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A subset of equine oral squamous cell carcinomas are associated with *Equus caballus* papillomavirus 2 infection

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Abstract

The cause(s) of oral squamous cell carcinoma (SCC) in horses is unknown, but papillomavirus infection as well as chronic periodontal disease are suspected to play a pathogenic role. In humans, some oropharyngeal cancers develop in association with human papillomaviruses (HPVs). Equine caballus papillomavirus 2 (EcPV2) is suspected to play a causal role in development of equine genital SCC. Given that association, we hypothesized that EcPV2 is associated with the development of oral SCC in horses. We performed standard polymerase chain reaction (PCR) and *in situ* hybridization (ISH) for EcPV2 on 31 formalin-fixed paraffin embedded equine oral SCCs (lingual, gingival, palate) and 10 equine non-SCC oral samples. PCR for EcPV2 was positive in 10/31 (32%) oral SCCs while all non-SCC oral samples were negative. Intense hybridization signals for EcPV2 nucleic acid were detected by ISH within neoplastic epithelial cells in 8/31 (26%) oral SCC but not in the adjacent normal oral mucosa. No hybridization signals were detected within control samples. This study provides additional support for a pathogenic association of EcPV2 in oral SCC in horses.

Keywords

Horses; squamous cell carcinoma; *Equus caballus* papillomavirus; oncogenic viruses; oral cavity; tongue; *in situ* hybridization

Oral squamous cell carcinoma (SCCs), while uncommon overall, are the most common oral malignancy in horses, typically occurring in older animals and arising from the gingiva, tongue, larynx, pharynx, or palate.[1] Treatment depends on how extensive the disease is at the time of diagnosis, but may include surgical resection, iridium-192 brachytherapy, 5-fluorouracil, or intralesional cisplatin.[2] While the prognosis for survival is good if complete removal of the tumor is possible, metastasis can occur at later stages of the disease.[2] An underlying cause for equine oral SCC has not been identified, although it is thought

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Conflict of interest

The authors report no conflicts of interest.

to be associated with chronic periodontal disease and potentially papillomavirus infection.[1, 3] In humans, an increasing number of oropharyngeal cancers have been demonstrated to be associated with human papillomaviruses (HPVs). Diagnostic confirmation of HPV status in these cancers significantly impacts prognosis and treatment, as oropharyngeal cancers associated with HPV infection are often less clinically aggressive and do not require the aggressive treatment measures recommended for non-HPV head and neck cancers [4, 5] In addition to oropharyngeal cancer, certain HPV types have a well-established causal association with cervical cancer, other genital cancers, and a subset of other head and neck cancers.[4] There is increasing evidence that equine papillomaviruses may play a similar role in cancer development in horses.[3] *Equus caballus* papillomavirus type 2 (EcPV2) likely plays a role in development of a subset of genital squamous cell carcinomas, as EcPV2 DNA as well as viral oncogene transcripts have been frequently detected within a subset of genital SCC and precursor lesions.[6, 7, 8, 9, 10, 11] EcPV2 DNA has also been identified within some equine head and neck cancers using PCR techniques[6, 9, 12] and within equine gastric SCC[13] using PCR and ISH, supporting a possible pathogenic role for this virus in the development of a subset of head and neck as well as gastric cancer.

Given the association of EcPV2 with genital SCC and detection of EcPV2 DNA using PCR within some head and neck SCCs, we hypothesized that EcPV2 nucleic acid would be identified within neoplastic cells of oral SCCs. We performed both standard polymerase chain reaction (PCR) and *in situ* hybridization (ISH) for EcPV2 E6 on 31 archived equine oral SCC cases, which included 3 from the maxillary gingiva; 6 from the mandibular gingiva; 1 oropharyngeal; 1 subepiglottal; 7 from the lip; 5 from the tongue; 2 from the palate; 2 gingival, not otherwise specified; and 4 oral, not otherwise specified (Table 1). ISH to detect viral nucleic acid is preferable to immunohistochemistry (IHC) for papillomavirus, as ISH is more sensitive at detecting the presence of small amounts of virus within advance tumors, where production of viral proteins may be limited and thus non-detected with IHC. Formalin fixed paraffin embedded (FFPE) tissue samples were collected from archived biopsies and necropsies from North Carolina State University's College of Veterinary Medicine (archives searched 1999–2021) and UC Davis's College of Veterinary Medicine (archives searched 1991–2021). Median age at time of diagnosis was 17 years old, with a range from 5 to 34 years old. Sex data was available for 28 animals and included 15 geldings and 13 females. Three horses underwent a surgical biopsy before the post-mortem examination; all of the other samples were obtained at the time of the postmortem examination. Tissue samples from ten control horses with no oral SCC were included (Controls 1–10, Suppl. Table S1), which spanned years 2005–2022 and were included from both institutions. Median age at time of surgical biopsy or necropsy for control samples was 14 years old, with an age range from 4 to 20 years old, and included 4 males and 4 females (sex data was not available for 2 animals).

Hematoxylin and eosin-stained slides were evaluated by two board certified pathologists (JL and BM) to confirm the diagnosis and further characterize the cases. The most commonly affected anatomic sites with SCC were gingiva/mandible (n=6), lip (n=7), tongue (n=5), and gingiva/maxilla (n=3) and the lesions therefore included both mucocutaneous and mucosal sites. Additional sites included palate (n=2), oropharyngeal (n=1), subepiglottal (n=1),

gingiva, location not otherwise specified (n=2), and oral, location not otherwise specified (n=4).

Microscopically, oral SCC lesions appeared typical for SCC at other sites, and were characterized by islands, trabeculae, and cords of neoplastic epithelial cells with squamous differentiation that were variably surrounded by a scirrhous fibrovascular stroma. In some cases, there was hyperplasia of the overlying epithelium with invasive islands of squamous epithelial cells extending into the subepithelial stroma. In other cases, the tissues were composed entirely of islands of neoplastic squamous epithelial cells within a supporting fibrous or desmoplastic stroma. Within the tumors, there were variable regions of keratinization, including concentric layers of keratin (keratin pearls), and intercellular bridges, although not all cases exhibited keratinization. Desmoplasia was variably present. Ulceration was apparent in some tumors, associated with granulation tissue deposition and abundant inflammation. Inflammation, characterized by infiltration of plasma cells, lymphocytes, and some neutrophils was also present within some tumors without evidence of overt ulceration. Anisocytosis and anisokaryosis varied widely from mild to marked within different tumors. No evidence of viral cytopathic effect (prominent keratohyaline granules, koilocytes, or intranuclear viral inclusions) was identified within the SCC tumors. Of the 14 cases with a full postmortem examination, 6 (43%) had evidence of metastasis to regional lymph nodes and/or lungs.

Standard PCR to amplify a 226-base pair (bp) product of the EcPV2 E6 gene was performed on total DNA isolated from four 10-μm-thick scrolls of formalin-fixed, paraffin-embedded (FFPE) tissue samples using a commercial DNA extraction kit (Qiagen QIAamp DNA FFPE tissue kit, Qiagen, Valencia, CA) following manufacturer's recommended protocols. PCR primers specific for the EcPV2 E6 gene and the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been previously described.[13] PCR products were run on a 1% agarose gel and visualized with SYBR Safe (ThermoFischer Scientific, Waltham, Massachusetts). Amplicons were identified within the positive control samples (known EcPV2 positive gastric SCC) for all primers and negative controls (no template DNA) yielded no amplicons. EcPV2 DNA was amplified from 10/31 (32%) oral SCC cases but none of the non-SCC control cases (Table 1). Equine GAPDH was amplified from all oral SCC samples and all non-SCC control samples, indicating adequate DNA isolation from all of the samples.[13]

In situ hybridization (ISH) was performed using a previously validated probe set designed to detect EcPV2 E6/E7 nucleic acid (probe binds both viral RNA and viral DNA) in genital SCCs (RNAscope; Advanced Cell Diagnostics (ACD), Hayward, CA).[11] A predesigned probe to the bacterial gene DapB (ACD) served as the negative control. A predesigned probe to the human reference gene Peptidylprolyl Isomerase B (PPIB) previously shown to react with equine samples[13] was purchased from ACD and run to ensure presence of adequate RNA within each sample. Tissues derived from an equine gastric SCC, previously shown to contain EcPV2 nucleic acid using this ISH probe, was included as a positive control. ISH was performed on 5-μm-thick FFPE sections using the RNAscope 2.5 RED assay kit according to manufacturer's recommended protocols and as previously described.[13] For three cases, high background staining of the negative control probe

DAB, which should be non-reactive with equine tissues, precluded clear determination of results of the EcPV2 probe. For these cases, additional ISH reactions were run using the RNAscope 2.5 BROWN assay kit, which produced decreased background staining using the negative control probe DAB to ensure accurate determination of EcPV2 hybridization results. Successful hybridization with either stain results in deposition of a red (or brown) stain which is in direct correlation with the amount of EcPV2 nucleic acid or PPIB mRNA.

ISH hybridization signals for EcPV2 were detected within 8 of 31 (26%) oral SCCs (Table 1). In two cases (Case 1 and 5), EcPV2 hybridization signals consisted of dot-like faint to moderately intense hybridization signals within the nucleus and cytoplasm of nearly all neoplastic epithelial cells (Fig 1). Within the other cases, intense diffuse nuclear hybridization signals were detected within some neoplastic cells in addition to robust dot-like nuclear and cytoplasmic staining in nearly all of the neoplastic cells (Fig 2). In one case (Case 6), the epithelium overlying the neoplasm was markedly exophytic and arranged in papillary projections, with invasion of neoplastic epithelial cells along the base of the papillary projections (Fig 3a). Hybridization signals within this exophytic region consisted of intense nuclear staining for EcPV2, predominantly within the upper layers of epithelium, with dot-like hybridization signals within the basal keratinocytes (Fig 3b). This spatial hybridization pattern is consistent with the productive papillomavirus life cycle, where vegetative viral replication takes place within keratinocytes in the upper layers of the mucosal epithelium (stratum spinosum and stratum granulosum), and expression of viral E6/ E7 is restricted to the basal keratinocytes.[4] In another case (Case 25), there was a discrete hyperplastic plaque adjacent to the SCC with a similar pattern of hybridization signals suggestive of the productive papillomavirus life cycle (Fig 4); within the regions of SCC, hybridization signals consisted of intense dot-like hybridization signals within nuclei and cytoplasm of neoplastic cells (Fig 5). In all cases, the ISH hybridization signals were restricted to the neoplastic or hyperplastic mucosal epithelial cells. Invasive neoplastic cells within all of the examined tumors contained variably intense dot-like hybridization signals within the nucleus and/or cytoplasm. For all cases, no signals were seen above background staining levels using the negative control probe. Twenty-three oral SCCs exhibited no hybridization signals for EcPV2 nucleic acid (Table 1). All cases revealed variably intense hybridization signals for PPIB (reference gene, Peptidylprolyl Isomerase B). ISH on the non-SCC oral samples revealed variably intense hybridization signals for PPIB but no hybridization signals for EcPV2 above background staining levels using the negative control probe (Supp. Table 1).

Seven cases were both ISH-positive and PCR-positive for EcPV2, as would be expected if EcPV2 were playing a role in lesion oncogenesis. Three cases were PCR-positive for EcPV2, but no hybridization signals were detected using the EcPV2 probe. In these cases, it is possible that the PCR detection of EcPV2 identified a surface contaminant, latent infection, or merely reflects the difference in sensitivity between the two assays. One case (Case 1) was PCR-negative for EcPV2 but positive for EcPV2 using ISH. As PCR is considered more sensitive than ISH, this was a somewhat unexpected result. One possible explanation is that processing and long-term storage differentially affected the DNA and RNA quality within the sample, or the detection methods in this particular case were more permissive to ISH than PCR. ISH-mediated detection of nucleic acid utilizes very

short probe sequences, which may be better suited if the RNA or DNA is significantly degraded. Three cases were from archived tissues over 23 years old (maximum 31 years). While hybridization signals in these older cases were fainter than more recently sampled tissues, all three cases yielded positive ISH hybridization signals for PPIB, and one case was additionally positive for EcPV2.

The overall detection of EcPV2 nucleic acid within oral SCC is comparable to other studies which have identified EcPV2 DNA in 15% (3/20), 26.6% (4/15), and 22% (11/49) of equine head and neck SCCs.[6, 9, 12] Within the latter two studies, if limiting the cases to only oral SCCs (not including nasal SCCs), 2/5 (40%) and 6/22 (27%) oral SCCs contained detectable EcPV2 DNA.[9, 12] Given that none of the non-SCC oral samples in this current study reported here contained detectable EcPV2 DNA, and that previous studies have shown EcPV2 detection rates of <10% in healthy equids, the detection of EcPV2 nucleic acid within tumor cells using ISH is highly suggestive of a pathogenic association between EcPV2 infection and oral SCC within at least a subset of equine oral SCC.[9, 10, 14] Since EcPV2 was not amplified within all SCC samples, it is possible that these samples are 1) infected with a different EcPV type, which would not be detected within this study 2) associated with other non-papillomavirus causes 3) sample nucleic acid was not of sufficient quality to allow detection or 4) EcPV2 is important in initiation but is not required for maintenance of the malignant phenotype, and thus is not always detected within well-established cancers.

In humans, a significant portion of head and neck cancers, which include cancers derived from the mucosal epithelium in the oral cavity, pharynx and larynx, are increasingly attributed to infections with high-risk HPV types.[15] Generally, pharyngeal cancers are more typically attributed to infection with HPV, with prevalence that ranges from 30% up to 90% depending on the study, while oral cavity and laryngeal cancers are more typically associated with tobacco consumption and/or alcohol abuse.[5] Detection of EcPV2 within only a subset of SCCs in our study raises the possibility that equine oral SCC may also be multifactorial. While the sample size in this study is too low to draw conclusions regarding whether SCC arising at specific sites are more likely to be associated EcPV2, it was notable that 3/5 (60%) lingual SCCs demonstrated presence of EcPV2 nucleic acid within the tumor cells. There was no EcPV2 detected within samples of SCC from the lip (0/7) or mandibular SCC (0/6). As mentioned previously, it is also possible that other equine papillomaviruses are associated with the EcPV2 negative oral SCCs this study; the methods used in this study were limited to detection of EcPV2, and would not have identified other genetically distinct papillomaviruses.

Identification of EcPV2 nucleic acid within a hyperplastic lesion adjacent to squamous cell carcinoma within one horse in this study and exophytic papillary projections overlying invasive SCC in another horse suggests the possibility that some equine oral SCCs may arise from EcPV-associated precursor lesions (viral plaques/ papillomas). Further, within the epithelium of the hyperplastic plaque, koilocytes were occasionally evident with rare amphophilic intranuclear viral inclusions, consistent with a productive viral infection. Progression of precursor viral hyperplastic lesions to SCC has been recognized in some equine genital lesions, where viral papillomas are thought to progress to SCC.[3] It is

unknown, however, how often this occurs or if the majority of genital SCC develop in this manner.[3]

In conclusion, this study provides support for an association between EcPV2 and a subset of equine oral SCC, particularly lingual SCC, by identifying EcPV2 nucleic acid within tumor cells using ISH. Further studies are necessary to determine the pathogenic role of EcPV2 in development of equine oral SCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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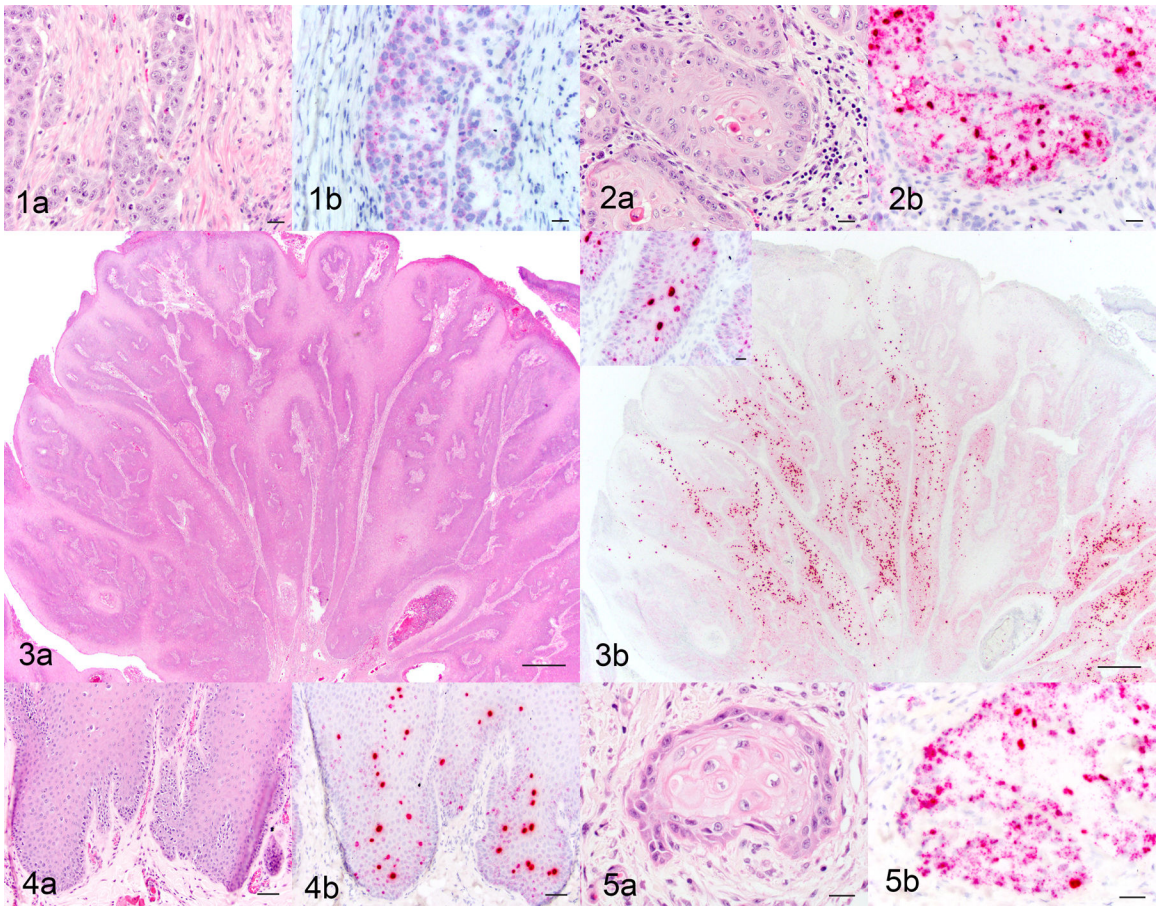

Figure 1.

Squamous cell carcinoma (SCC); Oral cavity; Horse No. 1. **Figure 1.** (a) High magnification of invasive island of neoplastic squamous epithelial cells within the subepithelial stroma. Hematoxylin and eosin (HE). Bar, 20μM. (b) Dot-like hybridization signals are present within the nuclei and cytoplasm of neoplastic cells. *In situ* hybridization (ISH), EcPV2 E6/E7 probe. Bar, 20μM. **Figure 2.** SCC; Tongue; Horse No. 13. **Figure 2.** (a) High magnification of invasive island of neoplastic squamous epithelial cells within the subepithelial stroma. HE. Bar, 20μM. (b) Strong dot-like hybridization signals are present within the nuclei and cytoplasm of neoplastic cells. Intense diffuse hybridization signals are present within the nuclei of some of the neoplastic cells. ISH, EcPV2 E6/E7 probe. Bar, 20μM. **Figure 3.** Exophytic hyperplasia; Oral cavity; Horse No. 6. **Figure 3.** (a) Low magnification of exophytic projections of hyperplastic epithelium. HE. Bar, 500μM. (b) Strong dot-like hybridization signals are present within the nuclei and cytoplasm of hyperplastic basal epithelial cells. Intense diffuse hybridization signals are present within the nuclei of some of hyperplastic epithelial cells within the upper layers of the epithelium. Bar, 500μM. Inset: High magnification demonstrating nuclear and cytoplasmic hybridization signals. Bar, 20μM. ISH, EcPV2 E6/E7 probe. **Figure 4.** Hyperplastic plaque; Oral cavity; Horse No. 25. **Figure 4.** (a) Hyperplastic oral epithelium. HE. Bar, 50μM. (b) Strong dot-like hybridization signals are present within the nuclei and cytoplasm of hyperplastic basal epithelial cells. Intense diffuse hybridization signals are present within the nuclei

of some of hyperplastic epithelial cells within the upper layers of the epithelium. ISH, EcPV2 E6/E7 probe. Bar, 50μM. **Figure 5.** SCC; Oral cavity; Horse No. 25. **Figure 5.** (a) High magnification of invasive island of neoplastic squamous epithelial cells within the subepithelial stroma. Hematoxylin and eosin (HE). Bar, 20μM. (b) Dot-like hybridization signals are present within the nuclei and cytoplasm of neoplastic cells but not in the adjacent fibrous stroma. *In situ* hybridization (ISH), EcPV2 E6/E7 probe. Bar, 20μM.

Table 1.

PCR and in situ hybridization for EcPV2 E6/E7 viral nucleic acid within equine oral squamous cell carcinomas (SCC).

Case #	Breed	Sex	Age (years)	Site	Sample type	PCR results	ISH results
1	UNK	UNK	UNK	Oral SCC with lymph node metastasis	Post mortem	-	+
2	UNK	UNK	UNK	Oral SCC	Biopsy	-	-
3	Quarterhorse	F	17	Oral SCC	Biopsy	-	-
4	Paint horse	F	14	Oral SCC	Biopsy	-	-
5	Paint horse	MC	18	Oropharyngeal SCC w/ lymph node and pulmonary metastasis	Post mortem	+	+
6	Thoroughbred	MC	34	Subepiglottal SCC	Biopsy	+	+
7	Quarterhorse	F	13	Buccal mucosal SCC	Biopsy	-	-
8	Arabian	MC	16	Soft and hard palate SCC	Biopsy/ Post mortem	+	+
9	Quarterhorse	MC	13	Hard palate SCC	Biopsy	-	-
10	Welsh Pony	MC	28	Gingival SCC	Biopsy	-	-
11	Quarterhorse	MC	24	Lingual SCC	Biopsy	+	+
12	Arabian	F	19	Lingual SCC with pulmonary metastasis	Post mortem	-	-
13	American Miniature horse	F	20	Lingual SCC	Biopsy/ Post mortem	+	+
14	Shetland Pony	MC	20	Lingual SCC	Post mortem	+	+
15	Quarterhorse	MC	28	Lingual SCC	Post mortem	+	-
16	Appaloosa	F	21	Lip mucocutaneous SCC w/ lymph node metastasis	Post mortem	-	-
17	Paint horse	MC	9	Lip mucocutaneous SCC	Biopsy	+	-
18	UNK	UNK	UNK	Lip mucocutaneous SCC	Biopsy	-	-
19	Mustang	F	5	Lip mucocutaneous SCC	Biopsy	-	-
20	Paint horse	F	14	Lip mucocutaneous SCC	Biopsy	-	-
21	Palomino	F	16	Lip mucocutaneous SCC	Biopsy	-	-
22	Gypsy Vanner Horse	MC	UNK	Lip mucocutaneous SCC	Biopsy	-	-
23	Quarterhorse	MC	15	Maxillary SCC	Biopsy	+	-
24	Quarterhorse	F	24	Maxillary SCC	Post mortem	-	-
25	Quarterhorse	MC	13	Oral cavity/ maxillary SCC with lymph node metastasis	Post mortem	+	+
26	Paint horse	MC	19	Mandibular SCC	Biopsy	-	-
27	Appaloosa	MC	20	Mandibular SCC	Biopsy/ Post mortem	-	-
28	Thoroughbred	F	7	Mandibular SCC	Post mortem	-	-
29	Quarterhorse	MC	27	Mandibular SCC	Post mortem	-	-
30	Mustang	F	6	Mandibular SCC w/ lymph node metastasis	Post mortem	-	-

Case #	Breed	Sex	Age (years)	Site	Sample type	PCR results	ISH results
31	Quarterhorse	F	14	Mandibular SCC	Biopsy	-	-

EcPV-2, *Equus caballus* papillomavirus-2; ISH, *in situ* hybridization; Unk, unknown