13
Knight et al. (2011a)	PV L1 broad-range PCR	Yes	SCC-related	20 penile SCC	9/20
			Other lesions	20 other penile lesions	1[a]/20
Bogaert et al. (2012)	EcPV2 E1-, L1- and LCR-E6-E7-specific PCR	No/Yes	SCC-related	4 penile papillomas (3/4 FF)	4/4
				8 PeIN	8/8
				16 penile SCC	15/16
				2 adjacent lymph nodes (FF)	1/2
				2 vulval SCC	2/2
				1 anal SCC	1/1
				1 oral SCC metastasis (FF)	1/1
			Normal	39 penile swabs	4/39
				20 vulvovaginal swabs	0/20
Sykora et al. (2012)	EcPV2 E6-specific PCR	No	SCC-related	1 penile papilloma	1/1[c]
				1 penile CIS	1/1[c]
				4 penile SCC	4/4[c]
				1 adjacent lymph node	1/1[c]
				1 vulval SCC	1/1[c]
			Normal	30 ocular swabs	1/30
				94 genital swabs	4/94
				54 semen samples	0/54
				15 milk samples	0/15
Lange et al. (2013a,b)	PV E1 and L1 broad-range PCR, EcPV2-specific PCR	Yes	SCC-related	7 penile papillomas	7/7
				7 penile CIS	7/7
				10 penile SCC	10/10
			Other lesions	11 other penile lesions	6/11[b]
Knight et al. (2013)	EcPV2 L1-specific qPCR	Yes	SCC-related	20 penile SCC	16/20
				20 oropharyngeal SCC	3/20
		Yes	Other lesions	19 other penile lesions	3/19
		No	Normal	32 penile mucosa	2/32
				40 vulval mucosa	1/40
				75 oral mucosa	2/75
				75 ocular mucosa	0/75
Newkirk et al. (2014)	EcPV2 E1-specific PCR	Yes	SCC-related	22 penile SCC	10/22
				42 ocular SCC	0/42
Fischer et al. (2014)	EcPV2 E7-E1-specific PCR	No	Normal	50 genital swabs	9/50
Van den Top et al. (2015)	EcPV2 E1- and L1-specific PCR	Yes	SCC-related	16 penile/preputial papillomas	14/16
				9 penile/preputial CIS	8/9
				78 penile/preputial SCC	69/78

FF, Formalin-fixed; SCC, Squamous cell carcinoma.
[a] A case of balanoposthitis.
[b] Case breakdown: $n = 3/4$ balanoposthitis, $n = 1$ melanoma, $n = 1$ amyloidosis, $n = 1$ follicular cyst.
[c] E6 DNA and mRNA.

cells as multiple episomes that replicate in synchrony with the cell cycle (Chambers et al., 2003), or integrate into the host cell genome (Doorbar et al., 2012).

Early research in natural animal models has revealed that PVs are usually species-specific and have a pronounced tropism for specific cellular environments, i.e. cutaneous and/or mucosal keratinocytes. Bovine Delta-papillomavirus types 1, 2 and 13 are an exception to this rule in that they can also infect fibroblasts and other ungulate species in addition to cattle (Campo, 2006a; Lunardi et al., 2013).

Thus far, more than 160 human PV (HPV) types have been sequenced completely[1] (De Villiers, 2013). From these, 15 types have been identified as carcinogenic in humans and are therefore termed high-risk HPVs (hrHPVs; Munoz et al., 2003; Parkin, 2006; De Martel et al., 2012; Forman et al., 2012). In contrast, only a limited number of equid PV types (1–7) are known to date (Scase et al., 2010; Lange et al., 2011, 2013b; Nasir and Brandt, 2013). Consequently, the aetiology of various potentially PV-induced cutaneous and mucosal cancers in equids remains to be established. In addition, there are currently no vaccines to protect horses from confirmed PV-induced tumours. In humans, it is currently accepted that infection by mucosal hrHPV types causes virtually all cervical cancers, most anal cancers, up to 50% of vulval, vaginal and penile cancers, and about 25% of head and neck SCCs (HNSCCs; Zur Hausen, 1996, 2000; Dayyani et al., 2010). E6 and E7 are the major viral oncoproteins driving HPV-induced carcinogenesis. Malignant transformation of persistently infected epidermal cells is achieved by complex interactions between these

[1] See: The PapillomaVirus Episteme. https://pave.niaid.nih.gov (accessed 16 May, 2017).

oncoproteins and cellular factors involved in cell-cycle regulation (Feller et al., 2010a,b). Cytologically and histologically, hrHPV-induced human genital precancerous lesions and SCC each display characteristic features. Precancerous lesions mainly include warts (condylomas) and intraepithelial neoplasia. Genital warts are usually benign exophytic lesions with koilocytosis, infected by low-risk (lr-), i.e. non-carcinogenic HPV types. However, some warts also show cytologic atypia, abnormal mitotic figures, contain intranuclear inclusion bodies and have the potential to progress to SCC. This subset of warts is hrHPV-induced and precancerous (Cubilla et al., 2016). HrHPV-associated penile intraepithelial lesions (PeIN) present as warty or basaloid neoplasms or, in case of warty-basaloid lesions, as a mixture of both, consisting of immature, atypical basaloid-type keratinocytes with intranuclear viral inclusion bodies, and a condylomatous surface (Stratton and Culkin, 2016). Interestingly, there is also emerging evidence for the existence of human penile plaques, i.e. hrHPV-positive flat whitish acanthotic lesions with mild koilocytosis, that could represent a link between penile warts and PeIN (Cubilla et al., 2004, 2016). Similarly, penile plaques have been recognised as SCC precursor lesions in horses (Scott and Miller, 2003; Van den Top et al., 2008b; Knottenbelt, 2009; Scase et al., 2010). Like human penile precancerous warts, equine genital papillomas show acanthosis and koilocytes and contain intranuclear bodies consistent with viral inclusions. Equine genital SCCs closely resemble their human hrHPV-associated counterpart in that they comprise hyperproliferative neoplastic keratinocytes of basaloid type that invade the subepithelial stroma. In combination, these findings suggest that equine genital SCC and precursor lesions, i.e. plaques, papillomas and CIS, are also induced by PVs (Scott and Miller, 2003; Cubilla et al., 2004; Scase et al., 2010; Chaux et al., 2012a,b; Velazquez et al., 2012).

Equine papillomavirus type 2 and equine genital SCC

In 2010, Scase et al. reported a novel equine papillomavirus, *Equus caballus* papillomavirus type 2 (EcPV2), from equine genital epithelial lesions. Sequence analysis revealed open reading frames for the oncoproteins E6 and E7, the regulatory proteins E1, E2 and E4, and the capsid proteins L1 and L2. Analyses of the predicted E6 and E7 oncoproteins demonstrated some of the amino acid sequence motifs observed in other PV species (Bernard et al., 2010). The E6 ORF contained two CXXC-X_{29}-CXXC motifs known to act as zinc finger DNA binding domains (Bolivar et al., 1999). Additionally, a PDZ binding domain (XS/TXV/L) was identified at the E6 C-terminus, a characteristic feature of hrHPV types, which is absent in low-risk HPVs (Gardiol et al., 1999). The predicted E7 protein sequence contained a single CXXC-X_{29}-CXXC motif enabling zinc finger DNA binding, but lacked the retinoblastoma binding domain (LXCXE) present in various carcinogenic PVs (Wise-Draper and Wells, 2008). Since no PV L1 sequence sharing more than 60% nucleotide homology with the EcPV2 L1 gene could be retrieved from Genbank, EcPV2 was assigned to a new PV genus, i.e. Dyoiota papillomavirus (Bernard et al., 2010; Scase et al., 2010).

Multi-institutional, international screening studies of equine genital SCCs and precursor lesions (plaques, papillomas, CIS) and smegma from SCC-affected horses revealed EcPV2 DNA and transcripts in up to 100% of genital lesions and smegma. However, EcPV2 DNA and transcripts were only demonstrated in a low percentage of samples obtained from healthy horses (Table 1). EcPV2 DNA was detected in 5/6 penile SCC and 1/1 clitoral SCC, but in 0/2 ocular SCC (by EcPV2 E6 in situ hybridisation; ISH), and in 16/21 equine genital premalignant lesions and SCC in the UK. In Australia, consensus PCR with degenerate PV L1-primers led to the identification of EcPV2 DNA in 10/14 genital SCCs and precursor lesions. In Austria, 4/4 penile SCCs and one adjacent lymph node, but 0/10 ocular SCCs were positive by EcPV2 E6-specific PCR. All 40 samples collected from healthy horses (27 smegma and 13 scrotal skin samples) included in that study tested negative for EcPV2 E6 DNA (Scase et al., 2010). Similar results were obtained in Belgium, where EcPV2-specific PCR-screening of 30 genital lesions revealed EcPV2 DNA in 29/30 tumours, but only 4/39 penile swabs and 0/20 vaginal swabs collected from apparently healthy horses tested EcPV2 DNA-positive using this assay (Bogaert et al., 2012). Similarly, only 5/193 samples (2.6%) obtained from tumour-free horses in Austria were positive by EcPV2 E6 PCR. Interestingly, EcPV2 infection in four of these five horses was associated with the animals being co-stabled with horses diagnosed with genital SCC. In the same study, 8/8 genital SCCs and precancerous lesions also tested positive for transcripts of the EcPV2 E6 oncogene, suggesting an active pathogenic role for EcPV2 in equine genital tumour development (Sykora et al., 2012). In 2015, van den Top et al. detected EcPV2 DNA in 91/101 preputial and penile lesions and demonstrated that E6, E2 and L1 were transcribed intralesionally. Additionally, p53 expression and metastasis to poorly differentiated SCC were positively correlated (Van den Top et al., 2015).

Other groups reported lower EcPV2 detection rates in equine genital lesions. Using degenerate PV L1 primers MY09 and MY11 for PCR screening, Knight et al. (2011a) detected EcPV2 DNA in 9/20 penile SCCs and 1/20 other SCC-unrelated penile tumours, i.e. a sample of chronic, ulcerative and granulomatous balanoposthitis (Knight et al., 2011a). The same group also described an interesting case of EcPV2-positive persistent and diffuse papilloma formation on the penis of a horse (Knight et al., 2011b). In 2013, this group evaluated the EcPV2 L1 DNA loads in equine penile and oropharyngeal SCC vs. SCC-unrelated penile lesions and genital, oral and ocular mucosa from healthy horses. The results of this study clearly confirmed earlier observations that EcPV2 is an oncogenic rather than a commensal virus, as 16/20 penile SCCs contained measurable to high amounts of viral DNA, while only 3/19 penile SCC-unrelated lesions and 5/222 mucosal samples obtained from tumour-free horses were positive by EcPV2 L1 qPCR. Interestingly, Knight et al. (2013) also detected the EcPV2 L1 gene in 3/20 oropharyngeal SCCs. Lange et al. (2013a,b) reported EcPV2 DNA detection in 24/24 genital SCC and precancerous lesions using EcPV2 ISH, and also in 6/11 penile SCC-unrelated lesions i.e. three cases of balanoposthitis, one melanoma, one follicular cyst and one case of amyloidosis, using PCR with degenerate PV E1 primers CP4 and CP5 and EcPV2-specific primers (Lange et al., 2013a). Detection of EcPV2 DNA in four cases of balanoposthitis (Knight et al., 2011a; Lange et al., 2013a) suggests that local inflammation might represent an initial stage of viral infection which induces a local cellular immune response in an attempt to clear the infection. The significance of EcPV2 DNA detection in the three other cases of SCC-unrelated genital lesions remains unclear. The latter could represent cases of transient or latent EcPV2 infection that have either been cleared or have reactivated. In 2014, Newkirk et al. reported that PCR with degenerate and EcPV2 E1- and BPV1-specific primers yielded EcPV2 sequences from 10/22 penile isolates, but none of 42 ocular SCC from formalin-fixed material. Finally, Zhu et al. (2015) used EcPV2 E6/E7 ISH to demonstrate EcPV2 DNA in 6/13 penile SCCs. The other 7/13 EcPV2-negative lesions were associated with UV radiation damage (Zhu et al., 2015). This finding is surprising because equine genitals are infrequently exposed to the sun because male horses keep the penis retracted in the sheath (Loeffler and Gaebel, 1971). Further work is needed to clarify the role of UV light as (co-)factor contributing to the development of equine genital SCC.

In summary, EcPV2 DNA has been detected in the vast majority of equine genital SCC lesions, but in only 6.3% of mucosal swabs or biopsies from apparently healthy individuals ($n = 315$; Table 1;

Scase et al., 2010; Knight et al., 2011a,b, 2013; Bogaert et al., 2012; Sykora et al., 2012; Lange et al., 2013a; Fischer et al., 2014; Newkirk et al., 2014; Zhu et al., 2015).

Reported variations between studies in PCR EcPV2 DNA detection rates in genital SCC lesions are probably attributable to differences in tissue processing and PCR protocols. Lower EcPV2 DNA detection rates have been reported for tumour DNA extracted from formalin-fixed material (Knight et al., 2011a; Newkirk et al., 2014). Prolonged (>1 week) storage of tissue in formaldehyde can lead to nucleic acid fragmentation into 300–4000 bp fragments; (Godfrey et al., 2000; Lehmann and Kreipe, 2001). Even when using PCR primers spanning short EcPV2 regions, formaldehyde-mediated DNA fragmentation can impair amplification and lead to false negative results (Lehmann and Kreipe, 2001). Additionally, several groups have used degenerate PCR primers that recognise conserved PV sequences within the L1 major capsid gene, such as primer pairs FAP59/FAP64 or MY09/MY11 (Knight et al., 2011a; Lange et al., 2013a; Newkirk et al., 2014) or ECPV2 L1-Specific primers (Knight et al., 2013). In hrHPV-induced human cervical, anogenital and oropharyngeal tumours, viral DNA has been shown to integrate into the host cell genome. Integration is usually accompanied by a loss of L1 and L2 capsid gene sequences, E2 and occasionally a portion of E1 (Thorland et al., 2003; Campo, 2006b; Xu et al., 2015). Preliminary PCR analysis of EcPV2 E6 and E2 qPCR also suggest that EcPV2 is integrated into the equine host cell genome (Sykora et al., 2017). If EcPV2 integration occurred in infected tumour cells, there could be loss of L1 and therefore failure to detect this major capsid gene using an L1 PCR system. Finally, there could have been variations in the sensitivity of the different PCR protocols used.

Interestingly, different variants of the EcPV2 E6 gene have been identified in genital SCCs and precursor lesions. These variants were not tumour type- or site-specific, but showed a clear geographical distribution (Kerscher, 2011). Almost half of the geldings presented between 2009 and 2016 at the Equine University Clinic in Vienna with genital SCC precursor lesions or SCC (16/34) for which EcPV2 screening was performed were of Icelandic breed. Of these, nine Icelandic horses had come from Iceland (S. Brandt, personal communication). Interestingly, all lesions from these imported horses contained a specific EcPV2 E6 variant (GenBank accession No. KX349721), which might have evolved in Iceland during 800 years of strict isolation on the island. It remains to be determined whether this variant has an adaption-mediated fitness advantage in Icelandic horses, and whether associated genital tumours are more common in Iceland than other European countries (Scase et al., 2010; Kerscher, 2011; Brandt et al., 2012).

To date, it is unclear how EcPV2 is transmitted between equids and how long the delay is between initial infection and tumour development. In humans, hrHPV infection is mainly transmitted by sexual contact. Most hrHPV infections are either cleared or take a subclinical course. HrHPV-induced neoplastic progression is a rare event that takes place many years after initial infection (Bosch et al., 2006). Reports of subclinical EcPV2 infection in healthy horses living in direct contact with horses with EcPV2-positive SCC suggests transmission of infection via contaminated fomites, flying insects feeding on lesions, and/or smegma (Sykora et al., 2012). This hypothesis is substantiated by the presence of virion-like structures in about 51% of tested genital tumour samples (0/1 penile plaque, 1/1 penile papilloma, 1/2 penile CIS, 8/15 penile SCCs and 0/1 related inguinal lymph node, 0/1 clitoral SCC swab, 2/2 vulval SCC). In addition, virion-like structures were detected in 2/4 smegma samples from EcPV2-positive equine penile papillomas or horses with SCC, and there were large amounts of viral DNA in these smegma samples (>400,000 and copies per cell in both cases; Sykora et al., 2017). Therefore, smegma could represent an important reservoir of infectious virions responsible for continuous re-exposure of the host or vector-mediated de novo infection of stable mates. This theory is supported by the early observation that regular genital hygiene can prevent precursor lesions from progressing to malignant SCC (Pascoe and Knottenbelt, 1999). Additionally or alternatively, contaminated fomites and/or flying insects feeding on lesions or tumour debris could act as a source of EcPV2 infection, as proposed for equine sarcoid-inducing bovine papillomaviruses of types 1 and 2 (BPV1, BPV2; Kemp-Symonds, 2000; Bogaert et al., 2005; Finlay et al., 2009). The finding of Iceland-specific EcPV2 E6 variants in genital SCC-affected Icelandic horses that had left the island many years before the onset of disease indicates a long delay between initial EcPV2 infection and tumour development, similar to what was shown retrospectively for HPV-infected human cervical cancer patients (Bosch, 2006; Sykora et al., 2012). The factors promoting a shift from latent infection to virally induced carcinogenesis are thought to be manifold and remain to be elucidated; in the horse this issue has not yet been addressed. EcPV2-specific antibody titres were identified by two groups using ELISA (Fischer et al., 2014) or pseudovirion (PsV) neutralisation assays (Schellenbacher et al., 2015) in 6/6 genital SCC-affected and 21/79 healthy horses (Table 2), indicating that equids might sometimes be able to clear infection by mounting effective humoral and cellular immune responses. This is similar to HPV infections, which are usually cleared in young women within 1 or 2 years (Bosch, 2006; Smola-Hess and Pfister, 2006; Stanley, 2006). In some equids however, EcPV2 can evade immune surveillance and establish a latent infection that can become acute in older animals with declining immune systems. This agrees with observations that the shift from latent to pathogenic hrHPV infections in humans can take at least 15 years (Smola-Hess and Pfister, 2006), and also fits with reports that genital SCCs mainly develop in older horses (Scott and Miller, 2003; Van den Top et al., 2008a; Knottenbelt, 2009; Nasir and Brandt, 2013). Current understanding of PV infections in humans and animals suggests that the immune system, notably cell-mediated immunity, has a major impact on the course of infection (Zur Hausen, 2000; Bosch et al., 2006; Smola-Hess and Pfister, 2006; Stanley, 2006; Stanley et al., 2008; Fischer et al., 2014; Schellenbacher et al., 2015; Brandt, 2016).

Table 2
Equus caballus papillomavirus type 2 (EcPV2) specific serum antibodies (Ab) in tumour-affected and healthy horses.

Study by	Sample source (*n*)	EcPV2 PCR		EcPV2 Ab detection	
		DNA source	Positive	Method	Positive
Fischer et al. (2014)	Tumour-free horses (50)	Genital swabs	9/50	ELISA	18/50[a]
Schellenbacher et al. (2015)	Horses with penile SCC (4)	SCC	4/4	PsV	4/4
	Horses with vulval SCC (4)	SCC	2/2	PsV	2/2
	Tumour-free horses (29)	Genital swabs	2/29	PsV	3/29[b]

SCC, Squamous cell carcinoma.
[a] Fourteen of 50 were EcPV2 PCR negative and 4/50 were EcPV2 PCR positive.
[b] Two of these three were EcPV2- positive Icelandic horses co-stabled with horses of the same breed with penile SCC (Sykora et al., 2012).

Histopathological findings, the presence of a PDZ binding domain in E6 (Scase et al., 2010) and intralesional transcription of this major oncogene (Sykora et al., 2012; Van den Top et al., 2015), and intralesional virus detection rates all indicate that EcPV2 might be causally involved in the development of genital precancerous lesions in male and female horses and their progression to SCC.

In 1992, Fairley et al. demonstrated PVs in equine aural plaques using immunohistochemistry and electron microscopy (Fairley and Haines, 1992). Since the discovery of EcPV2, five additional EcPV types (EcPV3-7) have been identified and fully sequenced in equine lesions, notably aural plaques (Lange et al., 2011, 2013b; Gorino et al., 2013; Taniwaki et al., 2013; Van den Top et al., 2015; Zakia et al., 2016). Interestingly, van den Top et al. also detected EcPV3 DNA in a subset of precancerous genital lesions and SCC (9/101; Van den Top et al., 2015). These findings suggest that EcPV3-7 also transforms lesions in equids. However, more work is needed to establish a causal relationship between these novel EcPV types and the development of specific epithelial lesions in equids. To date, EcPV2 is the only equine PV suspected to be of high-risk type, i.e. able to induce metastasising tumours in the equid host.

Papillomavirus infection and non-genital SCCs

SCCs involving the eyelid, the nictitating membrane and/or the conjunctiva are the most common carcinomas in horses. In contrast to genital SCCs, which are typically seen in older geldings, ocular SCCs mainly occur in younger horses of both sexes 6–12 years of age (Scott and Miller, 2003; Knottenbelt, 2009). Lesions commonly appear as pink or red proliferative masses that can be surgically removed. In severe cases, enucleation of the affected eye might be the therapeutic approach of choice (Scott and Miller, 2003; Knottenbelt, 2009). Excessive UV radiation, non-pigmented skin around the eyes, and genetic predisposition are supposed risk factors for the development of ocular SCC (Scott and Miller, 2003; Knottenbelt, 2009). Several groups have attempted to identify novel or previously identified PVs as causative agents of the disease. However, analyses of ocular SCCs ($n = 52$) in different countries failed to demonstrate PV DNA, and therefore ocular SCCs are currently considered as a different entity that might not respond to the same therapeutics used for PV-induced tumours in equids (Hewes and Sullins, 2006; Scase et al., 2010; Bogaert et al., 2012; Sykora et al., 2012; Knight et al., 2013; Newkirk et al., 2014; Haspeslagh et al., 2016).

In human patients, hrHPV infections account for about 25% of SCCs of the head and neck (HNSCCs; Dayyani et al., 2010). Similarly, EcPV2 DNA was detected in 3/20 formalin-fixed (15%), and 4/15 (26.6%) native equine SCCs of the head (HSCC) in two different studies. EcPV2-positive HSCCs were located in the oral cavity, nasal cavity, sinuses or pharynx (Knight et al., 2013; Sykora et al., 2017). In addition, virion-like structures were detected in two of four EcPV2-positive equine HSCCs and in smegma from an HSCC-affected horse from which no tumour material was available (Sykora et al., 2017). The proportions of equine HSCC-related EcPV2 infections detected thus far (mean, 20.8%) are comparable to those reported for hrHPV-associated HNSCC. In these tumours, hrHPV infection correlated with E7-mediated over-expression of the cyclin-dependent kinase inhibitor p16 and lack of p53 mutations (Dayyani et al., 2010). Correspondingly, analysis of EcPV2-positive equine HNSCCs to investigate oncoprotein transcription and p16 expression could help determine a possible aetiological relationship between EcPV2 and some equine HSCCs, given adequate sample size. While the method of entry of EcPV2 into the equine oral cavity and nasal sinuses is currently unknown, possibilities include direct contact with contaminated fomites, such as bedding or hay shared with a stable mate with genital SCC-, and/or flying biting/sucking insects that commonly infest equine mucocutaneous junctions such as the genitals, muzzle and nostrils and feed on body fluids.[2]

Prophylactic vaccines

Animal and human PV L1 capsid proteins generated in vitro self-assemble spontaneously to empty capsids termed virus-like particles (VLPs). VLPs induce high titres of PV type-specific neutralising antibodies and confer effective and long-lasting protection from infection by the PV type included in the vaccine (Giles and Garland, 2006). To date, three VLP-based vaccines for protection of humans against hrHPV-induced cancers are available and have been effective in reducing the worldwide prevalence of HrHPV infections and cervical cancer (Angioli et al., 2016; Thomas, 2016). Growing evidence that EcPV2 has an active role in the pathogenesis of equine genital SCC has encouraged the development of an EcPV2 L1 VLP-based vaccine for the prevention of EcPV2 infection in equids (Schellenbacher et al., 2015). Following in vitro generation and validation of EcPV2 L1 VLPs, their protective potential was assessed in a murine intravaginal challenge model (Roberts et al., 2007), using EcPV2 PsVs harbouring a reporter plasmid as a virus surrogate. New Zealand rabbits were immunised with EcPV2 L1 or control BPV1 and HPV16 L1 VLPs, and immune sera was passively transferred to immunocompetent mice that were subsequently challenged intra-vaginally with EcPV2 PsVs. Importantly, passive transfer of rabbit EcPV2 L1 VLP immune sera conferred complete protection from experimental EcPV2 PsV infection, but transfer of control immune sera had no cross-protective effect (Schellenbacher et al., 2015). Based on these promising data, the safety and immunogenicity of EcPV2 L1 VLPs was addressed in the equine model. Intramuscular prime boost immunisation of 14 horses with a bivalent vaccine containing equal amounts of BPV1 and EcPV2 L1 VLPs in adjuvant was well tolerated and induced a robust type-specific antibody response (Hainisch et al., 2017). These findings suggest that EcPV2 L1 VLPs have potential as components of a prophylactic vaccine against EcPV2 infection and also equine tumours for which a causal association with EcPV2 infection can be confirmed. As mentioned above, the identification of specific EcPV2 E6 sequences in penile SCCs from Icelandic horses that developed the disease many years after their transfer to Austria, and the prolonged hrHPV latency before disease onset in humans (Bosch et al., 2006), suggest that EcPV2-infection can occur early in life. As a consequence, and because the mode of transmission of EcPV2 between equids is still unclear, the most effective strategy for VLP-mediated protection from EcPV2-infection in populations of horses might be to immunise both male and female foals.

Conclusions

During the past few years, there has been growing evidence that EcPV2 infection has a major pathogenic role in equine genital SCC. EcPV2 DNA, transcripts and putative virions have been detected in genital SCC and precursor lesions in male and female horses, but only in a small proportion of apparently healthy individuals, indicating that EcPV2 infection actively contributes to disease onset and progression. As previously suggested, smegma may act as a source of infection as it can harbour multiple copies of viral DNA and probably infectious virions. Recent data support EcPV2 L1 VLPs as potent vaccine candidates for the prevention of EcPV2 infection and associated neoplasia. EcPV2 might also

[2] See: Kaufman, P.E., Koehler, P.G., Butler, J.F., 2015. External parasites on horses. University of Florida, USA, Institute of Food and Agricultural Sciences, UF/IFAS Extension #ENY-283. http://edis.ifas.ufl.edu/ig139 (accessed 16 May, 2017).

induce a subset of equine SCCs of the head, but more work is needed to confirm this hypothesis. Ocular SCCs seem to represent a different disease process of currently unknown aetiology, but to date, there is no evidence that PVs are involved in this type of ocular neoplasia.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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