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Title page

Immune cell analysis in equine penile papilloma, *in situ* squamous cell *carcinoma,* and invasive squamous cell carcinoma: FoxP3+ T regulatory lymphocytes differ according to equine papillomavirus 2 status

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Abstract

Equine penile tumors are common in horses and are often related to infection with equine papillomavirus type 2 (EcPV2). This study investigates the immune cell infiltrate (ICI) of these tumors in horses, focusing on the role of EcPV2. Using multiplex immunohistochemistry (mIHC) for CD3, CD20, and IBA-1 and immunohistochemistry (IHC) for FoxP3, 27 horses with papillomas (5/27), in situ carcinomas (CISs) (3/27), and squamous cell carcinomas (SCCs) (19/27) were evaluated. Eighteen cases tested positive for EcPV2 by either or both in situ hybridization (ISH) and PCR (18/27 by PCR only, of which 16/18 were ISH+). ICIs were more abundant in EcPV2-positive tumors, although differences were not statistically significant. The number of FoxP3+ regulatory T-cells were significantly higher in EcPV2+ tumors, both in intraepithelial and stromal compartments. There were higher IBA-1+ macrophage density densities in SCCs than in papillomas or CISs. p53 IHC was performed and non-basal positivity was associated with malignancy. *TP53* mutational analysis with next-generation sequencing revealed that 13/21 cases had a wild-type *TP53*, while *TP53* variants were detected in 4/21 cases. ICIs did not vary according to *TP53* status. Tumor proliferation was also assessed with Ki67, which indicated a progressively higher proliferation from benign to malignant tumors. In conclusion, although the number and distribution of B-cells, T-cells, and macrophages did not vary according to EcPV2 status, FoxP3 regulatory T-cells were observed in significantly higher numbers in EcPV2+ neoplasms, indicating a different immune landscape compared to EcPV2-negative tumors.

Keywords; EcPV2, FoxP3, lymphocyte, papilloma, penile, p53, squamous cell carcinoma, tumor microenvironment

Squamous cell carcinoma (SCC) is one of the most common tumors in horses, and it carries a poor prognosis due to its invasive and metastatic potential. One of the major risk factors associated with SCC is infection with equine papillomavirus type 2 (EcPV2).[6,13,14] In humans, penile SCC (hpSCC) is associated with persistent high-risk human papillomaviruses (hrHPV) infection in approximately 20-50% of cases.[4] HpSCCs can have devastating outcomes, but overall, infection with hrHPV represents a positive prognostic factor.[8]

The strong prognostic value for hrHPV infection reflects the existence of two different carcinogenic pathways: one is hrHPV-mediated, which is more immunogenic and associated with a better prognosis, while the other is HPV-independent and induced by chronic irritation, inflammation, and genetic alterations.[9]

Research on the tumor microenvironment in humans has shown that HPV-associated hpSCCs have different subsets of tumor-infiltrating immune cells compared to HPV-negative hpSCCs. Studies have shown that the average number of tumor-infiltrating T-cells is higher in hrHPV-associated than in hrHPV-negative cases, and in HPV-associated cases, the T-cells are strongly polarized towards a Th1 and cytotoxic immune response. In addition, HPV-positive SCCs are associated with significantly more tumor-infiltrating T lymphocytes and less stromal T lymphocytes than in HPV-negative carcinomas.[7]

Only one previous investigation has been performed to explore the characteristics of the tumor microenvironment in EcPV2-related penile SCC. Results indicated that there was an increased number of CD3+ lymphocytes, macrophages, plasma cells, and FoxP3+ lymphocytes in the intra/peritumoral stroma compared to the extratumoral tissue.[12] However, differences in infiltrating immune cells between EcPV2-positive and EcPV2-negative tumors have not been investigated previously.

Assuming that hrHPV and EcPV2-induced tumors may have analogous tumor microenvironment characteristics, we investigated the stromal and intratumoral immune

cell infiltrate in a series of penile tumors: papillomas, in situ carcinomas (CISs), and SCCs. The aim was to determine whether EcPV2-positive tumors are associated with a different tumor microenvironment compared to EcPV2-negative tumors, and thus whether the horse could represent a potential spontaneous animal model for hpSCC.

In hrHPV-related tumors, the protein E6 plays a critical role in inactivating p53, resulting in loss of tumor-suppressive functions.[17] To evaluate a potential involvement of p53 in the pathogenesis of equine penile tumors, and in particular to highlight potential pathogenetic mechanisms in relation to EcPV-2 infection, the expression and mutation of p53 and *TP53*, respectively, were investigated with immunohistochemistry and next-generation sequencing (NGS). In addition, Ki67 proliferative activity was investigated to highlight potential differences related to tumor malignancy and EcPV-2 infection.

Materials and Methods

Case Selection, Histology and Classification

Cases with a diagnosis of penile papilloma, CIS, or SCC diagnosed between 2018 and 2022 were retrieved from the digital records of two institutions, the Anatomic Pathology Section of the Department of Veterinary Medical Sciences of the University of Bologna (Italy) and the Department of Veterinary Anatomy Physiology and Pathology, Veterinary and Ecological Sciences, University of Liverpool (UK). Formalin-fixed, paraffin-embedded (FFPE) material and hematoxylin and eosin-stained slides were retrieved from the archives. For each case, patients' signalment and tumor location were recorded.

The histological sections were reviewed by 2 authors (BBa, GT), and cases were confirmed as papillomas, CISs, and SCCs according to previously published criteria.[13]

Cases from University of Liverpool were used under the VREC1277 ethical approval.

Immunohistochemistry (IHC)

IHC for Ki67, FoxP3, and p53 was performed in each case using 4 µm-thick consecutive sections. Sections were dewaxed and rehydrated. Endogenous peroxidase was blocked by immersion in 3% hydrogen peroxide for 30 minutes. Antigen retrieval was achieved using citrate buffer at pH 6.0, with heating for p53 in two 5-minute cycles in a microwave oven at 750 W; for Ki67 and FoxP3, 20 minutes in a pressure cooker at 100-110°C. Primary antibodies used were p53 (monoclonal, clone PAb240, BD Pharmingen, 1:200 dilution), Ki67 (monoclonal, clone MIB-1, Dako Denmark, 1:600 dilution), and FoxP3 (monoclonal, clone FJK-16s, eBioscience, Invitrogen, 1:400 dilution). All antibodies were incubated with the tissue sections overnight at 4°C. The reactions were amplified by the avidin-biotin method (ABC kit elite, Vector) and visualized with 3,3'-diaminobenzidine (DAB, 0.04% for 4 minutes). Sections were counterstained with Harris' hematoxylin, rinsed in tap water, dehydrated, and coverslipped. Sections of equine squamous cell carcinoma with known p53 expression were used as positive controls. For Ki67 and FoxP3, normal equine intestine and lymph node were used as positive controls, respectively. Negative controls consisted of sections incubated with the omission of the primary antibody.
p53 labeling was evaluated semiquantitatively and considered positive when >10% of neoplastic cell nuclei exhibited positivity, as previously reported.[16] Cases were classified as follows: 0 (negative) or 1 (positive); and p53-positive cases were subcategorized as: B (predominantly basal) or NB (non-basal. i.e. suprabasal or diffuse positivity).

Multiplex Immunohistochemistry (mIHC)

The entire mIHC procedure was performed in an automated immunostainer (Discovery Ultra Roche Diagnostics) on 3 µm-thick sections from FFPE samples. Sections were dewaxed with EZ prep solution (Roche Diagnostics) and antigen retrieval was performed with CC1 solution (Roche Diagnostics) at 95°C for 40 minutes. A triple IHC protocol was used to identify T lymphocytes, B lymphocytes, and macrophages using CD3 (CONFIRM anti-CD3,

clone 2GV6, concentration 0.4µg/ml, Roche Diagnostics), CD20 (clone L26, dilution 1:300, Invitrogen) and IBA-1 (clone NB100-1028, dilution 1:2500, Novus Biological) respectively as primary antibodies. After incubation with CD3, sections were treated with rabbit multimer (Rb-HRP) and subsequently with Discovery ChromoMap DAB Kit (Roche Diagnostics). Sections were then incubated with CD20 and treated with mouse multimer (Ms-HRP) (Roche Diagnostics) and Discovery Teal HRP Kit (Roche Diagnostics). Finally, sections were incubated with IBA-1 and rabbit multimer as secondary antibody, followed by the Discovery Purple HPRP staining kit (Roche Diagnostics). Positive controls consisted of a normal equine lymph node. Negative controls consisted of sections incubated with the omission of the primary antibodies.

In Situ Hybridization (ISH)

ISH using RNAscope probes (Advanced Cell Diagnostics, ACD) was performed using probes targeting *E6/E7* oncogenes of EcPV2 according to the manufacturer's protocol. Briefly, FFPE samples were hybridized with *E6/E7* V-EcPV2 RNAscope probe. Six amplifying solutions were used for each amplification steps: AMP-1, AMP-3, and AMP-5 for 15 min each and AMP-2, AMP-4, and AMP-6 for 30 min.
Amplified signal was detected using Fast RED (Advanced Cell Diagnostics). Then nuclei were counterstained with Gill's haematoxylin.

Polymerase Chain Reaction (PCR)

Total DNA was extracted from 5 µm-thick FFPE tissue samples using the QIAamp DNA FFPE Tissue Kit (Qiagen, Germany) following manufacturer's instructions. DNA integrity and the presence of inhibitors were checked by amplification of a fragment of the horse *beta actin* gene.[11] Detection of EcPV2 DNA was performed using an end-point PCR with primers specifically designed on the E7 protein-encoding gene. The newly designed primers

(EcPV2_E7_F AGC GTG TTG CAG AGG AGG ACC TGG; EcPV2_E7_R GCC CCT CTT GTG ACG CGC AGT CCGC) amplified a 200-nucleotide fragment using the Taq DNA Polymerase kit (QIAGEN, Germany). The amplification program consisted of an initial denaturation step of 94°C for 5 minutes, followed by 40 thermal cycles: 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 30 seconds, and a final elongation step of 72°C for 5 min. In each amplification reaction, a DNA extract of an EcPV2+ sample was used as positive control while ultrapure water was used as no template control. PCR products were separated by electrophoresis in a 2.0% (W/V) agarose gel in Tris-acetate-EDTA buffer and visualized by UV light after staining with Midori Green Advance DNA Stain (Nippon Genetics, Düren, Germany). Amplicons of the expected size were considered positive.

Next-Generation Sequencing

NGS was performed at the Molecular Pathology Laboratory of Solid Tumor of the Policlinico Sant'Orsola-Malpighi, Bologna, Italy. Tumor areas with a high (>50%) tumor cellular component were selected, and the DNA was extracted from two to three 10 µm-thick sections using the Quick Extract FFPE Kit (Lucigen, LGC Biosearch Technologies, Hoddesdon, UK). DNA was then quantified using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA was amplified using a laboratory-developed NGS panel. The custom panel was designed using the Ion AmpliSeq Designer Tool (Thermo Fisher Scientific) covering the coding sequences of the following genomic regions (reference: Equus Caballus 2.0, total of 92 amplicons between 125 and 175 bp in length, panel size: 22.41 kb): *TP53* (start chr11:50952714, end chr11: 50959353) and *myosin* (start chr9: 45496284, end chr11: 45512499). The sequencing was performed using the GeneStudio S5 Prime Sequencer (Ion 530 Chip), and the results were analyzed using the Ion Reporter plugin Variant Caller (Thermo Fisher Scientific) and the Golden Helix GenomeBrowse (v3.0,

https://www.goldenhelix.com/products/GenomeBrowse/). To avoid false positive results, only the nucleotide variants with a variant allele frequency higher than 10% were used for the mutational call. The functional effect of each variant was evaluated using the PolyPhen2 tool (http://genetics.bwh.harvard.edu/pph2/), performing the query with the amino acid sequence of equus reference and the corresponding positions of the variants.

Digital Image Analysis

All immunohistochemical slides were scanned with Grundium Ocus 20 (Tampere, Finland) using a 20x objective (0.25 µm/pixel) to obtain a whole-slide image. The digital images were analyzed with the open-source software QuPath v0.5.0.3.[2]

Ki67 scores were determined in the whole-slide image (global score) by counting the number of Ki67-positive cells using the automatic positive cell counter tool in QuPath. Only the tumor area was selected using the *magic wand* tool, avoiding areas of necrosis, inflammation, and artifacts. The positive cell detection tool was applied to the selected area of the whole-slide image to detect positive and negative nuclei. The tool is based on a combination of thresholding for hematoxylin and DAB, morphological features (cell shape and size), and texture. The tool was used as follows: *Analyze → Cell analysis → Positive cell detection*. Parameters were interactively adjusted for each case using the following settings: detection image was set as optical density sum; requested pixel size = 0.5; background radius = 8; median filter radius = 0; sigma = 1.5; min/max area = 8/100 µm; threshold = 0.02-0.08; maximum background intensity = 3.0; and cell expansion = 0 µm. For each case, a single DAB threshold value was created for Ki67-staining (nucleus DAB optical density mean, threshold = 0.05-0.2), which was assessed and adjusted manually to refine detection and classification. Before each analysis, one or more randomly sized squares were drawn to adjust settings and establish a threshold for positive and negative

cell detection. To separate positive nuclei of tumor and non-tumor (stromal and inflammatory) cells, the *object classifier* tool was applied. A random trees object classifier was created by annotating representative tumor cells and non-tumor cells (training), and applied to the whole section to separate non-tumor and tumor nuclei. Due to the heterogeneity of tumors, annotations were added until a visually acceptable discrimination between epithelial tumor cell nuclei and stromal nuclei was achieved for each whole-slide image. The Ki67 score (% of positive cells out of the total number of nuclei) for tumor-only cells was recorded.

For FoxP3, the positive cell detection tool was used as described for Ki67 (Fig.1a). A random trees object classifier was created by annotating representative tumor cells and stromal cells (training) and applied to the whole section (Fig.1b). Positive cells were recoded as number of cells per mm^2, which was obtained by dividing the number of cells by the total area and was assessed and calculated using the *pixel classifier* tool (Fig.1c). Details on digital image analysis are included in the Supplemental Materials.

To evaluate mIHC positivity, the *pixel classifier* tool (Artificial Neural Network ANN_MLP) was used, to assess the total area occupied by each marker (CD3, brown; CD20, -teal; IBA-1, purple). Before the analysis, validation of the area-based analysis was performed by comparing manual cell counts performed by 4 different scorers and the areas occupied by each marker (Supplemental Materials, Supplemental Figures S1-4, Supplemental Table S1). Separate analyses were performed for tumor stroma (s-) and intraepithelial (tumor) (t-) compartments by manual annotations. To train the classifier, example annotations of each cell type were manually entered, predicting the area occupied by each cell type through color coding. The classifier was loaded to each whole-slide image after manual tumor-stroma segmentation, and when satisfactory prediction quality was reached for each case, the percentage of area occupied by each marker was recorded separately for tumor

and stroma (Fig. 2). Visual evaluation of image analysis results was performed for each analysis by an experienced pathologist (BBa).

Statistical Analysis

The clinical and histopathologic characteristics were summarized using descriptive statistics. Normality of continuous data was assessed with the Shapiro-Wilk test. All continuous data had a non-normal distribution. For non-normally distributed data, the median and range (min-max) were reported; for normally distributed variables, the mean and standard deviation (SD) were reported. To compare continuous variables between groups, the non-parametric Wilcoxon rank sum test was performed. To quantify the correlation between continuous variables, Spearman's correlation coefficient was used. To assess associations between categorical variables, Fisher's test was performed. Results were considered statistically significant at a threshold of $p \leq 0.05$. Statistical analysis was performed with the language for statistical computing R, v4.2.0 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Patient Characteristics, Histological Classification and EcPV2 Status

Twenty-seven penile tumors from 27 horses were included in the study. Of these, 5 (19%) were classified as papillomas, 3 (11%) as CISs, and 19 (70%) as SCCs. EcPV2 status was considered positive when either or both PCR and ISH were positive. EcPV2 positivity was found in 18/27 cases by PCR only, of which 16/18 cases were positive with ISH. All CISs and papillomas were EcPV2-positive, while only 10/19 SCCs were positive (Table 1). With ISH, positivity was observed only within neoplastic epithelial cells, never within the stromal or inflammatory cells (Fig. 3a).

Immune Cell Density and Topography

Area-based quantification of the mIHC revealed that CD3+ T-cells were the most abundant component of the inflammatory cell infiltrate both in the stromal and in the intraepithelial compartments (median: 11.23% and 7.25%, respectively). Approximately equal densities of IBA-1+ macrophages were present in the stromal and intraepithelial compartments (median: 2.65% and 2.71%, respectively). CD20+ B-cells were the least represented, with a median value of 0.12% and 0.11% of the intraepithelial and stromal areas respectively (Table 2, Fig.4).

Different numbers of immune cells were observed in EcPV2+ tumors compared to EcPV2- tumors, although no statistically significant differences were observed in density of tumoral or stromal T-cells, B-cells, or macrophages according to EcPV2 status (Table 3, Fig. 5). In detail, CD3+ cells represented 13.45% of the stromal area in EcPV2+ tumors, while in negative tumors, the median area percentage was 9.20 (p=0.085). By contrast, CD3+ cells were similar in the negative and positive cases within the intraepithelial compartment (median: 7.87% in EcPV2- versus 6.50% in EcPV2+ tumors, p=0.719).

CD20+ B-cells occupied a median value of 0.46% of the stromal area in the EcPV2-negative cases and 0.10% in the positive cases. In the intraepithelial compartment, B-cells occupied a median value of 0.11% of the area in the EcPV2- cases, and 0.14% in the EcPV2+ cases. These differences were not statistically significant (p=0.502 and 0.857, respectively).

Similar densities of IBA-1+ macrophages were detected in the two groups. The area occupied by stromal macrophages had a median value of 1.87% in the EcPV2- cases, and 2.76% in the positive cases (*p*=0.487). In the intraepithelial compartment, macrophages represented 3.58% of the area in the EcPV2- tumors, and 2.58% in the EcPV2+ ones (*p*=0.292).

FoxP3+ regulatory T-cells (Tregs) were detected in higher numbers in the stroma compared to the intraepithelial compartment (287 cells/mm^2 and 49 cells/mm^2, respectively). FoxP3+ cell densities were markedly higher in EcPV2+ tumors, compared to EcPV2- neoplasms, both in the intraepithelial (28 cells/mm^2 in EcPV2- versus 61 cells/mm^2 in EcPV2+) and in the stromal (95 cells/mm^2 in EcPV2- versus 395 cells/mm^2 in EcPV2+) areas (p=0.035 and 0.024, respectively). (Table 3, Fig. 6).

The infiltration by T-cells, B-cells, and macrophages was also evaluated in relation to each other. Scatterplots demonstrated only a modest correlation between CD3+ and FoxP3+ cells in the intraepithelial compartment (Supplemental Figures S5, S6).

ICIs were also evaluated based on histological classification, regardless of EcPV2 status, but statistical significance could not be calculated due to small sample sizes (Table 4). All cells were detected in similar densities in papillomas, CISs, and SCCs, except for tumor IBA-1+ macrophages, which were markedly denser in SCCs than in papillomas and CISs (Table 4). Stromal and tumor CD3+ lymphocyte concentrations were different according to diagnosis, with papillomas showing the highest density and SCCs having the lowest density. FoxP3+ Tregs were detected in similar numbers regardless of malignancy.

ICI analysis was also restricted to SCC cases. Results are summarized in Table 5. Briefly, immune cell infiltration mirrored the infiltrates of all cases, being higher in the EcPV-2+ tumors, although the difference in FoxP3+ Tregs was more striking. Specifically, Tregs were significantly more numerous in the EcPV2+ SCCs, both in the stromal (95.47 cells/mm^2 in EcPV2- versus 538.90 cells/mm^2 in EcPV2+) and epithelial (28.48 cells/mm^2 in EcPV2- 109.68 cells/mm^2 in EcPV2+) compartments (p=0.022 and 0.011, respectively).

p53 Expression/*TP53 Status*

p53 status was evaluated with both IHC and NGS to assess protein expression and gene mutation respectively. p53 was detected in 4/5 (80%) papillomas, 3/3 (100%) CISs, and

9/19 (47%) SCCs. Of these, 1/4 papillomas and 9/9 SCCs had non-basal positivity; hence, the diagnosis of SCC was associated with non-basal positivity (Table 6, Fig. 1c). *TP53* was assessed with NGS and was found to be non-evaluable (inadequate) in 6 cases due to DNA degradation. Of the remaining 21 cases, in 13 cases (62%) *TP53* was wild-type, while variations were detected in 8 cases (38%). Specifically, 4 *TP53* mutations were considered pathogenic with the Poliphen2 score, while the other 4 were classified as benign (3) or silent (1). All pathogenic mutations occurred in SCCs (Table 7), and not in CISs or papillomas. There was no association between p53 expression and EcPV2 status. Of the 13 p53+ cases, 4 were EcPV2+ and 9 were EcPV2-. Finally, immune cell infiltrates did not differ according to p53 expression or *TP53* mutation status; however, stromal Treg numbers were markedly higher in cases with pathogenic mutations compared to wild-type cases and cases with benign/silent mutations (Supplemental Table S2).

Ki67 Score

The mean Ki67 score was 17.22 (SD:8.85). Similar Ki67 scores were identified in papillomas (mean:15.16, SD:6.98), CISs (mean 18.60, SD:10.49), and SCCs (mean 17.18, SD:9.07). No association was found between Ki67 scores and p53/TP53 status. However, Ki67 scores were lower in the viral-associated tumors. The EcPV2- tumors had a mean Ki67 score of 21.29 (SD:10.34), while EcPV2+ tumors had a mean Ki67 score of 15.18 (SD: 7.52).

Discussion

The association between equine penile tumors and EcPV2 infection is well established[19] and is similar to that in humans, in which penile cancer is associated with hrHPV infections.[8] In men, hrHPV-associated penile tumors carry a better prognosis compared to hrHPV-negative neoplasms. One of the potential explanations for this difference is related to the tumor microenvironment. In fact, recent studies have demonstrated that hrHPV-related and hrHPV-unrelated penile tumors harbor a different tumor microenvironment.[7,9]

However, so far this question has not been addressed in equine penile tumors, and very little is known about potential differences between EcPV2-associated and viral-independent lesions in horses. With the aid of a supervised machine learning method in this study, immune cell populations were quantified and localized in a series of equine penile tumors. Although T-cells were found in higher densities in in EcPV2-related versus unrelated tumors, both in the stromal and intraepithelial compartment, these differences were not statistically significant. This finding overlaps with studies in hpSCC, which have demonstrated a higher number of immune cells in HPV-related cases, particularly in the stroma.[7,9] Moreover, in our case series, all immune cells varied according to malignancy; in fact, SCCs had lower immune cell density compared to CISs and papillomas. When the analysis was carried out based on diagnosis, differences were in fact more marked. Papillomas had the highest density of both stromal and intraepithelial CD3+ T-cells and FoxP3+ Tregs, which was lower in CISs and further decreased in SCCs. Spontaneous regression of papillomas is a well-known phenomenon related to intratumoral infiltration with cytotoxic T lymphocytes, hence lymphocyte infiltration in papilloma is regarded as a positive prognostic factor.[15]

Although results in the literature on hrHPV-related tumors are conflicting, results from previous studies in human hrHPV-related tumors parallel our findings.[7,10]

Tumor-associated macrophages play a major role in the tumor microenvironment by increasing angiogenesis, enhancing tumor cell mobility, and modulating immunotolerance.[3] Recent studies found a positive correlation between CD68+ macrophages (both M1 and M2 phenotypes) and tumor progression in human cervical and oral SCC. [9] Specifically for penile SCC, research on tumor-associated macrophages is limited. One study found no difference in CD163+ macrophages between hrHPV-related and hrHPV-unrelated SCCs,[1,5] while in another study, high intra-tumoral numbers of CD163+ correlated with lymph node metastasis.[9] In our study, macrophage density tended

362 to increase with malignancy, particularly in the stromal tissue, but despite higher density in
363 EcPV2+ tumors, differences were not significant. The role of macrophages in cancer
364 biology is well known, and our finding confirm that tumor-associated macrophages may
365 play a role in cancer progression.[3] To better evaluate the role of macrophages in equine
366 penile tumors, further investigation with additional M1 and M2 markers are needed.
367 P53 was also investigated with NGS and IHC. Because of the commonly observed
368 absence of p53 detection in different types of hrHPV-positive SCCs, expression of p53 in
369 SCC has been suggested as a surrogate marker for the absence of hrHPV infection.[16] In
370 our cohort, p53 protein expression, either as basal or supra-basal, did not predict EcPV2
371 status, Dysregulation of p53 was previously assessed in EcPV2-associated penile lesions;
372 immunohistochemical expression of the protein was limited to the basal layer in
373 papillomas, but was found in upper keratinocyte layers of CISs and SCCs.[16] In our cases,
374 p53 expression was not exclusively observed in malignant cases, although supra-basal
375 localization was almost exclusively observed in SCCs. This partially supports previous
376 investigations, in which p53 was indicated as a potential marker to define benign versus
377 malignant cases.[13,16] However, comparison of IHC and NGS analyses also indicated that,
378 in equine penile SCC, immunohistochemical expression and distribution of p53 is not
379 predictive of *TP53* mutation. Since the tumor mutational status can also influence immune
380 cell infiltrates in tumors, and ICIs in hpSCC are linked to *TP53* mutations,[1,18] potential
381 differences in ICI distribution were assessed according to p53 status, but no significant
382 differences were found. While pathogenic *TP53* mutations were observed exclusively in
383 malignant cases, mutations occurred equally in EcPV2+ and EcPV2- cases.
384 Data on proliferation were also assessed with Ki67. Results indicate that EcPV2+ penile
385 carcinomas have a lower proliferative activity, although this difference was not statistically
386 significant. It could be speculated that EcPV2+ tumors have lower malignant potential;

however, prospective studies with follow-up data and survival are needed to confirm this hypothesis.

Additional considerations need to be made for the digital quantification of immune cells. Digital image analysis has important advantages, mostly related to objectivity and speed of the analysis; however, there are a number of limitations. Firstly, minor inaccuracies can be expected for the tumor/stroma segmentation, since this step was performed manually for each case to allow a separate analysis of the two compartments for the triple IHC. Since CD3, CD20, and IBA-1 were performed by multiplex immunohistochemistry, these were assessed by evaluating the area occupied by each cell type with a pixel classifier, a machine learning tool integrated in the digital image analysis software. Hence, the area occupied by each cell type was an indirect measure of their number relative to the area and to each other. It is possible that IBA-1 positivity interpretation was slightly overestimated compared to the actual cell count, due to the normally larger size of macrophages compared to lymphocytes, also in relation to their increase in size upon activation. Overall, validation assays demonstrated a good correlation between manual and digital analysis, supporting the reliability of this method to analyse immunohistochemical positivity. FoxP3+ Tregs were evaluated in the whole tumor section, then averaged per mm^2. The majority of the previous studies evaluate a representative areas of 1 mm size, while in this case series, evaluation of whole tumor sections was performed, which allowed a presumably more representative quantification of cell density and topography, not being biased by the subjectivity of the area selection.

Limitations of this study must also be considered, in particular in relation to sample sizes. Although an equal number of EcPV2+ and EcPV2- cases was included which allowed statistical significance to be reached, the low number of papillomas and CISs precluded

more conclusive results, particularly regarding how the tumor microenvironment changes in relation to malignancy.

In conclusion, this study suggests that B-cell, T-cell, and macrophage infiltrates are not influenced by EcPV2 status. However, a higher number of Tregs was observed in EcPV2+ tumors, indicating that EcPV2+ infection elicits a different immune response compared to EcPV2-unrelated tumors, but future studies will be needed to elucidate the prognostic impact of tumor-infiltrating immune cells in equine penile tumors.

References

1. Ahmed ME, Falasiri S, Hajiran A, Chahoud J, Spiess PE. Review the immune microenvironment in penile cancer and rationale for immunotherapy. Vol. 9, *Journal of Clinical Medicine*. MDPI 2020:1–17.
2. Bankhead P, Loughrey MB, Fernández JA, et al. QuPath: Open source software for digital pathology image analysis. *Sci Rep*. 2017;**7**(1).
3. Chen XJ, Han LF, Wu XG, et al. Clinical significance of CD163+ and CD68+ tumor-associated macrophages in high-risk HPV-related cervical cancer. *J Cancer*. 2017;**8**(18):3868–3875.
4. Fernández-Nestosa MJ, Clavero O, Sánchez DF, et al. Penile intraepithelial neoplasia: Distribution of subtypes, HPV genotypes and p16INK4a in 84 international cases. *Hum Pathol*. 2023;**131**:1–8.
5. Hladek L, Bankov K, von der Grün J, et al. Tumor-associated immune cell infiltrate density in penile squamous cell carcinomas. *Virchows Arch*. 2022;**480**(6):1159–1169.
6. Knight CG, Munday JS, Peters J, Dunowska M. Equine penile squamous cell carcinomas are associated with the presence of equine papillomavirus type 2 dna sequences. *Vet Pathol*. 2011;**48**(6):1190–1194.

436 7. Lohneis P, Boral S, Kaufmann AM, et al. Human papilloma virus status of
437 penile squamous cell carcinoma is associated with differences in tumour-
438 infiltrating T lymphocytes. *Virchows Arch*. 2015;**466**(3):323–331.
439 8. Moch H, Cubilla AL, Humphrey PA, Reuter VE, Ulbright TM. The 2016 WHO
440 Classification of Tumours of the Urinary System and Male Genital Organs—
441 Part A: Renal, Penile, and Testicular Tumours. *Eur Urol*. 2016;**70**(1):93–105.
442 9. Ottenhof SR, Djajadiningrat RS, Thygesen HH, et al. The prognostic value of
443 immune factors in the tumor microenvironment of penile squamous cell
444 carcinoma. *Front Immunol*. 2018;**9**(JUN).
445 10. Partlová S, Bouček J, Kloudová K, et al. Distinct patterns of intratumoral
446 immune cell infiltrates in patients with HPV-associated compared to non-virally
447 induced head and neck squamous cell carcinoma. *Oncoimmunology*.
448 2015;**4**(1):965570.
449 11. Pietra M, Peli A, Bonato A, Ducci A, Cinotti S. Equine bronchoalveolar lavage
450 cytokines in the development of recurrent airway obstruction. *Vet Res*
451 *Commun*. 2007;**31**(SUPPL. 1):313–316.
452 12. Porcellato I, Mecocci S, Mechelli L, et al. Equine Penile Squamous Cell
453 Carcinomas as a Model for Human Disease: A Preliminary Investigation on
454 Tumor Immune Microenvironment. *Cells*. 2020;**9**(11):1–17.
455 13. Ramsauer AS, Wachoski-Dark GL, Fraefel C, et al. Paving the way for more
456 precise diagnosis of EcPV2-associated equine penile lesions. *BMC Vet Res*.
457 2019;**15**(1):1–13.
458 14. Sykora S, Brandt S. Papillomavirus infection and squamous cell carcinoma in
459 horses. *Vet J.* 2017;**223**:48–54.

15. Thaiwong T, Sledge DG, Wise AG, Olstad K, Maes RK, Kiupel M. Malignant transformation of canine oral papillomavirus (CPV1)-associated papillomas in dogs: An emerging concern? *Papillomavirus Res*. 2018;**6**:83–89.
16. van den Top JGB, Harkema L, Lange C, et al. Expression of p53, Ki67, EcPV2- and EcPV3 DNA, and viral genes in relation to metastasis and outcome in equine penile and preputial squamous cell carcinoma. *Equine Vet J*. 2015;**47**(2):188–195.
17. Trias I, Saco A, Marimon L, et al. P53 in Penile Squamous Cell Carcinoma: A Pattern-Based Immunohistochemical Framework with Molecular Correlation. *Cancers (Basel)*. 2023;**15**(10).
18. Zargar-Shoshtari K, Spiess PE, Berglund AE, et al. Clinical Significance of p53 and p16ink4a Status in a Contemporary North American Penile Carcinoma Cohort. *Clin Genitourin Cancer*. 2016;**14**(4):346–351.
19. Zhu KW, Affolter VK, Gaynor AM, Dela Cruz FN, Pesavento PA. Equine Genital Squamous Cell Carcinoma: In Situ Hybridization Identifies a Distinct Subset Containing Equus caballus Papillomavirus 2. *Vet Pathol*. 2015;**52**(6):1067–1072.

Figure legends

Figure 1. Penis, horse. Image analysis for the quantification of FoxP3+ T-cells, immunohistochemistry (IHC). a) IHC showing intratumoral FoxP3+ T lymphocytes. b) Automatic nuclei detection of intratumoral FoxP3+ cells (red), intratumoral FoxP3- cells (blue), and stromal FoxP3+ cells (green). Inset: high magnification showing FoxP3+ intratumoral nuclei (red outline), intratumoral FoxP3- cells (blue outline), and FoxP3+ stromal nuclei (green) c) Area quantification of tumor and stromal compartments by pixel classification. Red, tumor; green, stroma.

Figure 2. Penis, horse. Image analysis for the quantification of immune cells in multiplex immunohistochemistry. a) Low magnification of a papilloma in a whole-slide image. b) Manual area selection to separate intraepithelial (tumor) from stromal compartment (red outline). c) Pixel classification of CD3+ cells in brown, CD20+ cells in teal, and IBA-1+ cells in purple, and tumor in green. d) Higher magnification of annotated area. e) Higher magnification of quantification with pixel classification.

Figure 3. Penis, horse. a) Squamous cell carcinoma showing intracytoplasmic positivity for equine papillomavirus type 2. In situ hybridization. b) Basal positivity of equine penile papilloma for p53. p53 immunohistochemistry (IHC). c) Non-basal positivity of neoplastic cells for p53 in a penile squamous cell carcinoma. p53 IHC.

Figure 4. Penis, horse. Squamous cell carcinoma showing an infiltrate of CD3+ cells (brown), CD20+ cells (teal), and IBA-1+ cells (purple). Inset: high magnification. Multiplex immunohistochemistry.

Figure 5. Boxplot graph showing quantification of CD3, CD20 and IBA-1 positivity separated according to tumoral and stromal compartments, and according to equine papillomavirus type 2 (EcPV2) status. Immune cells are detected in similar amounts in EcPV2+ tumors compared to EcPV2- tumors.

Figure 6. Boxplot graph showing quantification of FoxP3 positivity separated according to tumoral and stromal compartments, and according to equine papillomavirus type 2 (EcPV2) status. Both intraepithelial and stromal FoxP3+ cell densities are significantly higher in EcPV2+ tumors.