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AUTHORS: Suárez-Bonnet, A; Willis, C; Pittaway, R; Smith, K; Mair, T; Priestnall, S L

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# MOLECULAR CARCINOGENESIS IN EQUINE PENILE CANCER: A POTENTIAL ANIMAL MODEL FOR HUMAN PENILE CANCER

Alejandro Suárez-Bonnet[1], Claire Willis[1], Rachel Pittaway[1], Ken Smith[1], Tim Mair[2], Simon L. Priestnall[1]

[1]Department of Pathobiology and Population Sciences, The Royal Veterinary College, Hawkshead Lane, Hatfield, Hertfordshire AL9 7TA, UK

[2]Bell Equine Veterinary Clinic, Mereworth, Maidstone, Kent, ME18 5GS, UK

Key words: animal model, equine, epithelial-mesenchymal transition, invasion front, penile cancer

Corresponding author.

*E-mail address*: asuarezbonnet@rvc.ac.uk

## Abstract

**Objectives**: To evaluate the expression of COX-2, E-cadherin, vimentin, 14-3-3σ, and PTEN tumor-related proteins in equine penile papillomas (ePP) and squamous cell carcinomas (ePSCC), the occurrence of epithelial-mesenchymal transition (EMT) at the invasion front (IF) and compare our findings with current knowledge on human penile squamous cell carcinoma (hPSCC).

**Material and Methods**: We analyzed, by immunohistochemistry in 45 penile proliferative epithelial lesions, the expression of COX-2, E-cadherin, vimentin, 14-3-3σ, and PTEN using monoclonal antibodies. Tumors were histopathologically classified as well-differentiated or poorly-differentiated using the IF grading scheme. Semiquantitative analysis was performed to determine down or up-regulation of the proteins and association with histopathological characteristics were statistically investigated using Mann-Whitney U test and/or Spearman's tests.

**Results**: COX-2 was neo-expressed in 86.6% of the cases and expression progressively increased from ePP to ePSCC ($p=0.0003$) and from well to poorly-differentiated ($p=0.033$). High COX-2 expression was associated with a high mitotic index (MI) ($p=0.026$). In contrast to normal epidermis, ePSCC had very low E-cadherin expression in 64% of the cases ($p=0.0005$). Vimentin was neo-expressed in 65% of poorly-differentiated ePSCC at the IF indicating EMT. Cytoplasmic 14-3-3σ protein expression was reduced in 42% of the ePSCC and additionally, nuclear expression of 14-3-3σ in neoplastic keratinocytes and in the cytoplasm of stromal fibroblasts at the IF were features only found in ePSCC. PTEN protein showed a tendency to be decreased or lost in ePSCC.

**Conclusions**: Our study provides evidence of molecular abnormalities in ePSCC similar to those reported for human PSCC. The occurrence of EMT at the IF is a common event in ePSCC. Naturally-occurring ePSCC could serve as a valuable preclinical animal model to explore upcoming therapeutic options for hPSCC.

## 1. Introduction

Human penile cancer is a rare disease in developed countries with an incidence rate of around 1 per 100,000 [1]. In contrast, it is more common in developing countries accounting for up to 10-17% of cancer in males with an incidence that reaches 4 per 100,000 [1, 2]. Squamous cell carcinoma (SCC) is the predominant cancer type and accounts for over 95% of penile cancer cases [3-5]. The main treatment for advanced tumors is phalectomy and, when inguinal lymph node metastasis is present, bilateral inguinal lymphadenectomy is also performed. These procedures are associated with high psychological morbidity and negatively impact the quality of life of affected patients [4]. In the UK, it is reported that there has been an increase in incidence of approximately 25% since 1985 [6, 7]. The pathogenesis of human penile SCC (hPSCC) remains unclear; lack of circumcision, phimosis, cigarette-smoking and obesity have been widely discussed as potentially contributing to its development. The hypothesis of a bimodal pathogenesis of hPSCC is accepted, with human papillomavirus- (HPV) and non-HPV-related cases. HPV infection plays a role in hPSCC carcinogenesis and high-risk HPV-DNA is present in 12% to 72% of cancers [7].

As a result of its rarity, most treatment options are based on the results of small clinical trials. Experimental models for human diseases, including *in vitro* studies using cancer cell lines, and animal models using non-spontaneous/induced tumors or xenografts, are valuable resources in biomedical research when evaluating new potential therapies [8]. However, these models have limitations and often they do not resemble morphologically, or at the molecular level, the disease in humans. Currently, there is a lack of commercially available hPSCC cell lines and no reliable xenograft models have been produced [9].

In humans, a variety of proteins involved in hPSCC have been studied and associated with pathological features and prognosis. Overexpression of the enzyme COX-2 is associated with proliferation, angiogenesis, invasion and metastasis [10, 11]. Phosphatase and tensin homolog (*PTEN)*, a tumor suppressor gene, is frequently mutated in many cancers, including hPSCC, leading to reduced production of its transcription product, PTEN protein, which holds important functions preventing cell proliferation [12]. E-cadherin and vimentin are proteins associated with the epithelial-

mesenchymal transition (EMT) process, which have been associated with a poorer prognosis [13, 14]. Additionally, 14-3-3σ is an oncoprotein that promotes cell proliferation, survival, invasiveness, metastasis and chemoresistance by binding and modulating various molecular pathways in vulvar, cervical and head and neck SCC [15-18].

Naturally occurring tumors in domestic animals represent a unique opportunity to study cancer *in vivo.* Yet, few studies have been done to establish a specific animal cancer as a model for its human counterpart [8]. SCC is the most common penile neoplasm in the horse and a novel type of equine papillomavirus (EcPV-2) is suggested to play a role as initiator in this cancer, acting in a similar way to high-risk HPV-16 in humans [19]. Currently, there is a significant paucity of knowledge on the molecular events leading to the initiation and progression of PSCC in the horse and, given the clinical and biological similarities with hPSCC, we hypothesize they could share similar protein aberrations and altered molecular mechanisms, particularly EMT. Equine PSCC (ePSCC) could therefore serve as a highly relevant spontaneous animal model in the context of translational research.

The objectives of this study were to analyze the expression of COX-2, 14-3-3σ, E-cadherin, vimentin and PTEN proteins in equine penile papilloma (ePP) and ePSCC and compare their expression with histopathological features and in comparison with hPSCC. Furthermore, we aimed to analyze the occurrence of EMT by examining E-cadherin and vimentin expression.

## 2. Methods

### *2.1. Cases and histological assessment*

A search in the database of the Diagnostic Pathology Service of the Royal Veterinary College and the University of Bristol was performed using the following key words; ‘equine’, ‘papilloma’ and ‘squamous cell carcinoma’. A total of 58 cases were identified. Epithelial proliferative lesions from the skin, ocular or vulvar regions were excluded and finally a total of 45 cases of ePP or ePSCC were selected. Hematoxylin and eosin archived tissue sections were available and were re-examined by two veterinary pathologists (ASB and SLP). The neoplasms were analyzed in both the core

and at the invasive front (IF) (defined as the deepest area of the tumor and the boundary between the infiltrating neoplastic cells and the dermis). Histological examination, using a modified two-tier grading system based on the Bryne system, classified ePSCC as either well-differentiated or poorly-differentiated (Table 1) [20]. The presence of vascular invasion (presence of neoplastic cells within lymphatic or blood vessels) was also investigated. Clinical follow-up data was available for 6 cases.

Table 1. Invasive Front Grading System for use in equine penile squamous cell carcinoma*

| Morphological Feature | Score | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Degree of keratinization | Highly keratinized (>50% of cells) | Moderately keratinized (20–50% of cells) | Minimal keratinization (5–20% of cells) | No keratinization (0–5% of cells) |
| Pattern of invasion | Pushing, well delineated infiltrating borders | Infiltrating, solid cords, bands and/or strands | Small groups or cords of infiltrating cells | Marked and wide-spread cellular dissociation in small groups and/or in single cells (n < 15) |
| Desmoplasia | None | Slight | Moderate | Marked |
| Nuclear polymorphism | Little nuclear polymorphism (>75% mature cells) | Moderately abundant nuclear polymorphism (50–75% mature cells) | Abundant nuclear polymorphism (25–50% mature cells) | Extreme nuclear polymorphism (0–25% mature cells) |
| Mitotic index (mitoses in ten 400x fields) | 0–1 | 2–3 | 4-5 | >5 |

*This grading is a slightly modified version of the Bryne's system. Scores for each parameter provide a score from a minimum of 5 to a maximum score of 20. The tumor grade is based on the total score as follows: well-differentiated (5-14), poorly-differentiated (15-20).

### *2.2. Immunohistochemistry*

Tissues had been fixed in 10% neutral-buffered formalin for 24 to 48 hours at room temperature, processed according to standard procedures, and embedded in paraffin wax. Four-micrometer-thick fresh-cut tissue sections on positively charged slides were obtained. Immunohistochemical analysis was performed on a BondMax Autostainer (Leica, UK). The following monoclonal antibodies were used; anti-COX-2 (clone SP-

21, 1:50, Thermo, UK), 14-3-3σ (clone 5D7, 1:40, Santa Cruz Biotechnology, Germany), PTEN (clone G-6, 1:100, Santa Cruz Biotechnology, Germany), E-cadherin (NCH-38, Dako, UK), vimentin (clone V9, 1:500; Dako, UK) and PTEN. Heat-induced antigen retrieval was performed using a pH 6 (Bond ER1; Leica, UK) or pH 9 buffer (Bond ER2; Leica, UK). The Bond Polymer Refine Detection kit (Leica, UK) was used for visualization. A positive reaction was indicated by the presence of distinct brown cytoplasmic labelling. As positive control, normal equine glabrous skin (for 14-3-3σ, E-cadherin, vimentin and PTEN) and normal equine kidney (for COX-2) were used. Negative controls were prepared by replacing the primary antibody with Leica Antibody Diluent (Leica, UK) only.

*2.3. Immunohistochemical evaluation*

Samples were analyzed by two of the authors (ASB and SLP) at a double-headed light microscope and discrepancies were discussed and a consensus reached. Analysis was made independently of other histopathological features and blinded to clinical data. All proteins were semi-quantitatively analyzed in the entire sample in comparison with normal penile squamous epithelium by using previously published scoring systems for human or veterinary medicine studies. All cell markers were evaluated within the tumor core and at the IF. For COX-2, an immunohistochemical score (IHS) was assigned based on the intensity of staining (none, 0; weak, 1; moderate, 2; strong, 3) and percentage of positive tumor cells (0%, 0; <25%, 1; 25-50%, 2; 51-75%, 3; >75%, 4) [21]. Positive cells at the tumor surface were excluded from the IHS as they were likely reactive/inflamed cells due to surface trauma. The product of the intensity and distribution scores gave a total IHS ranging from 0 to 12. For COX-2 and for statistical analysis purposes a cut-off value of ≥4 designated a positive case. 14-3-3σ cytoplasmic labelling in neoplastic cells was scored based on intensity (none/weak, 1; moderate, 2; strong, 3) and percentage of positive tumor cells (<10%, 1; 10-50%, 2; >50%, 3). A total IHS from 1 to 9 was obtained by multiplying the values of these two parameters. Cases were classified as Low (IHS <9) or High (IHS=9) categories. Any labeling of the neoplastic cell nuclei and/or stroma was considered to indicate positive 14-3-3σ protein expression [15, 17]. PTEN was evaluated following a previously published quantification system which used the IHS, 0 (no staining), 1 (weak), 2

(medium) and 3 (strong). An IHS of ≤1 designated loss of PTEN [12]. For E-cadherin evaluation, cases with ≤ 65% of positive cells with a membranous staining were considered negative. For vimentin, cytoplasmic immunoreactivity in more than 5% of neoplastic cells designated the tumor as positive [14].

*2.3. Statistical analysis*

Expression of COX-2, 14-3-3σ, E-cadherin and vimentin were compared with histological subtype, MI, necrosis, and inflammation. All statistical analysis was completed in Graphpad Prism (version 7, GraphPad Software, La Jolla California, USA) using a Mann-Whitney U test and/or Spearman's correlation when appropriate. $P<0.05$ was considered significant for all statistical tests.

## 3. Results

*3.1. Epidemiological data*

Clinical follow-up was available for 6/45 cases. Four horses, in which excisional biopsies or penile amputation were performed, were originally diagnosed as ePSCC. In this group three horses were humanely euthanized due to tumor recurrence and tumor-related clinical disease (one had lymph node and pulmonary metastasis). The mean survival time for these 4 cases was 786 days (187-1178 days). A fifth case was originally diagnosed with ePP, recurred two times as ePSCC and died due to gastric colic (presumably unrelated to SCC although autopsy was not performed). The sixth case, for which follow-up was available, was originally diagnosed as ePP, recurred twice as papillomas and is still in remission. Surgical treatment included conventional resection plus cryotherapy.

*3.2. Histopathological findings.*

In all cases the tumor cells were compared with those of the normal epidermis to characterize their degree of differentiation. Additionally, of the 45 samples, 23 contained histologically normal epidermis adjacent to the neoplasm. 11/45 (25%) penile lesions were classified as ePP. On low power, they were characterized as thick plaques or broad-based papillary projections supported by a small amount of fibrovascular stroma and frequently orthokeratotic hyperkeratosis was present. A

variable degree of superficial erosion (self-induced trauma) with intraepithelial neutrophilic exocytosis was observed at the surface of the lesions. At the dermo-epidermal junction, the basal layer often formed anastomosing rete ridges. Cases of papilloma did not invade beyond the basement membrane and the keratinocyte maturation was orderly. Benign neoplastic cells had minimal cellular atypia and occasionally exhibited a clear and swollen cytoplasm. MI in this group ranged from 1-30 with a mean value of 8.8 (Fig.1).

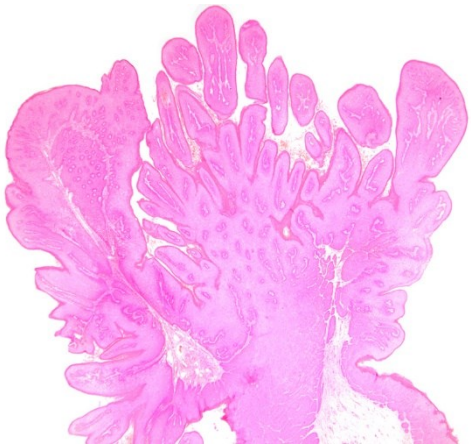

Fig. 1. An ePP exhibits multiple papillary projections of acanthotic and dysplastic epidermis radiating from a central fibrovascular stalk.

In 34/45 (75%) cases the penile lesions had greater cellular atypia and there was an obvious breaching of the basement membrane. These lesions were considered malignant and designated as ePSCC. In well-differentiated ePSCC (N=14) the IF consisted of broad and anastomosing trabeculae, cords and/or islands supported by variable amounts of connective tissue. These later arrangements variably contained central accumulations of concentrically laminated keratin (‘keratin pearls’) (Fig.2). A scirrhous reaction (desmoplasia) was not observed in well-differentiated ePSCC. Anisokaryosis and anisocytosis were mild and the mitotic index ranged from 2-22 with a mean value of 9. Some degree of inflammation was observed in almost all well-differentiated ePSCC consisting mainly of infiltrates of lymphocytes and plasma cells with vary rare Mott cells. Only one case exhibited neutrophilic inflammation within the supporting stroma.

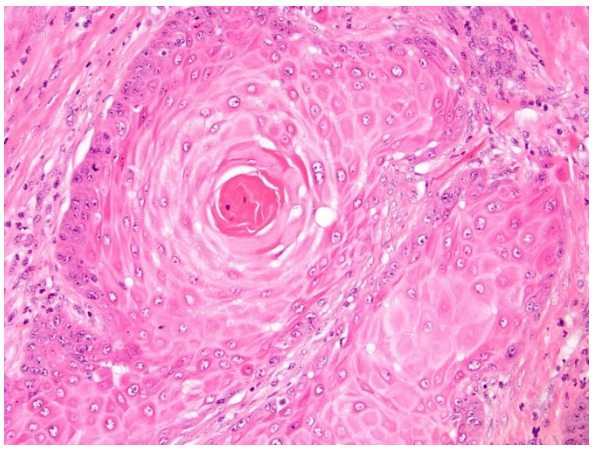

Fig. 2. Formation of a ‘keratin pearl’ in a well-differentiated ePSCC.

Twenty poorly-differentiated ePSCC (N=20) were diagnosed based on the histopathological characteristics present at the invasion front (IF). Neoplastic cells at the IF were arranged forming irregular trabeculae, nests and clusters of few tumor cells which exhibited moderate to marked cellular atypia with no keratinization (Fig. 3). A moderate to marked scirrhous reaction was present in all cases at the IF. They contained irregularly round nuclei (nuclear pleomorphism) with one or more hyperchromatic and prominent nucleoli. Additionally, this group also had nests or isolated individual keratinized (dyskeratotic) cells, which consisted of prominent or shrunken hypereosinophilic cells with a hyperchromatic nucleus. Moderate to marked desmoplasia was observed in all cases within this group. Anisokaryosis and anisocytosis were moderate to marked and the MI ranged from 3-66 with a mean value of 23. MI was positively correlated with malignancy (ePP vs ePSCC, (p=0.04)) and tumor differentiation (well vs poorly differentiated ePSCC, (p=0.001)). Vascular invasion (presence of tumoral cells within blood or lymphatic vessels) was observed in 7 (20%) poorly-differentiated ePSCC. In two cases of poorly-differentiated ePSCC, either lymph node or lung were submitted for histopathological analysis. In both cases, tumor metastasis was observed with neoplastic cells displaying the same characteristics as the primary tumor (Fig. 4).

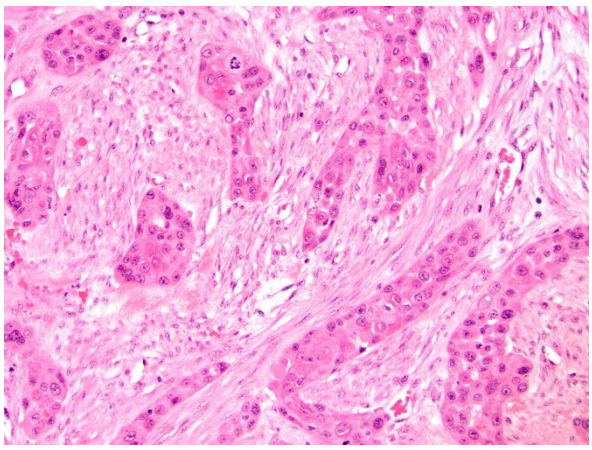

Fig. 3. Formation of bizarre cords and nests of neoplastic cells in a poorly-differentiated ePSCC.

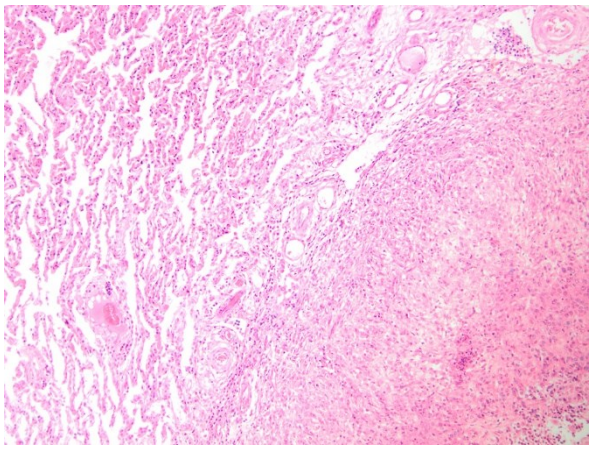

Fig. 4. The lung parenchyma is infiltrated by a metastatic ePSCC.

### *3.3. Immunohistochemical findings*

The main immunohistochemical results are summarized in Table 2.

Table 2

Summary of protein expression results in ePSCC in comparison with known protein expression levels in hPSCC and other human carcinomas

| Protein | Equine (this study) | Human | Cell compartment | Reference |
|---|---|---|---|---|
| COX-2 | *Up-regulated in 86.6% | Up-regulated in 100% | Cytoplasm | 30 |
| 14-3-3σ | Up-regulated in 79% | Up-regulated in 61.7% | Nucleus | 17 |
| | | Up-regulated in 75% | Nucleus | 15 |
| | Down-regulated in 42% | Down-regulated in 28% | Cytoplasm | 15 |
| | Up-regulated in 88% (stroma) | Up-regulated in 80% (stroma) | Cytoplasm | 40 |
| E-cadherin | Down-regulated in 64% | Down-regulated in 48% | Membrane | 14 |
| | | Down-regulated in 71.8% | Membrane | 3 |
| Vimentin | Up-regulated in 65% | Up-regulated in 33% | Cytoplasm | 14 |
| | | Up-regulated 44% | Cytoplasm | 3 |
| PTEN | Down-regulated in 50% | Down-regulated in 62% | Cytoplasm | 12 |

*Up-regulated and down-regulated designation in relation with normal penile tissues

### *3.3.1. COX-2 expression*

Equine kidney exhibited intense immunolabeling of the macula densa that served as a positive control for this antibody. Additionally, healthy equine epidermis was always negative. When the surface of an ePP or an ePSCC was eroded and inflamed, intense labelling was observed but this not assessed to produce an IHS. In this group, 44 cases were analyzed and 39 (86.6%) were positive and distributed as follows; 10/11 (90.9%) ePP and 29/33 (87.9%) ePSCC. Total IHS for ePP ranged from 0-6, and ePSCC from 0-12 (maximum). The mean IHS was 3.5 for all cases, 2.5 for ePP and 3.9 for ePSCC. An IHS ≥4 was considered strongly COX-2 positive and ≤3 weakly positive (Fig. 5). There was a significant difference between ePP and ePSCC IHS ($p=0.0003$). Mean IHS for well-differentiated ePSCC was 2.4 (range 0-6) and 4.9 (range 0-12) for poorly-differentiated ePSCC ($p=0.033$). MI was positively correlated with COX-2 expression ($p=0.026$).

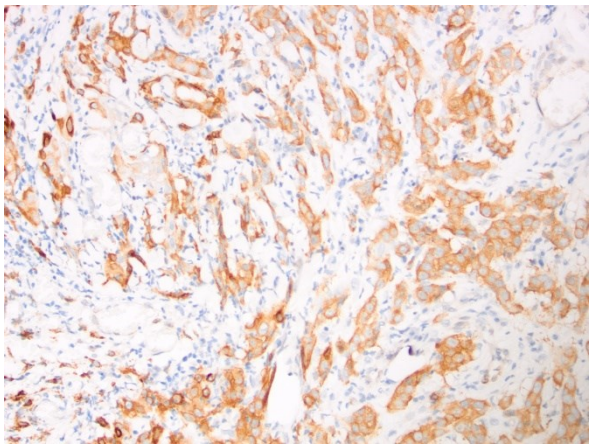

Fig.5. Multiple bizarre cords of neoplastic epithelial cells labelling strongly for COX-2 in a poorly-differentiated ePSCC.

### *3.3.2. 14-3-3σ expression*

14-3-3σ expression was analyzed in 41 cases: 8 ePP and 33 ePSCC. Keratinocytes in all layers of the epidermis exhibited intense cytoplasmic immunolabeling whilst cell nuclei were negative. The stratum corneum and subjacent dermal fibroblasts were negative in areas of normal epidermis (Fig. 6). Total IHS for both ePP and ePSCC ranged from 6-9 (maximum). The mean IHS was 7.9 for all cases, 8.6 for ePP and 7.7 for ePSCC. An IHS <9 was considered low expression and an IHS of 9 was considered high expression. Seven (88%) ePP showed high expression whilst in the ePSCC group there was a reduction of the protein in 14 cases (42%). Nuclear immunolabeling, detected in 2 ePP (25%) and in 26 ePSCC (79%), was significantly associated with malignancy ($p=0.0073$) but not with tumor grade ($r=0.350$). Stromal fibroblasts were negative in all ePP and positive in 29 ePSCC (88%) cases. No significant difference was observed between stromal expression and tumor differentiation. Positive stromal fibroblasts were particularly located at the IF while the stroma distant to the IF was consistently negative. Additionally, neoplastic cells at the IF largely exhibited cytoplasmic and nuclear immunostaining (Fig.7). MI was not correlated with 14-3-3σ expression variables. In the metastatic lung lesion, the protein was highly expressed

by neoplastic cells but also by adjacent reactive stromal fibroblasts while the remaining lung parenchyma was completely negative.

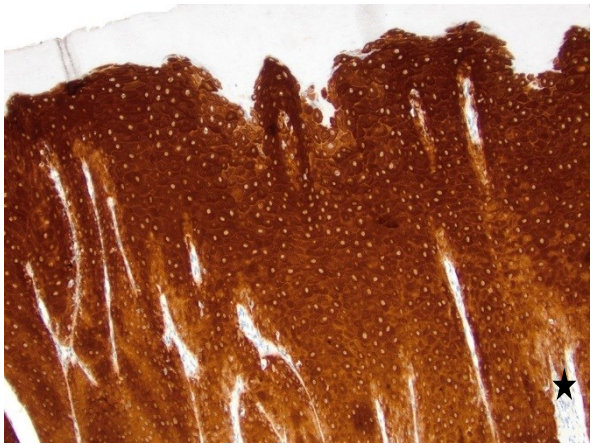

Fig. 6. The cytoplasm of normal keratinocytes strongly expresses 14-3-3σ. Cell nuclei are negative. The subjacent dermis (star) does not express 14-3-3σ.

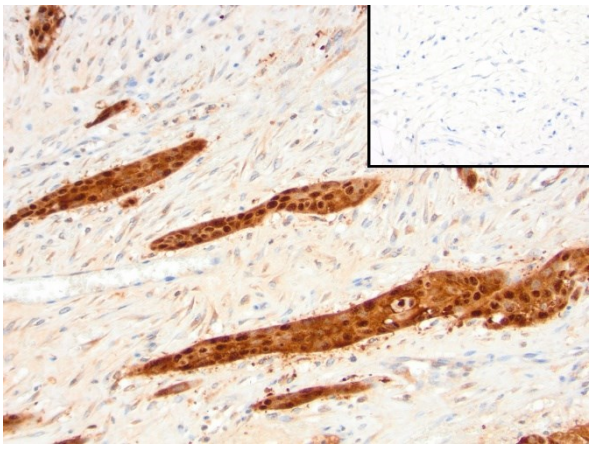

Fig. 7. Multiple bizarre cords of neoplastic epithelial cells exhibit slightly weaker cytoplasmic 14-3-3σ expression. Cell nuclei are strongly positive. The supportive connective tissue weakly expresses 14-3-3σ protein. Inset: An area of connective tissue away from the invasion front of the same case is completely negative.

*3.3.3. E-cadherin expression*

E-cadherin expression was assessed in 43 samples: 10 ePP and 33 ePSCC. Normal epidermis and all ePP samples displayed the same staining intensity as the cell membrane (Fig. 8). Conversely, 21 (63.6%) ePSCC showed significantly decreased (downregulation) E-cadherin labelling (p=0.0005) (Fig. 9). Downregulation was also significantly associated with poorly-differentiated ePSCC (p=0.027). Additionally, 27 (81.8%) ePSCC exhibited abnormal cytoplasmic expression with 7 (21.1%) having lost all typical membranous expression (p=<0.0001). Furthermore, this was correlated with histological differentiation: all poorly-differentiated ePSCC exhibited cytoplasmic expression compared to only 7/13 (53.8%) well-differentiated tumors (p=0.002). A negative correlation between E-cadherin expression and MI was noted (p=0.0022), in addition to a positive correlation between MI and atypical distribution of labelling (p=0.0017). A positive correlation between cytoplasmic E-cadherin translocation and nuclear 14-3-3σ expression was detected (p=0.0008).

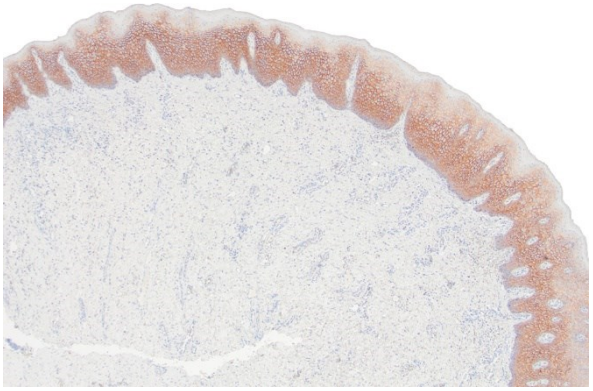

Fig. 8. E-cadherin antibody uniformly highlights the normal penile epidermis. The subjacent stroma is negative.

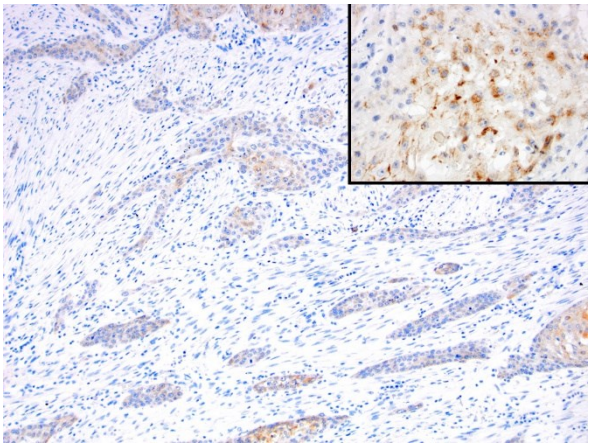

Fig. 9. Multiple bizarre cords of neoplastic epithelial cells show loss of E-cadherin expression in a poorly-differentiated ePSCC. *Inset*: Neoplastic cells exhibit cytoplasmic E-cadherin.

### *3.3.4 Vimentin expression*

Vimentin expression was assessed in 41 cases. In normal skin, ePP and well-differentiated ePSCC this antibody highlighted mesenchymal components of the dermis (blood vessels, fibrocytes, adipocytes, mononuclear inflammatory cells) whilst the epithelial cells were consistently negative. In 13 out of 20 poorly-differentiated ePSCC (65%) vimentin was expressed in more than 10% of neoplastic cells at the IF and was statistically associated with malignancy ($p=0.0001$) and with tumor differentiation ($p<0.0001$) (Fig.10). Groups of neoplastic cells within lymph node and lung metastases neo-expressed vimentin intermediate filaments. Vimentin neoexpression was correlated with decreased expression of E-cadherin and but not with E-cadherin nuclear expression. No other significant correlations with the remaining markers were observed.

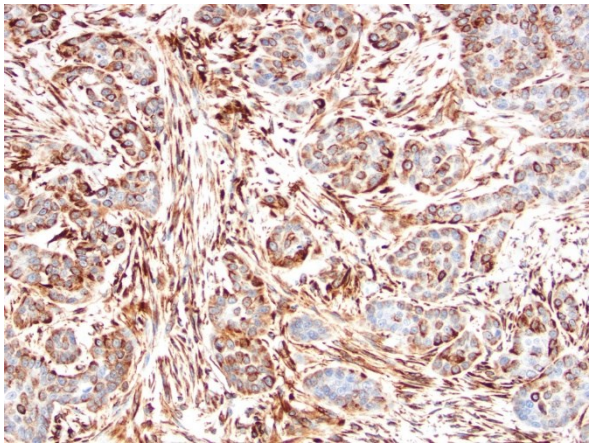

Fig. 10. Multiple nests of neoplastic epithelial cells labelling strongly for vimentin in a poorly-differentiated ePSCC. Fibroblasts within the supporting stroma are also positive.

### *3.3.4. PTEN expression*

A subset of 4 ePP, 2 well- and 2 poorly-differentiated ePSCC were analyzed. In areas of histologically normal skin, as well as in the four ePP, PTEN was present in the cytoplasm of keratinocytes and variably in fibroblasts. The canine mammary gland used as positive control stained appropriately. The 2 well-differentiated ePSCC showed areas of medium or weak immunoreaction while the 2 poorly-differentiated ePSCC were negative (Fig. 11 and Fig. 12).

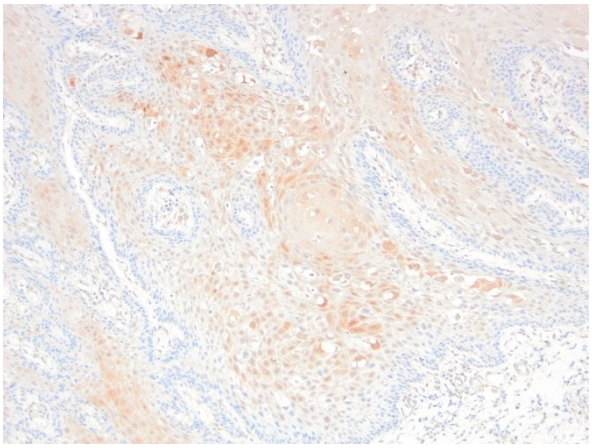

Fig. 11. PTEN is expressed by keratinocytes in an ePP. Fibroblasts within the subjacent dermis are moderately positive.

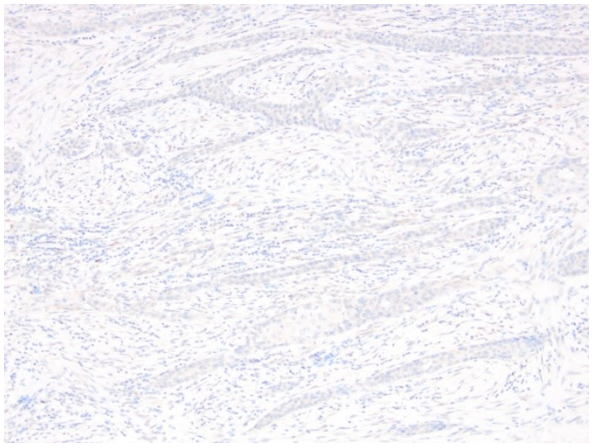

Fig. 12. Multiple bizarre cords of neoplastic epithelial cells show total loss of PTEN expression in a poorly-differentiated ePSCC.

## 4. Discussion

Around 10% of all neoplasms in the horse affect the external genitalia and ePSCC is the most common with a reported incidence of 50-83% which greatly exceeds the incidence of this cancer in humans [22-24]. In both humans and horses the disease produces severe discomfort, spreads to regional lymph nodes leading to distant metastases and death. In humans its low incidence and the lack of a reliable inducible

animal model has limited significant breakthroughs regarding pathogenesis and potential therapeutic targets [2, 7, 22, 25]. In this study we have analyzed histologically and immunohistochemically 45 cases of ePSCC to investigate the expression of cancer-related proteins and correlated them with the histomorphological features of these tumors. Furthermore, this study aimed to analyze, for the first time, the occurrence of EMT at the IF of ePSCC as has been documented in hPSCC [3, 13, 14].

Histological examination identified 11 ePP and 34 ePSCC. In the latter group a two-tier grading system of the IF identified 14 well-differentiated and 20 poorly-differentiated ePSCC. The histological appearance of ePP resembles the human 'differentiated' and 'warty' subtypes of penile intraepithelial neoplasia (PeIN) and ePSCC is virtually identical to the most common subtype of PSCC termed 'usual type' [4, 26]. The new WHO classification for epithelial penile tumors is based on both morphological features and immunoreactivity of the biopsy sample for p16 HPV [5, 26]. Although there is recent evidence that EcPV-2 is integrated in the host genome in approximately 20-40% of SCC, a similar classification system, based on morphology and EcPV-2-status, has not been established for horses [19, 27]. This is probably due, in part, to the lack of a commercially available anti-EcPV-2 antibody but other potential limitations such as laboratory costs and reticence of owners may be involved. Future multi-institutional studies in a large cohort of samples should focus on addressing a possible association between EcPV-2 infection status and histomorphological variants not yet described.

Overexpression of COX-2 in different types of carcinomas in humans and domestic animals is associated with an anti-apoptotic, pro-angiogenic, invasive and metastatic phenotype. Regarding ePSCC, this study is the largest to significantly associate COX-2 expression with malignancy, tumor differentiation and MI, all important prognostic factors for survival and metastasis. In the present study 86% of the examined cases exhibited COX-2 immunoreaction which is in agreement with a previous study [28], but differs from another that reported only 50% of cases being positive with the majority showing less than 1% of immunoreactive neoplastic cells [21]. In these studies, correlation between COX-2 overexpression, tumor differentiation and MI was not analyzed or showed no statistical association. A third study using Western-Blot found ubiquitous COX-2 expression in both normal epidermis and ePSCC samples [29]. This

variability of results could be attributed to differences in methodology, antibodies, different thresholds employed or unknown pre-sampling treatment using non-steroidal anti-inflammatory drugs (NSAIDs). Interestingly, our study shows that ePSCCs overexpress COX-2 in a high proportion of cases as occurs in invasive hPSCC but also esophageal SCC [30, 31]. The use of COX-2 inhibitors alone or as part of a multimodal therapy to aid in the treatment of the disease is a field of current research in human and veterinary medicine [10, 11, 32]. To illustrate this, in veterinary medicine NSAIDs are routinely used in the adjunct treatment of canine urothelial carcinomas, a well-recognized spontaneous model for human bladder cancer [33]. Furthermore, a single case reported complete remission of an equine head and neck carcinoma with anti-COX-2 therapy [34]. Given that in humans, inhibition of COX-2 as part of the therapeutic plan is currently under debate, we propose that the horse could represent a reliable and accessible spontaneous model to test novel COX-2 selective inhibitors to treat this type of cancer.

In various human carcinomas, including those of the head and neck, cervical and vulvar carcinomas, altered 14-3-3σ expression is associated with greater invasiveness, proliferation and metastasis [15, 17, 35]. Furthermore, in canine inflammatory mammary carcinoma, a very aggressive type of carcinoma that resembles inflammatory breast carcinoma in women, and in canine renal cell carcinomas, there is a significant neo-expression of 14-3-3σ protein and it is an independent prognostic indicator of shorter survival time [36, 37]. Expression of 14-3-3σ in tumoral cells is usually cytoplasmic, however in very aggressive neoplasms nuclear translocation can occur and is associated with cell proliferation. In normal conditions, 14-3-3σ sequesters CDK2/cyclin B1 in the cytoplasm resulting in G/M2 arrest. Consequently, 14-3-3σ nuclear translocation could play a role in the progression of SCC. Interestingly, in our study none of the samples of normal epidermis showed nuclear 14-3-3σ expression whilst the opposite was true in 80% ePSCC. Similar results have been published in human esophageal and vulvar SCC [16, 17]. 14-3-3σ was originally identified as an epithelial marker constitutively expressed in various types of epithelium. On the other hand, several studies have demonstrated that 14-3-3σ is secreted into the extracellular milieu by keratinocytes and induces fibroblasts to produce and secrete matrix metalloproteinases which allow the tumor to invade and metastasize [38, 39]. Recently stromal expression of 14-3-3σ

has been demonstrated in endometrial carcinomas and, similar to our results, the neoexpression by fibroblasts in areas of desmoplasia at the IF could be indicative of an active proinvasive environment within the extracellular matrix [40]. To provide more evidence of the role of 14-3-3σ in cancer development, recently it has been shown that 14-3-3σ provides chemoresistance properties to SCC and this fact, along with the anti-apoptotic properties of 14-3-3σ in tumoral cells, highlights the potential of this protein as a very promising biomarker and therapeutic target for penile SCC [18, 41-43].

PTEN expression could only be analyzed in a small subset of benign and malignant penile lesions. We observed a complete absence of this tumor suppressor protein in poorly-differentiated ePSCC whilst areas of normal epidermis, ePP and well-differentiated ePSCC were all variably positive. Obviously the sample size is too small to draw any conclusion regarding the role of PTEN in ePSCC, however based on our results we suggest a loss of PTEN tumor suppressor protein in a similar way as occurs in human PSCC [12].

In order to suggest a particular naturally-occurring neoplasm in a companion animal species, as a reliable model to study its human counterpart, the neoplasm should closely mimic the hallmarks of multistep cancer development which include; a similar initiator agent, the capacity to evade anti-proliferative signals, the production of anti-apoptotic and pro-proliferative proteins, the activation of angiogenesis, and the acquisition of pro-invasive and metastatic abilities. Metastasis is the main complication and cause of death in human patients with advanced hPSCC and horses with advanced disease are humanely euthanized to avoid suffering. Recently a number of publications have studied the occurrence of the EMT phenotype in hPSCC [3, 13, 14]. EMT is a complex process that involves the loss of epithelial characteristics, which includes E-cadherin downregulation, and the acquisition of a mesenchymal phenotype, mainly vimentin neoexpression. EMT is a normal and essentially physiologic event during embryogenesis and wound healing but in cancer is directly associated with apoptosis resistance, stromal and lymphovascular invasion, metastasis and poor prognosis [44]. To the authors' knowledge this is the first study to analyze the EMT process in an equine cancer, and provides evidence of dramatic E-cadherin downregulation and significant vimentin neoexpression, the latter a canonical marker of EMT, which was found in 65% of poorly-differentiated ePSCC.

Neoplastic cells neoexpressing vimentin had very low or absence of membranous E-cadherin or, interestingly, they exhibited cytoplasmic internalization of the protein. Aberrant cytoplasmic and nuclear E-cadherin expression has been recently related with a more aggressive behavior in cancer due to increasing signaling through activation of AKT/MAPK pathways via PI3K interaction [45, 46]. Additionally, we observed that neoplastic cells neo-expressing vimentin highly expressed 14-3-3σ. Our findings are in agreement with previous investigations that showed co-expression of 14-3-3σ and vimentin in EMT areas at the IF and the formation of a 14-3-3σ-vimentin complex that inhibits autophagy via an AKT-dependent mechanism, consequently promoting cell survival in cancerous cells [47, 48].

## 5. Conclusions

In summary, our study is the first to recognize the histological and immunophenotypical similarities between ePSCC and hPSCC. Additionally, we have demonstrated that when the IF exhibits features of a poorly-differentiated PSCC, EMT is a usual event. In light of these findings and in the era of the 'one-pathology' concept, we suggest that further research comparing equine and human PSCC could represent an outstanding opportunity to develop a preclinical animal model from which both species could obtain substantial benefit.

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