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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

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Summary

Reasons for performing study: Equine penile and preputial squamous cell carcinoma (SCC) is a potentially lethal disease of which little is known regarding the relationship between tumour characteristics and prognosis.
Objectives: To assess the relationship between tumour differentiation grade (tumour subtype), presence of papillomaviruses, expression of viral genes (E2, E6, L1), nuclear proteins p53 and Ki67 and metastasis in equine penile and preputial SCC and to assess the relationship of tumour subtype, presence of papillomavirus *type 2*, p53 and Ki67 with survival.
Study design: Retrospective case–control study using archived material.
Methods: Samples (n = 103) from 87 horses with penile and/or preputial intraepithelial neoplasia (PIN), papilloma or SCC and corresponding case files were evaluated. Tumours were graded microscopically and p53 and Ki67 expression evaluated immunohistochemically. Equine papillomavirus (EcPV) *types 2* and *3* DNA was detected by conventional PCR. Real-time PCR was used for quantification of E2, E6 and L1 mRNA.
Results: Equine papillomavirus *type 2* DNA was detected in 89.4% and EcPV3 in 1.5% of horses. No differences in quantitative expression of E2, E6 and L1 oncogenes between subtypes were found. Expression of p53 and occurrence of metastasis were positively correlated to a less differentiated subtype ($r = 0.429$, $P<0.001$ and $r = 0.769$, $P = 0.001$, respectively). Differences in survival between subtypes were significant (log Rank $P<0.001$); horses with less differentiated tumours were more likely to die of the disease (papilloma 8.3%; G1 26.1%; G2 26.3%; G3 63.3%).
Conclusions: In equine penile and preputial SCC, tumour grading is an important prognosticator for survival and a predictor for presence of metastases. Expression of p53 and Ki67 and presence or expression of EcPV2 and EcPV3 do not appear to be important prognosticators.

Keywords: horse; squamous cell carcinoma; penis; grading; p53; Ki67; EcPV2; EcPV3; immunohistochemistry

Introduction

Squamous cell carcinoma (SCC) is the most common neoplasm of the external genitalia in horses, with an incidence of 49–82.5% [1–3]. Breed, nonpigmented genitalia and virus infection are risk factors for the development of SCCs in the horse [3–8].

In human penile cancer, risk factors for lymph node metastases include tumour depth or thickness, anatomic site, size, growth pattern, certain pathological subtypes (based on histopathological grading), and perineural and lymphovascular invasion, with the latter 3 being the most important predictors [9]. In horses, histological grade has been reported to have predictive potential for nodal metastases and therapeutic success rates of penile SCC [10]. In man, there is strong evidence that certain human papillomavirus types (HPV), especially HPV16 and-18 with their oncoproteins (E6 and E7), are causative agents for malignancies [11]. In horses, the presence of *Equus caballus* papillomavirus *type 2* (EcPV2) in penile intraepithelial neoplasia (PIN) lesions, papillomas and SCCs has been reported [6–8,12,13]. Recently, *Equus caballus* papillomavirus *type 3* (EcPV3) has been described in aural plaques [14]. It is not known whether this novel papilloma virus can be present in penile and preputial neoplasms as well.

In man, several nuclear markers have been evaluated as potential prognosticators [15]. Deregulated p53 function is involved in many types of cancer, as the protein plays a role in cell proliferation, apoptosis, response to DNA damage and genome stability. Interactions with viral oncoproteins can either stabilise wild type p53 protein or accelerate its degradation, resulting in loss of p53 function and uncontrolled cell growth [16]. In human penile cancer patients, p53 expression is an independent predictor of lymph node metastasis and survival [15], Ki67 is a nuclear protein that is associated with, and may be necessary for, cellular proliferation. The expression of Ki67 in human penile cancer has been shown to be correlated to nodal metastasis but is not deemed a good prognosticator for survival [17–19].

The objective of the current study was to assess the relationship between tumour differentiation grade (tumour subtype), presence of papillomaviruses, expression of viral genes (E2, E6, L1), nuclear proteins p53 and Ki67 and metastasis in equine penile and preputial SCC and to assess the relationship of tumour subtype, presence of papillomavirus *type 2* and nuclear proteins p53 and Ki67 with survival.

Materials and methods

Patients

Case records of male horses with SCC of the external genitalia referred to the Utrecht University Department of Equine Sciences (from 1988 to 2011) and the Department of Surgery and Anaesthesiology of Domestic Animals, Faculty of Veterinary Medicine, Ghent University (from 2000 to 2011) were evaluated. Data recorded included castration status, time of diagnosis, treatment, follow-up time and outcome. Cases with a sufficient amount of archived paraffin-embedded tumour tissue were selected for further histological and immunohistochemical (IHC) analyses.

Histological grading

The original haematoxylin and eosin (H&E) stained sections from which the diagnosis had been made were independently reviewed by 2 of the authors (J.G.B.v.d.T. and L.H.) using standard light microscopy. Penile

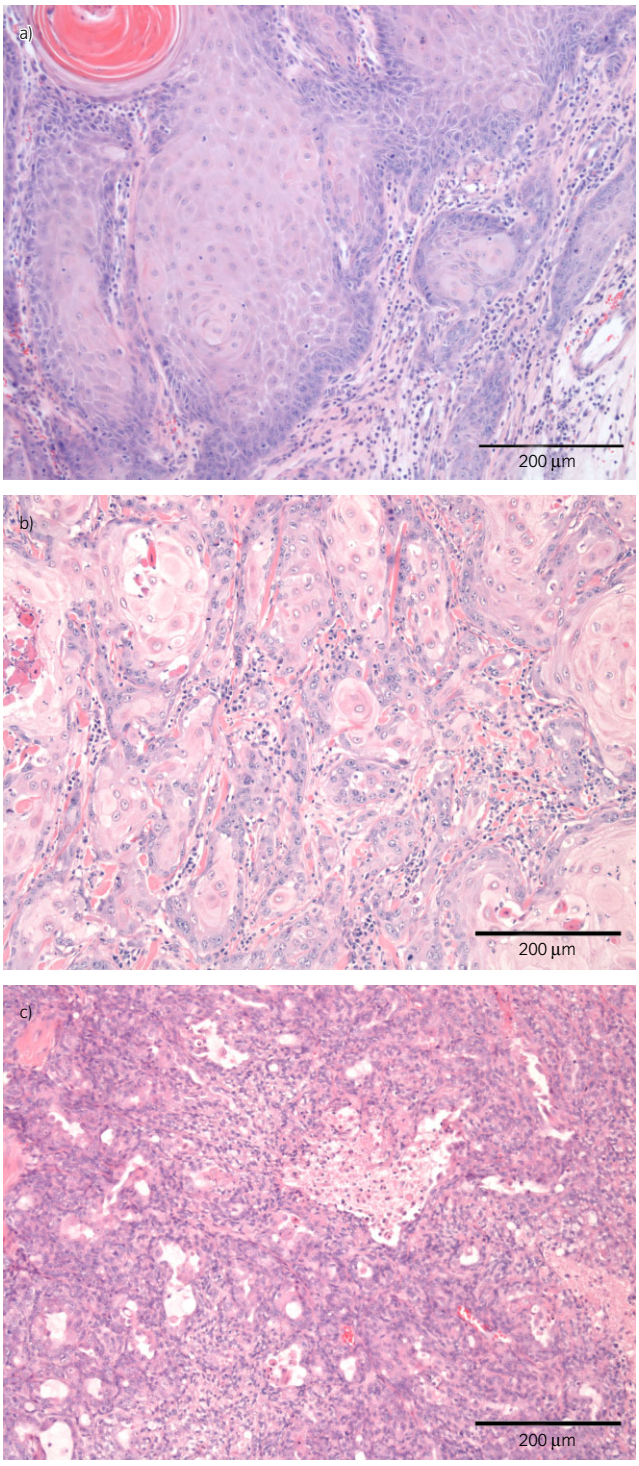

Fig 1: a) H&E stained section of a well-differentiated squamous cell carcinoma (SCC) (G1). b) H&E stained section of a moderately differentiated SCC (G2). c) H&E stained section of a poorly differentiated SCC (G3).

intraepithelial neoplasia, papilloma and SCC were distinguished. For SCC, an earlier described 3-tier grading system was used [20]. In summary, grades were classified as well differentiated tumours (*grade 1*; G1; Fig 1a), moderately differentiated (*grade 2*; G2; Fig 1b) or poorly differentiated (*grade 3*; G3; Fig 1c). In well-differentiated tumours, neoplastic cells were almost undistinguishable from normal/hyperplastic squamous cells, except for the presence of minimal basal/parabasal atypia. Tumours were considered G3 when showing any proportion of anaplastic cells. High grade areas were identified at low power either as solid sheets of irregular small aggregates, cords or nests of cells. At higher power, they were characterised by little or no keratinisation, high nuclear/cytoplasmic ratio, a thick nuclear membrane, nuclear pleomorphism, clumped chromatin, prominent nucleoli and numerous mitoses. Moderately differentiated neoplasms (G2) were the remainder of cases, not fitting into criteria described for G1 and G3. Compared with G1 tumours, they were more disorganised and showed less keratinisation, higher nuclear cytoplasmic ratio, a thickened nuclear membrane, moderate nuclear pleomorphism, evident nucleoli, more obvious mitoses and variable clumped chromatin. The poorest differentiated part of the tissue was decisive for the grade. In cases of different grading by the 2 observers, definitive grading was achieved by consensus after discussion.

Additionally, of the available lymph nodes sections, the presence or absence of metastasis was recorded.

P53 and Ki67

Immunohistochemistry: Formalin-fixed, paraffin-embedded tissues were mounted on silane coated slides (KP plus)[a]. Slides were treated with 1:200 monoclonal mouse anti-p53 (DO-1, SC-126)[b] and 1:50 monoclonal mouse anti-Ki67 (MIB-1, SC-101861)[b] overnight at 4°C. Goat anti-mouse biotinylated immunoglobulin G (Vector BA-9200)[c] was used as secondary antibody at a concentration of 1:125. The immune reaction was visualised with a standard ABC/peroxidase kit (Vector PK-4000)[c] for p53 and an elite ABC/peroxidase kit (Vector PK-6100)[c] for Ki67, with diaminobenzidine (DAB) as a chromogen. The sections were counterstained with haematoxylin.

An SCC with known p53 expression served as a positive control, equine colonic mucosa served as a positive control for Ki67; negative controls were made by omitting the primary antibody. Slides with melanin staining in the epithelium (11 normal control tissues, 1 PIN and 1 SCC G3) were bleached [21].

Immunohistochemical analysis: Slide scanning was performed on 3 XT Scanscope scanners[d] with an Olympus 20/0.75 Plan Apo objective[e]. Immunohistochemical analyses were performed using a personal computer (HP Z400, Windows XP)[f,g] with help of Imagescope viewing software (version 10.2.2.2319)[d], comprising an IHC nuclear image analyses algorithm (version v8 10.0.0.1798)[d] and a colour deconvolution algorithm (version v9 v10.0.0.1798)[d]. Stain colour parameters were calculated automatically using the colour deconvolution algorithm. The positive control was a slide with only expression of the stain (DAB) and no nuclear counter-staining. The negative control was a slide only expressing the nuclear counterstain (haematoxylin), with no positive staining. An SCC and colon with known expression of p53 and Ki67 respectively were used for this purpose. For further details on the calibration of the nuclear image analysis algorithm, see Item S1.

Next, a standard grid with multiple rectangles (1E6–2E6 μm^2) was projected over the slide and used to define the areas to be counted. The nuclear image analyses algorithm was subsequently used to quantify the percentage of positive nuclear staining and the average staining intensity (1 = weak, 2 = moderate, 3 = intense) of the nuclei within the predefined rectangles. An expression score was calculated by multiplying the percentage of staining by the average staining intensity (e.g. expression score = 10.4% x 2 = 21).

EcPV2 and EcPV3

Detection of EcPV2 DNA and EcPV3 DNA: Rolls of paraffin-embedded tissue (6 x 20 µm) were harvested for DNA extraction. Between different samples the microtome was cleaned with alcohol 96%, DNA Away[h] and knives were changed. Between every 5 tumour samples, a negative control sample was cut (normal equine testicle, kidney and liver). Using a DNeasy Blood and Tissue kit[i] DNA was isolated according to the manufacturer's recommendations.

Equine glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as a housekeeper gene [22]. To screen for EcPV2 2 different primer sets were chosen, the first PCR amplifying a fragment from the E1 gene, the

TABLE 1: Primers and conditions for conventional and real-time polymerase chain reaction (PCR) assays

Target and primer names	Sequences	PCR conditions
EcPV2 E1 (679nt)		
E1 EcPV2-NB f	5′-GCGGACTGCGCGTCACAAGAGGGGC-3′	95°C for 5 min, 35 cycles at 95°C for 20 s, 68°C for 40 s, 72°C for 1 min, 72°C for 10 min
E1 EcPV2-NB r	5′-ACGCAAGCACCACCCACTGCTTGGCA –3′	
EcPV2 L1 (57nt)		
L1 EcPV-SP1 f	5′-CGTGCACAGGGGCAAAAC-3′	95°C for 5 min, 30 cycles at 95°C for 1 min, 56.5°C for 1 min, 72°C for 1 min, 72°C for 10 min
L1 EcPV-SP1 r	5′-AACTGTAACATACACCTTGTC-3′	
EcPV3 L2 (290nt)		
We_F03	5′-TCTGGGTAGCCGCAAATATC-3′	94°C for 3 min, 40 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s
We_R09	5′-GCACCTGAGGTCCTATCTGG-3′	
Equine GAPDH gene		
EcPV2 E2 (real-time PCR)		
E2-2791f	5′-GATGCACTGGCCGAAAGACA-3′	95°C for 10 min, 40 cycles at 95°C for 15 s, 59°C for 60 s
E2-2855r	5′-TCTCTGACAAAACCACAGGTCCA-3′	
EcPV2 E6 (real-time PCR)		
E6-461f	5′-TCAGTGTACCGCGTCAGTGTCC-3′	95°C for 10 min, 40 cycles at 95°C for 15 s, 65°C for 60 s
E6-526r	5′-CGCCCTCGATCTCAATCCAC-3′	
EcPV2 L1 (real-time PCR)		
L1-6857f	5′-CATGGACCCAGACATTCTTGAGG-3′	95°C for 10 min, 40 cycles at 95°C for 15 s, 60°C for 60 s
L1-6904r	5′-TGAGAGCTGCGAGGACATGG-3′	

second a fragment from the L1 gene of EcPV2 [7] (Table 1). For both primer sets PCR was performed in a 10 μl reaction mixture, containing 200 μmol of each dNTP, 0.5 μmol of forward and reverse primer, 0.5 U FastStart *Taq* DNA polymerase[l] and 1.5 mmol/l $MgCl_2$ with 2.5 μl of template DNA. To test for EcPV3 DNA a primer set amplifying a fragment of the L2 gene of EcPV3 was designed and evaluated. Prior to sample testing the detection level of the PCR was evaluated, which showed that about a single template molecule per reaction could be detected. Red Taq ready reaction mix (Sigma-Aldrich)[k] was used according to the manufacturer's recommendations.

All samples were tested undiluted and as a 1:10 dilution. Polymerase chain reaction products were separated by electrophoresis on an agarose gel and visualised by ethidium bromide staining. Negative extraction controls with DNA from normal equine testicle, kidney and liver as well as a no template control with H_2O were included in each experiment.

Quantitative EcPV2 mRNA analysis: Sample collection, RNA extraction, cDNA synthesis and quantitative analysis: Twenty-six samples from 18 horses were also stored in RNAlater (Ambion)[l] for subsequent mRNA analysis. Total RNA isolation and subsequent first strand cDNA synthesis were performed as described previously [22]. Polymerase chain reaction primers for detection of EcPV2 E2, E6 and L1 are presented in Table 1. Specificity was tested using BLAST analysis against the genomic NCBI database. The amplicon and surrounding sequences were characterised using Mfold [23] to take into account possible secondary structures at the primer binding site which might influence the PCR efficiency. The PCR products were cloned and sequenced for verification. For detailed information on the procedure, see Supporting Information Item S2. Obtained data were normalised against a set of 3 reliable reference genes (Ubiquitine, RPL32 and hypoxantine phosphoribosyltransferase [HPRT]), determined according to [22].

Outcome and follow-up

Nine horses were subjected to euthanasia at first presentation because of the severity of the disease (n = 7) or because of post surgical complications (n = 2). For the horses that were discharged from the hospital follow-up information was obtained using a questionnaire that was filled in at the clinic by the attending clinician (J.G.B.v.d.T. or A.M.) when horses returned for re-examination, or through written or telephone correspondence with the owners when horses did not return during the study period. A maximum of 4 attempts were made to contact the owner. The follow-up data from an earlier study [3] formed the core of the data used for the present study. The follow-up time was 6–168 months (mean 43.0 months; median 37.5 months; s.d. 34.1 months).

Data analyses

The level of agreement of tumour grading was calculated with Kappa analysis.

Data of real-time PCR, p53- and Ki67 expression scores were log-transformed to achieve a normal distribution (checked by graphical analysis of p-plot). Differences between subtypes in presence of EcPV2 in ln expression of E2, E6 and L1 genes, ln p53 and ln Ki67 expression scores and occurrence of metastasis were analysed with restricted maximum likelihood by use of a linear mixed model with tumour composition and presence of EcPV2 as factors.

To account for repeated measures (analysis of multiple tumours in one horse) the 'horse-subject' was introduced as a random effect factor in the linear mixed model.

Correlations between all single independent variables (subtype, tumour composition and presence of EcPV2) and the dependent variables (ln p53- and ln Ki67 expression scores) were calculated in a multiple regression analysis (method enter) using Pearson's correlation coefficient. Correlation between subtypes and metastasis was calculated using Spearman's rank correlation coefficient.

Survival was calculated using Kaplan–Meier survival analysis with dependent variable time-to-event and with as independent variables subtype, presence of EcPV2, and p53 and Ki67 expression scores. Log Rank statistics were used to compare survival curves. Horses were censored when dying of another cause than penile and preputial SCC, lost to follow-up or when horses were still alive at the end of the study.

For all tests, differences were considered significant at $P \leq 0.05$. All statistical analyses were performed with SPSS 20[m].

Results

Patients

Case records from 134 horses with penile and preputial tumours were identified. Forty-seven cases were excluded because of the inability to locate the owner (31 cases), incomplete records and/or the absence of formalin fixed tissue samples (16 cases). Tissue samples and follow-up information were available for 87 horses of various breeds (Item S3). In total, 103 samples from the 87 horses were included. Six horses were

TABLE 2: Differentiation grade (subtype), composition, presence of viral antigen, metastases and follow-up data from 103 samples of penile and preputial tumours in 87 horses, with respect to tumour subtypes

	Conclusive tumour subtypes					
	PIN	P	G1	G2	G3	Total
Total tumours examined	9	16	24	19	35	103
Primary tumours at first presentation	3	12	23	19	30	87
Composition of total tumours examined						
Homogeneous	9	16	8	6	6	45
Papilloma/well			16			16
Papilloma/moderate				1		1
Well/moderate				12		12
Moderate/poor					16	16
Well/moderate/poor					13	13
EcPV2, EcPV3#						
Positive samples	8	14(#1)	24	16	29	91
Negative samples	0	1	0	3	6	10
No GADPH	1	1				2
Occurrence of metastases						
Detected				1	12	13
Not detected		1		4	1	6
Follow-up status after first presentation						
No evidence of disease	3	5	9	7	6	30
Alive with disease		2		1	1	4
Dead of other cause		4	8	6	4	22
Dead of disease		1	6	5	19	31

PIN = penile intraepithelial neoplasia; P = papilloma; G1 = well-differentiated tumour; G2 = moderately differentiated tumour; G3 = poorly differentiated tumour; well = well differentiated tumour cells; moderate = moderately differentiated tumour cells; poor = poorly differentiated tumour cells.

stallions and 77 geldings; the castration status of 4 horses was unknown. The median age at first presentation was 18.0 years (mean 18.9 years; range 7–33 years).

Grading

Initially, both observers (J.G.B.v.d.T. and L.H.) independently graded 63.1% of the tumours identically, which represents a moderate level of agreement (κ = 0.529, P<0.001). The other 36.9% of the tumours were graded by consensus after discussion. Primary tumours at first admission were graded as PIN (n = 3), papilloma (n = 12), SCC G1 (n = 23), SCC G2 (n = 19) and SCC G3 (n = 30). Of the total number of tumours examined (including recurrences) 45 (43.7%) had a homogeneous and 58 (56.3%) a heterogeneous composition (Table 2).

Horses diagnosed the first time with PIN lesions had a lower median age than horses with other lesions: 11.5 years (mean 12.4; range 7–17 years). The median age of horses diagnosed with papilloma was 17.5 years (mean 17.8; range 12–27 years), with G1 SCC 19.5 (mean 20.1; range 9–30), with G2 SCC 17.5 years (mean 17.8; range 9–31 years) and with G3 SCC 20.5 years (mean 20.6; range 12–27).

Virus detection

In 2 of the 103 samples no GADPH DNA could be detected. Of the GADPH-positive samples, EcPV2 was detected in 91 (90.1%) samples and 10 (9.9%) samples were negative. Of all horses, 76 (89.4%) were positive for EcPV2, there were no significant differences in EcPV2 presence between tumour subtypes. Eighty-nine lesions of 67 horses were examined for the presence of EcPV3, of which 2 were negative for GADPH. EcPV3 was found in only one papilloma (1.1% of lesions; 1.5% of horses); EcPV2 was also present in that lesion. Of the 12 normal penile and preputial skin samples, one (8.3%) sample was positive for EcPV2, which was significantly less than in all tumour subtypes (P<0.001).

Expression of viral E2, E6 and L1 genes

There were no statistically significant differences between the logarithmically transformed values of the expression of the 3 EcPV2 genes (E2, E6 and L1) in noninvasive lesions (PIN, papilloma) and invasive lesions (SCC G1, -G2 and -G3) (data not shown). None of the control samples was positive. The correlation between ln expression of E2 and L1 in lesions was moderate, but significant (r = 0.491 P = 0.011).

P53 and Ki67 expression

The correlations between observers and the nuclear image analyses algorithm for p53 and Ki67 detection were r≥0.932 (P<0.010).

The ln p53 expression score of G2 (mean 3.3, s.d. 1.5) was significantly higher (P = 0.045) than the score of normal skin (mean 1.6, s.d. 0.4) (Fig 2). Scores of G2 and G3 SCCs were significantly higher than of PIN lesions and also than the G1 score (Fig 2). There was a significant difference (P<0.001) of ln p53 expression scores between EcPV2 negative (mean 3.0, s.d. 1.7) and EcPV2 positive (mean 2.6, s.d. 1.5) lesions.

A moderate, significant correlation was found between ln p53 expression score and subtype (r = 0.429 P<0.001) and a weak, but significant correlation between ln p53 expression score and tumour composition (r = 0.275 P<0.032). In the multiple regression model (predictors: subtype, tumour composition and presence of EcPV2), subtype (β = 0.485 P<0.001) and presence of EcPV2 (β = 0.266 P<0.003) were significant predictors. This means subtype and presence of EcPV2 have significant effect on p53 expression: 'p53 expression score' = 0.485* 'subtype' + 0.050 * 'tumour composition' + 0.266 * 'presence of EcPV2'. The level of variance that can be explained (r^2) by this model was 0.228 (22.8%).

There were no correlations between ln p53 and ln E2, E6 or L1 expression.

There were no differences in ln Ki67 expression scores between subtype groups (P = 1.000) (Fig 2), or significant correlations between ln Ki67 expression scores and any other parameter.

Occurrence of metastasis

Lymph nodes were histopathologically examined in 19 horses and metastases detected in 13. The primary tumour diagnosed in these horses was an SCC G3 in 12 (92.3%) and an SCC G2 in 1 (7.7%) case. A strong correlation was found between subtype and occurrence of metastases (r = 0.769, P = 0.001).

There was no difference of ln p53 (P = 0.261) or ln Ki67 expression score (P = 0.765) in lesions of horses with or without metastasis.

Follow-up

Follow-up information was obtained for 6 cases by questionnaire when the owners visited the clinic, for 62 cases by telephone and 19 cases by mail.

All horses with a PIN lesion as primary lesion survived (Fig 3). One horse with a papilloma (8.3% of the total horses diagnosed with papilloma), 6 (26.1%) with a G1 SCC, 5 (26.3%) with a G2 SCC and 19 (63.3%) horses with a G3 SCC died of the disease. In total, 31 (35.6%) horses died of the disease (with or without treatment). Differences in survival between subtypes were significant (log Rank P<0.001). Only one (11.1%) of 9 EcPV2 negative horses died of the disease against 30 (39.5%) of 76 infected horses; however, this difference was not significant (log Rank P = 0.108).

There is a trend towards better survival in horses having a non-log transformed p53 expression score of ≤100 than in horses with score >100 (log Rank P = 0.063) (Fig 4). Ki67 expression score was not found to be related to survival.

Discussion

Tumour subtype (histopathological differentiation grade) was a significant predictor for survival based on the Kaplan–Meier survival analysis. Survival

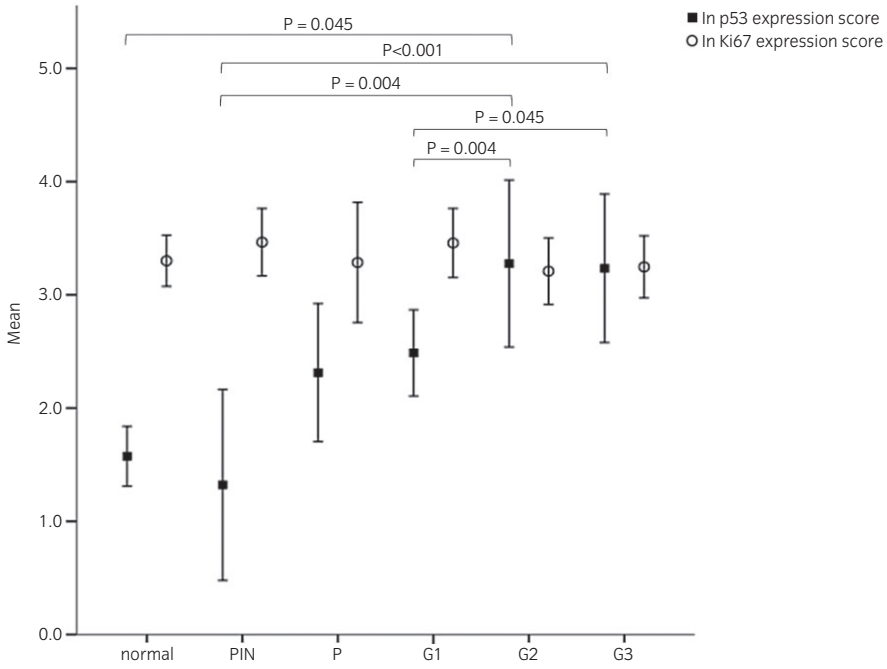

Fig 2: Expression score of p53 and Ki67 on a logarithmic scale related to subtype. Dots and squares represent means and bars represent 95% confidence interval. PIN = penile intraepithelial neoplasia; P = papilloma; G1 = well-differentiated tumour; G2 = moderately differentiated tumour; G3 = poorly differentiated tumour.

was high for animals affected with PIN or papilloma, but the chance of survival decreased considerably in animals with G1 and G2 tumours and was worst in animals with G3 tumours. These observations are in line with earlier findings that in horses with poorly differentiated lesions treatment is more likely to be unsuccessful [10], which had prompted the proposal that in equine medicine, as in human medicine, tumour grading should be part of a protocolled approach for this type of tumour [24]. However, it was noted that, to provide a firmer base for this recommendation, further investigations, preferably of multicentre character, into the relationship of tumour grade with outcome in larger populations would be desirable. The present study provides further evidence and support for this recommendation.

In man, metastatic rate is significantly higher in tumours harbouring any proportion of *grade 3* tissue [20]. In the current study, a similarly strong correlation between subtype and metastasis (r = 0.769 P = 0.001) was found, providing a scientific basis to extend the human recommendation [20] to carefully screen for metastases in *grade 3* SCCs to horses.

There are various indications that infection with EcPV2 plays an important role in the pathogenesis of penile cancer in the horse [6–8,12,13]. The current study supports this, as 90.1% of the samples were positive against only one positive result in 12 samples from normal penile skin. In man, HPV presence in penile SCC is seen as a positive prognosticator for survival [25], but we could not confirm this for horses. The most likely explanation is that EcPV2 presence in equine urogenital

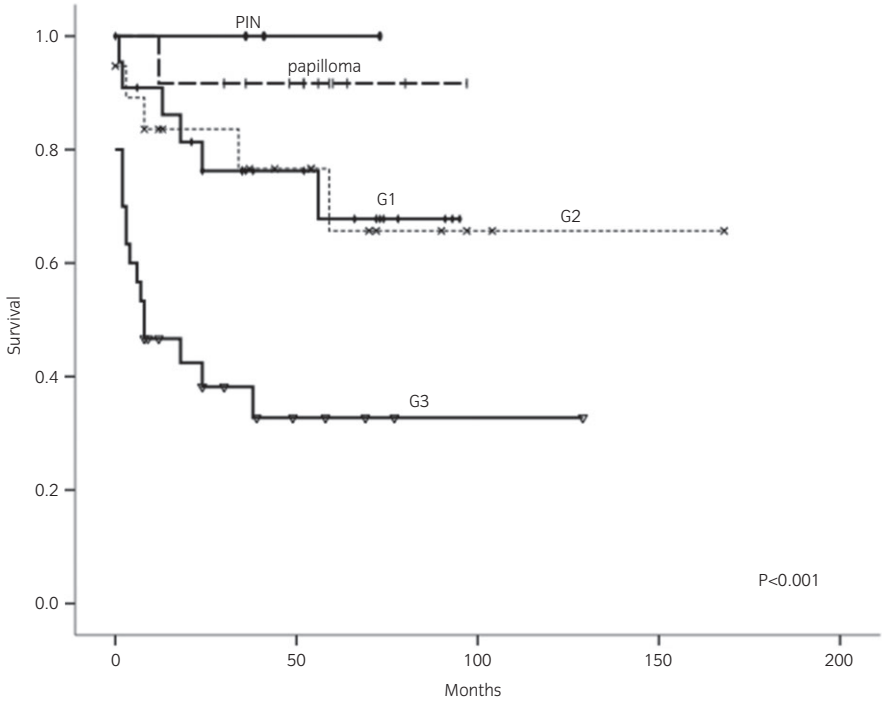

Fig 3: Overall survival from first presentation related to tumour subtype. PIN = penile intraepithelial neoplasia; P = papilloma; G1 = well-differentiated tumour; G2 = moderately differentiated tumour; G3 = poorly differentiated tumour.

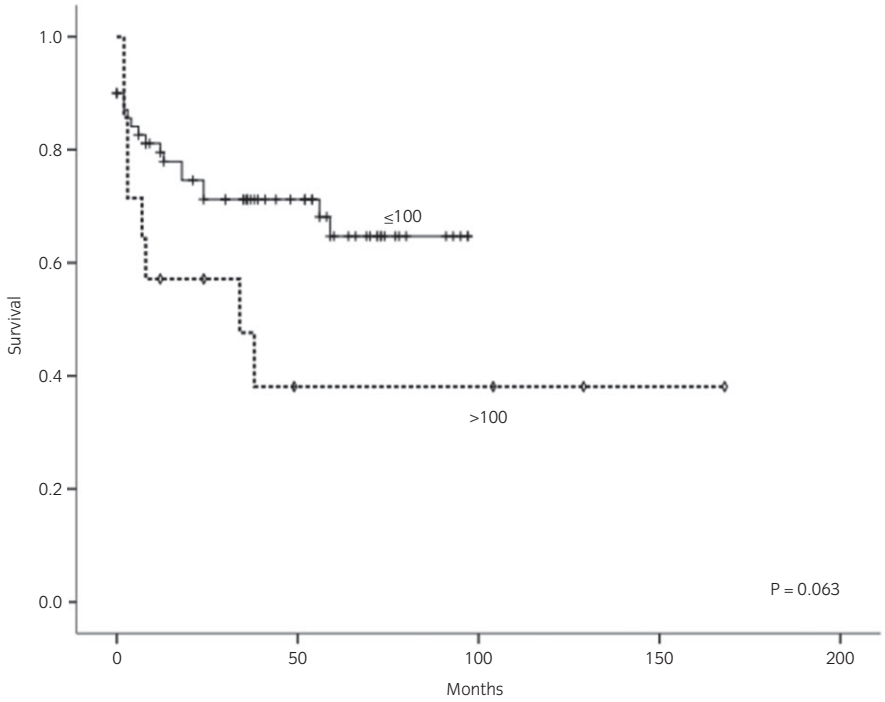

Fig 4: Overall survival from first presentation related to p53 expression score.

SCC is so common that this parameter is not discriminative enough. As for EcPV3, this study does not provide any evidence for a role in equine penile and preputial tumours.

Expression of p53 was higher in less differentiated SCC, indicating some value of this parameter for predicting disease severity. Further, lesions negative for EcPV2 had a significantly higher nuclear p53 expression score than EcPV2-positive lesions. This may be explained by viral E6 proteins that facilitate p53 degradation of wild type p53 in man [16]. We could not confirm this hypothesis by demonstrating a negative correlation between ln p53 expression score and E6 mRNA quantity. However, the group of animals in which E2, E6 and L1 expression was evaluated was relatively small and did not permit drawing of any definitive conclusions.

Another reason for the high expression of p53 might be the formation of a mutant form of p53. Mutations and aberrant expression of the p53 gene have been detected in domestic animals [26–30] and mutated p53 protein was found in penile tumours in 5 of 6 horses [30]. The altered protein has a 10- to 20-fold greater half-life than wild type p53 and accumulates within the nuclei of tumour cells in amounts that can be detected immunohistochemically [31], whereas the concentration of the wild type p53 in normal cells is too low to be demonstrated by this method. As the antibody used in this study detected both wild type and mutant p53, no discrimination could be made.

In man, p53 is a prognostic factor for metastatic disease [32], which was not confirmed in the present study, but group size was only 19 horses and possibly not all metastases were identified.

In man, a positive correlation between percentage of Ki67-stained cells and tumour grade and risk of metastasis has been suggested [17], but this could not be confirmed in the horse. However, the group of horses with metastases was relatively small and the high percentage (56.3%) of heterogeneous tumours may explain the lack of differences in Ki67 expression between subtypes. As tumours were graded according to the least differentiated field, large fields of well-differentiated cells with low Ki67 expression could be present together with small fields of poorly differentiated cells with high Ki67 expression, leading to a low overall Ki67 expression score and a high grade.

There were no differences in ln mRNA expression of the viral genes E2, E6 and L1 between noninvasive and invasive lesions. The lower expression of E2, E5, E6 and E7 genes in fibroblastic sarcoids compared with nodular sarcoids has been explained by a possible 'hit and run' action of bovine papilloma virus (BPV) with the virus itself inciting tumour growth, but being less important for tumour progression [28]. It is conceivable that a similar 'hit and run' action of EcPV2 might lead to a PIN or a papilloma in the horse, followed by progression to SCC by other mechanisms. However, no difference was found in ln mRNA expression between the noninvasive tumour subtypes and the more advanced subtypes (invasive SCCs) in the present study. This may be due to the relative small number of samples examined, but it is also possible that the above mentioned 'hit and run' action is not a hallmark of the type of tumours investigated in the current study. Further investigation on mRNA expression in a larger number of samples seems warranted.

The current study has several limitations. In horses, tumours are often very large at time of first examination whereas only a relatively small tissue sample is studied in detail. The presence of a well-differentiated tumour does not necessarily exclude presence of poorer differentiated tissue elsewhere. Also, a few poorly differentiated cells can be easily overseen. In this restrospective study, missing or incomplete data reduced the population of this study to only 64.9% of the potential candidates (87/134) which limits its power with relatively low numbers in various subgroups. It is especially difficult to draw definitive conclusions on the absence of effects or relationships.

It can be concluded that in equine penile and preputial SCC histopathological grading is an important prognosticator for survival and presence of metastases. Infection with EcPV2 is important in the development of these tumours, but EcPV3 is probably not involved. Expression of p53 increases with decreased differentiation of the tumour, but presence of EcPV2, p53 and Ki67 cannot be used as prognosticators. There is thus far no indication that expression of the viral genes E2, E6 and L1 can be used as prognosticator.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

Ethical review not currently required by this journal: the study was performed on archived material collected previously during clinical

procedures. Explicit owner informed consent for participation in the study was not obtained.

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Authorship

J.G.B. van den Top and A. Martens contributed to study design, study execution and data analysis and interpretation. L. Harkema, C. Lange and A. Gröne contributed to study design and study execution. J.M. Ensink and A. Barneveld contributed to study design. C.H.A. van de Lest and P.R. van Weeren contributed to study execution and data analysis and interpretation. All authors contributed to the preparation of the manuscript and gave their final approval of the manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Item S1: Details on nuclear image analysis algorithm.
Item S2: Detailed information on quantitative EcPV2 mRNA analysis.
Item S3: Population overview: breed distribution of 87 horses with penile or preputial tumours and mean age, country of origin and number of primary tumour subtypes per breed.