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Increased p16^{CDKN2A}, but not p53, immunostaining is predictive of longer survival time in cats with oral squamous cell carcinomas

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ABSTRACT

Although oral squamous cell carcinomas (SCCs) are common in cats there are currently few prognostic markers for these cancers. This study used 52 feline oral SCCs to determine if prognosis can be predicted by the age or sex of the cat, the presence of bone within the diagnostic sample, or the anatomic location of the SCC. Additionally, as p16^{CDKN2A} protein (p16) and p53 are prognostic for human oral SCCs, p16 and p53 immunostaining was evaluated.

Only SCC location and p16 immunostaining were prognostic. Cats with oropharyngeal SCCs had an estimated median survival time (MST) of 151 days which was significantly longer than cats with maxillary (51 days $P = 0.017$), sublingual (33 days $P = 0.011$) and mandibular (34 days $P = 0.029$) SCCs. Overall, 19% of oral SCCs were p16-positive with intense nuclear and cytoplasmic immunostaining within most neoplastic cells, 69% had cytoplasmic immunostaining that was confined to the periphery of nests of neoplastic cells, and 12% had no p16 immunostaining. Cats with p16-positive SCCs had a MST of 87 days, which was significantly longer than cats with p16-peripheral SCCs (MST 37 days, $P = 0.03$), but not longer than cats with p16-negative SCCs (MST 51 days, $P = 0.72$). No papillomaviral DNA was amplified from the p16-positive SCCs. Twenty (39%) SCCs contained immunostaining for p53, but this was not prognostic ($P = 0.31$). These results suggest that feline oral SCCs develop by cellular mechanisms that result in one of three patterns of p16 immunostaining. Cancers which develop due to these mechanisms appear to have different clinical behaviors and p16 immunostaining predicts the behavior of these common feline cancers.

Introduction

Squamous cell carcinomas are the most common neoplasms of the oral cavity of cats and are the fourth most common feline cancer (Munday et al., 2017). While cats with these neoplasms have median survival times (MSTs) of around 40 days, 5–10% of cats survive a year after diagnosis (Postorino Reeves et al., 1993; Hayes et al., 2007; Bergkvist et al., 2011). However, there are few prognostic factors that have been established for feline oral SCCs. One study revealed that cats with SCCs from the maxillary region survived longer (Gendler et al., 2010), another reported oropharyngeal SCCs were associated with the longest survival times (Klobukowska and Munday, 2016), while other studies reported no significant differences in survival times between cats with SCCs from different locations in the mouth (Hayes et al., 2007). The prognostic potential of a small number of immunohistochemical markers have been previously evaluated. Stromal smooth muscle actin and cyclooxygenase immunostaining were found to be prognostic, immunostaining for Ki67 was inconsistent, and immunostaining for epidermal growth factor receptor did not predict survival times (Hayes et al., 2007; Bergkvist et al., 2011; Yoshikawa et al., 2012; Klobukowska and Munday, 2016).

Human patients also frequently develop oral SCCs and these neoplasms are generally thought to be caused either by exposure to tobacco and alcohol or by infection by high-risk human papillomaviruses (PVs). One of the best predictors of prognosis for human oral SCCs is p16^{CDKN2A} protein (p16) immunostaining (Karpathiou et al., 2016). The p16 protein prevents cell replication by regulating the function of the retinoblastoma (pRb) protein (Foulkes et al., 1997). Loss of pRb promotes uncontrolled cell replication and markedly increases p16 within the cell (Parry et al., 1995). Human oral SCCs caused by tobacco develop by alterations in pathways that do not affect pRb and increased p16 immunostaining is not present in these cancers (Smeets et al., 2007).

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However, the mutations that tobacco causes result in a more aggressive cancer phenotype and these SCCs have a worse prognosis (Psyrri et al., 2009). In contrast, the high-risk alpha human PVs consistently cause cancer by degrading pRb and these SCCs have marked p16 immunostaining (Smeets et al., 2007). As the genetic alterations caused by PVs result in a less aggressive cancer phenotype, p16 immunostaining indicates a more favorable prognosis (Psyrri et al., 2009). Interestingly, p16-positive human oral SCCs that are not caused by PV infection also have a more favorable prognosis (Lewis et al., 2010).

The cause of feline oral SCCs is currently unknown with no evidence that PVs cause these neoplasms (Munday, 2014). However, PVs are an important cause of feline cutaneous SCCs and p16 immunostaining is consistently present in feline PV-induced skin cancers (Munday et al., 2011b). Furthermore, p16 immunostaining was found to be prognostic in feline cutaneous SCCs (Munday et al., 2013). As variable p16 immunostaining has been reported in feline oral SCCs (Munday et al., 2011c) the first aim of the present study was to determine if p16 immunostaining could predict the survival times of cats with oral SCCs

The p53 protein is an important tumor suppressor protein and p53 mutations are more common in human oral SCCs that have been caused by tobacco exposure than in SCCs caused by PV infection (Brennan et al., 1995; Gendler et al., 2010). While this suggests p53 immunostaining could be a useful prognostic marker for human oral SCCs, immunostaining has only variably been found to be prognostic for these cancers (Geisler et al., 2002; Ritta et al., 2009; Karpathiou et al., 2016). As variable p53 immunostaining has been previously reported in feline oral SCCs, (Snyder et al., 2004) the potential to use p53 immunostaining as a prognostic marker was also evaluated.

Materials and methods

Identification of cases and inclusion criteria

Databases of two diagnostic laboratories (New Zealand Veterinary Pathology Ltd. and Gribbles Veterinary Pathology) were searched for histology samples of feline oral SCCs submitted between 2008 and 2014. Samples that had been collected with the aim of achieving a surgical cure were not included in the study. Surveys were sent to the submitting veterinarians to determine the age and sex of the affected cat, the neoplasm location, treatments given after biopsy, and the time and cause of death. Cats were excluded if they had been subject to euthanasia within 5 days of the biopsy being taken. This was to exclude cases in which euthanasia was performed on confirmation of the clinical diagnosis of oral SCC. Cats that were subject to euthanasia for reasons other than the oral SCC and cats that received treatments aimed at curing the SCCs were also excluded, although cats that received palliative treatments such as anti-inflammatories or antibiotics were included. Cats were censored at the time of the last visit to the veterinary clinic in cases that were subsequently lost to follow up.

Histological assessment

Histological sections from all cases were evaluated to confirm the diagnosis of SCC and to determine if the neoplasm had infiltrated bone. Oral SCCs were also classified into subtypes using previously described criteria (Munday et al., 2017).

Immunohistochemistry

Immunohistochemistry was performed to detect p16 and p53 as previously described (Munday et al., 2011a; Munday and Aberdein, 2012) using a mouse anti-human p16 clone G175-405 antibody and a mouse anti-human p53 clone pAb 240 antibody (both from BD Biosciences, San Jose, CA). The p16 antibody positive control was a feline cutaneous Bowenoid *in situ* carcinoma while a feline cutaneous SCC was the positive control for the p53 immunostaining. Normal epithelium within the sections was used as an internal negative control along with sections for which the primary antibody was omitted.

The SCCs were classified as p16-positive, p16-peripheral, and p16-negative. Neoplasms that were p16-positive had intense nuclear and cytoplasmic immunostaining in over 80% of the neoplastic cells. Immunostaining in SCCs that were classified as p16-peripheral was of moderate intensity and was confined to the cytoplasm of cells on the periphery of nests and trabeculae of neoplastic cells. Finally, neoplastic cells within p16-negative SCCs contained scant immunostaining that was rarely visible within cells on the periphery of nests of neoplastic cells. Oral SCCs were classified as p53-positive if greater than 50% of the cells contained immunostaining and as p53-negative if less than 50% of the neoplastic cells demonstrated p53 immunostaining.

Polymerase chain reaction to detect papillomavirus DNA

Total DNA was extracted from samples and the presence of amplifiable DNA confirmed by amplifying a section of the feline p53 gene as previously described (Kidney et al., 2001). Two consensus PCR primers, FAP59/64 and CP4/5, were used to detect the presence of PV DNA in the extracted DNA as previously described (Munday et al., 2007, 2015). The positive controls for the primers were a Bowenoid *in situ* carcinoma that was known to contain FcaPV-2 for the FAP59/64 primers and a feline oral papilloma known to contain FcaPV-1 for the CP4/5 primers.

Statistical analyses

Differences between groups were investigated by Pearson chi-squared or Fisher's exact tests and survival times were investigated using Kaplan–Meier and Cox regression analyses using IBM SBSS Statistics v25 (IBM).

Results

Cases included and histological evaluation of SCCs

A total of 52 SCCs were included in this study. Cats had an average age of 13.5 years (range 6–18 years). Thirty-two cats were male and 20 were female. Twenty one (40%) SCCs developed in the gingiva of the maxilla, 12 (23%) were sublingual, 12 (23%) were mandibular, and 7 (13%) oropharyngeal. Forty three of 52 (83%) SCCs were conventional, six of 52 (11%) acantholytic and three of 52 (6%) basaloid subtypes. Bone was visible in 21 of the 52 samples including 10 of 21 (48%) maxillary, 4 of 12 (33%) mandibular, 3 of 12 (25%) sublingual, and 1 of 7 (14%) oropharyngeal SCCs.

Immunohistochemistry evaluation

Immunostaining for p16 was performed on 48 oral SCCs. Overall, 9 of 48 (19%) SCCs were classified as p16-positive (Fig. 1), 33 of 48 (69%) p16-peripheral (Fig. 2) and 6 of 48 (12%) p16-negative (Fig. 3; Table 1). Neither the age ($P = 0.9$) or the sex ($P = 0.1$) of the cat was significantly associated with the p16 classification of the oral SCC. However, there were significant differences in p16 classification between SCCs from different locations ($P = 0.013$). Interestingly the highest rate of both p16-positive and p16-negative SCCs were from the oropharyngeal region with just one of the seven oropharyngeal SCCs being classified as p16-peripheral.

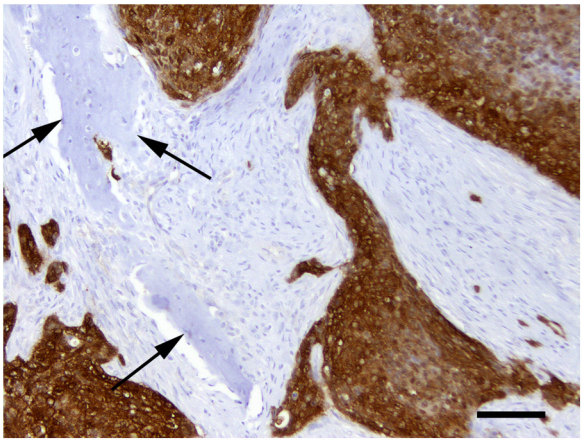

Fig. 1. Photomicrograph of a feline oral squamous cell carcinoma. Intense intranuclear and intracytoplasmic $p16^{CDKN2A}$ immunostaining is visible within almost all of the neoplastic cells. Spicules of bone are visible adjacent to the neoplastic cell nests and trabeculae (arrows). Haematoxylin counterstain, bar = 50 μm.

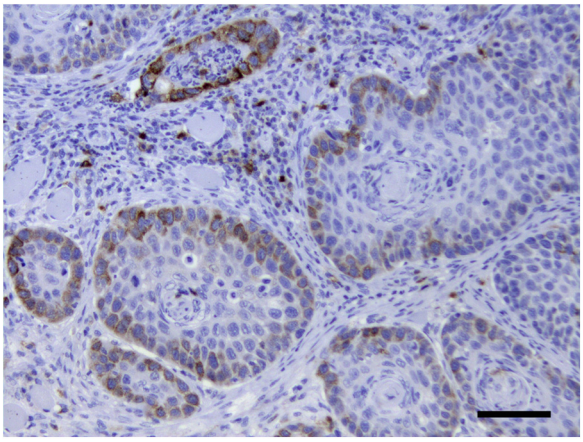

Fig. 2. Photomicrograph of a feline oral squamous cell carcinoma. Cytoplasmic $p16^{CDKN2A}$ immunostaining is confined to cells at the periphery of neoplastic nests and trabeculae. Haematoxylin counterstain, bar = 50 μm.

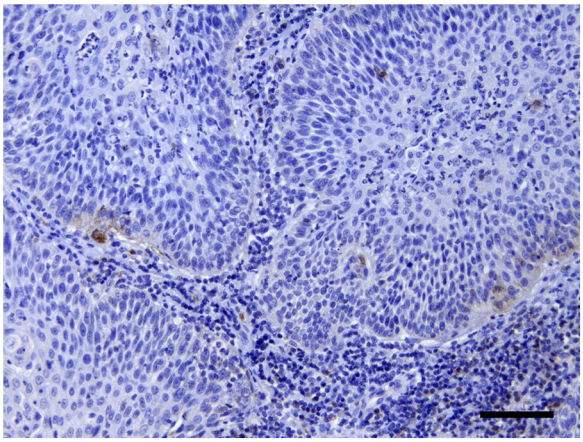

Fig. 3. Photomicrograph of a feline oral squamous cell carcinoma. Minimal intracytoplasmic $p16^{CDKN2A}$ immunostaining is rarely visible within neoplastic cells on the periphery of the neoplastic cell nests. Much of the immunostaining visible is present within surrounding stromal cells. Haematoxylin counterstain, bar = 50 μm.

The presence of bone within the diagnostic sample was not significantly associated with p16 immunostaining ($P = 0.13$).

Immunostaining for p53 was performed in 51 SCCs. Two patterns of p53 immunostaining were visible within the SCCs with sections either showing prominent nuclear immunostaining visible in over 80% of the cells (p53-positive; Fig. 4) or faint nuclear immunostaining visible in 5% or less of the cells (p53-negative; Fig. 5). Twenty of 51 (39%) SCCs were p53-positive and 31 of 51 (61%) were p53-negative. Immunostaining for p53 was not significantly associated with the age of the cat ($P = 0.9$), sex of the cat ($P = 0.35$), location of the SCC ($P = 0.19$), or the presence of bone within the sample ($P = 0.48$). There was no significant association between p53 and p16 classification within the SCCs ($P = 0.39$).

Survival of cats with oral SCCs

The overall estimated MST of the 52 cats in this study was 45 days (95% confidence intervals [CI] 29.9–60.1 days). Neither the age or the sex of the cat was significantly associated with MST ($P = 0.5$ and $P = 0.76$ respectively) (Table 2). However, cats with oropharyngeal SCCs survived for 151 days which was significantly longer than cats with maxillary (51 days $P = 0.017$), sublingual (33 days $P = 0.011$) and mandibular (34 days $P = 0.029$) SCCs. There were no significant differences between the MST of cats with maxillary, sublingual and mandibular SCCs.

The MST of cats with p16-positive SCCs was 87 days which was significantly longer than the MST of cats with p16-peripheral SCCs (37 days, $P = 0.03$), but not significantly different to the MST of cats with p16-negative SCCs (51 days, $P = 0.72$). There was no significant difference in MST between cats with p16-peripheral SCCs and p16-negative SCCs ($P = 0.09$, Fig. 6). When only maxillary, mandibular, and sublingual SCCs were considered, the estimated MST of cats with p16-positive SCCs was 80 days which was significantly longer than the survival of cats with p16-peripheral SCCs ($P = 0.034$), but not longer than cats with p16-negative SCCs ($P = 0.12$). When only the seven oropharyngeal SCCs were considered there were no significant differences in the survival times of cats with SCCs with different p16 classifications. Cats with p53-positive SCCs had a MST of 37 days which was not significantly different to the MST of cats with p53-negative SCCs (51 days, $P = 0.31$, Fig. 7).

PCR evaluation of p16-positive cases

While the p53 gene was amplified, neither the MY09/11 or CP4/5 primers amplified PV DNA any of the p16-positive oral SCCs.

Discussion

Cats with p16-positive oral SCCs survived significantly longer than cats with p16-peripheral SCCs. This suggests that p16 immunostaining can provide useful prognostic information for these common feline neoplasms. The p16-positivity of the SCCs was not due to a papillomaviral etiology. However, the results are consistent with studies of human oral SCCs that show p16-positivity to confer a better prognosis regardless of the cause of the SCC (Sedghizadeh et al., 2016). No significant differences in survival were observed between p16-positive and p16-negative SCCs in the present study, although only small numbers of SCCs with each immunostaining pattern were identified.

As in a previous study of feline oral SCCs (Munday et al., 2011c), three distinct patterns of p16 immunostaining were observed in the present study. The most common pattern was visible in around 70% of SCCs and consisted of moderate p16 immunostaining that was confined to the cells lining neoplastic cell nests or trabeculae. As this immunostaining pattern is similar to that seen in normal epithelium, this suggests that the pRb-p16 pathway was intact and

Table 1
Summary of immunostaining observed within the feline oral squamous cell carcinomas (SCCs).

	$p16^{CDKN2A}$ protein immunostaining			p53 immunostaining	
	Positive (%)	Peripheral (%)	Negative (%)	Positive (%)	Negative (%)
All SCCs	9 (19)	33 (69)	6 (12)	20 (39)	31 (61)
Sex					
Male	6 (20)	18 (60)	6 (20)	11 (35)	20 (65)
Female	3 (17)	15 (83)	0 (0)	9 (45)	11 (55)
Location					
Maxilla	4 (22)	12 (67)	2 (11)	6 (29)	15 (71)
Sublingual	0 (0)	12 (100)	0 (0)	7 (58)	5 (42)
Mandible	2 (18)	8 (73)	1 (9)	6 (50)	6 (50)
Oropharynx	3 (43)	1 (14)	3 (43)	1 (17)	5 (83)
Bone					
No	3 (10)	22 (73)	5 (17)	14 (42)	19 (58)
Yes	5 (27)	12 (67)	1 (6)	6 (33)	12 (66)

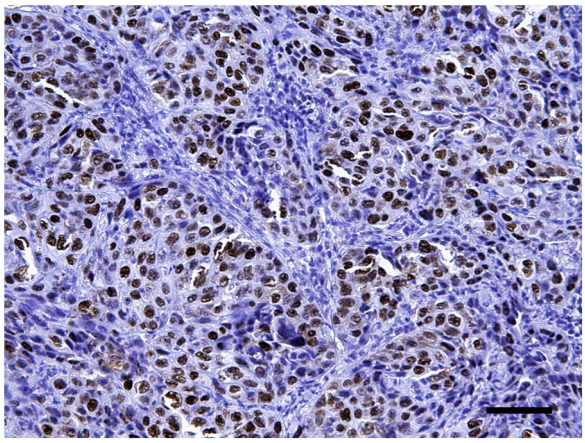

Fig. 4. Photomicrograph of a feline oral squamous cell carcinoma. Intense nuclear p53 immunostaining is visible within most of the neoplastic cells. Haematoxylin counterstain, bar = 50 μm.

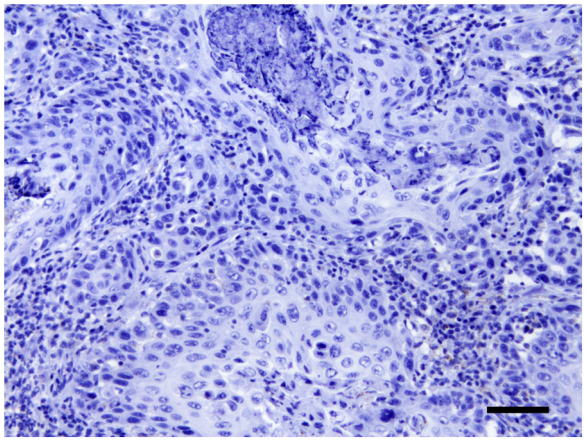

Fig. 5. Photomicrograph of a feline oral squamous cell carcinoma. No significant immunostaining for p53 is visible within the neoplastic cells. Haematoxylin counterstain, bar = 50 μm.

that the SCC developed due to alterations in unrelated genes or proteins. The second pattern was seen in around 20% of SCCs and appeared as intense nuclear and cytoplasmic p16 immunostaining in almost all neoplastic cells. These SCCs likely contained alterations that prevented the production of pRb (Parry et al., 1995). Finally, the least common pattern was an almost complete loss of p16 immunostaining within the neoplastic cells. Previous studies have revealed promotor methylation, homozygous deletion of the entire *p16* locus, and the presence of missense mutations as all potential causes of the loss of p16 production by the neoplastic cells (Mochizuki et al., 2017). As these three patterns of p16 immunostaining were associated with differences in survival times it suggests that, as in human oral SCCs, the cellular mechanisms that lead to the development of a feline oral SCC influence the subsequent behavior of that neoplasm.

The location of the SCC in the mouth was also prognostic with cats with oropharyngeal SCCs surviving longer than cats with oral SCCs in other locations. Oropharyngeal SCCs were also more likely to be p16-positive suggesting that p16 immunostaining could have simply been a marker of a SCC from the oropharynx. To exclude this possibility, the association between p16 immunostaining and prognosis was evaluated in the 41 SCCs from the maxilla, mandible, and sublingual regions. As p16 immunostaining continued to predict survival, it appears useful as a prognostic marker for feline non-oropharyngeal oral SCCs.

Immunostaining to detect p53 was not prognostic in the feline oral SCCs. The presence of p53 immunostaining in a cell usually indicates the presence of a missense mutation within the p53 gene that results in an accumulation of non-functional p53 within the cell. While interpretation of increased immunostaining is simple, it is harder to interpret reduced p53 immunostaining within a sample. This is because normal p53 protein is rapidly removed from the cell and cells with normal p53 function will demonstrate little, if any, p53 immunostaining. Likewise, the presence of nonsense mutations or deletions within the p53 gene will prevent protein production also resulting in scant p53 immunostaining within the samples (Karpathiou et al., 2016). It is possible that the inability to differentiate normal protein function and loss of p53 protein function within the cells contributed to the inability of p53 immunostaining to predict prognosis in this study.

In the present study, around 40% of the oral SCCs had intense p53 immunostaining, indicative of the presence of missense p53 mutations. Previously, a potential association between environmental tobacco smoke and p53 mutation was investigated in 41 feline SCCs. While p53 immunostaining was detected in 7 (17%) neoplasms, immunostaining was not significantly associated with tobacco smoke exposure (Snyder et al., 2004).

The p16 status of a SCC was not associated with the p53 status in the feline oral SCCs. This is in contrast to human oral SCCs that either have increased p53 if caused by tobacco or increased p16 if caused by papillomavirus infection (Karpathiou et al., 2016). The lack of association between p16 and p53 immunostaining in the feline oral SCCs suggests the presence of disruptions to multiple different cellular control pathways, indicating that these cancers develop due to a number of different mechanisms. The lack of association supports previous studies that suggest neither tobacco

Table 2
Survival times (95% confidence intervals, CI) of cats with oral squamous cell carcinomas. Kaplan–Meier and Cox regression analyses were used to determine differences in survival of cats with squamous cell carcinomas (SCCs) with different properties. For the location, the *P* values given are compared to survival times of cats with SCCs of the oropharynx while I values for the p16 classification are compared to cats with SCCs that were p16-positive.

	Number	Estimated median survival time (95% CI), days	*P* value
Sex			0.76
Male	32	38 (25.8–50.2)	
Female	20	47 (23.2–70.7)	
Location			
Oropharynx	7	151 (104.8–197.2)	
Maxilla	21	51 (34.6–67.4)	0.017
Sublingual	12	33 (23–42)	0.011
Mandible	12	34 (18.7–49.3)	0.029
Bone present			0.88
No	34	42 (27.7–54.3)	
Yes	18	47 (26.2–67.8)	
p16CDKN2A immunostaining			
Positive	9	87 (66.5–107.5)	
Peripheral	33	37 (28.3–45.7)	0.03
Negative	6	51 (0–155.4)	0.72
p53 immunostaining			0.31
Positive	20	37 (28.6–45.4)	
Negative	31	51 (33.5–68.4)	

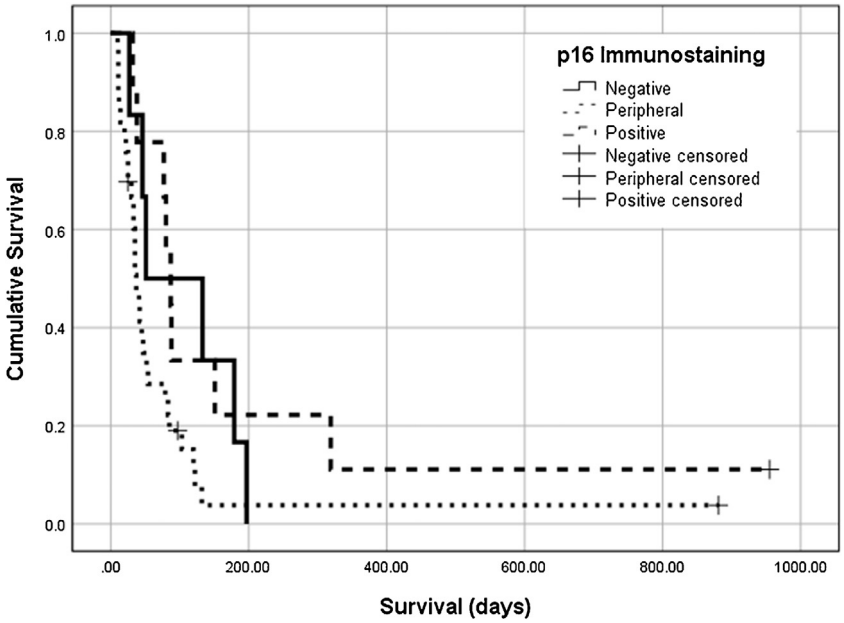

Fig. 6. Kaplan–Meier survival curve for 48 cats with oral squamous cell carcinomas (SCCs) illustrating the survival times of cats with p16^{CDKN2A}protein (p16)-positive, p16-negative, or p16-peripheral cancers. Cats with p16-positive SCCs had an estimated median survival time of 87 days which was significantly longer than cats with p16-peripheral SCCs (37 days, $P = 0.03$).

smoke or papillomaviruses strongly predispose to feline oral SCCs (Munday et al., 2009; Munday and French, 2015; Snyder et al., 2004).

Papillomaviral DNA was not amplified from the p16-positive SCCs, although the presence of a novel non-amplifiable type cannot be excluded. The cause of oral SCCs in cats is currently unknown, although evidence from humans suggests chronic dental disease could potentially be important in cancer development (Munday et al., 2017).

The age of the cat was not prognostic in this study. In contrast, age is a significant prognostic factor in human oral SCCs, possibly because younger patients are more likely to have PV-induced SCCs (Windon et al., 2018) and younger patients are able to receive more intensive treatment (Massa et al., 2019). The cats in the present study only received palliative treatment excluding the possibility that survival times could be influenced by treatment. In contrast to humans (Woolgar, 2006), the histological subtype of the oral SCC was not prognostic in the present study. However, non-conventional oral SCCs were rare and additional studies that include larger numbers of SCCs are required. The presence of bone within the oral SCC was not prognostic for the feline oral SCCs. This was surprising as this feature was expected to be a marker for the invasiveness of the neoplasm. The absence of prognostic significance may be because all feline oral SCCs are invasive, regardless of whether or not bone is visible within the diagnostic sample.

A prognostic marker must consistently predict prognosis when used by different pathologists in different diagnostic laboratories (Meuten et al., 2018). While many prognostic markers have been proposed in veterinary pathology, these are often subsequently found to inconsistently predict prognosis because of inter-pathologist variability when subjectivity evaluating histological criteria (Northrup et al., 2005). Immunostaining for p16 was easy to interpret

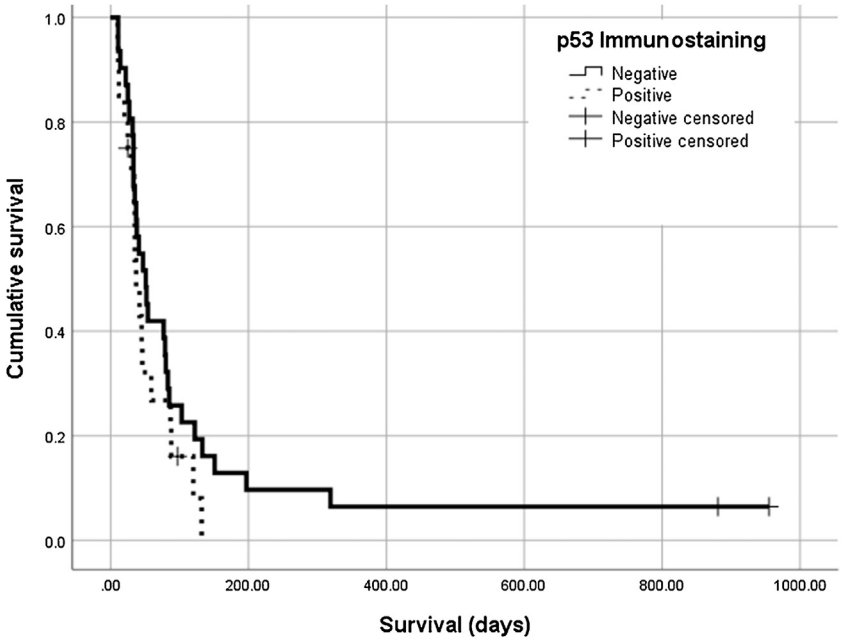

Fig. 7. Kaplan–Meier survival curve for 49 cats with oral squamous cell carcinomas (SCCs) illustrating the survival times of cats with p53-positive or p53-negative cancers. There was no significant difference in survival times observed ($P = 0.31$).

in the feline SCCs and this ease of interpretation has probably significantly contributed to the successful application of p16 immunostaining for predicting the behaviour of oral SCCs in people.

Like all studies of prognostic markers in veterinary medicine, the present study is limited by the use of euthanasia as the determinant of survival time. Owners determine the time of euthanasia due to many factors, including some that may not be directly due to the cancer. While the influence of euthanasia is not expected to introduce bias, it probably increased the variability in the data used in this study. Other limitations of the study include the retrospective design and the absence of necropsy examinations to confirm that the cats had been subject to euthanasia due to the oral SCC.

Conclusions

Feline oral SCCs had variable, but well-defined patterns of p16 immunostaining and this immunostaining could be used to predict survival times. These results suggest that feline oral SCCs develop by a variety of different cellular mechanisms and neoplasms that contain increased p16 may be less aggressive and have a more favourable clinical behavior.

Conflict of interest statement

None of the authors has any financial or personal relationships that could influence or bias the content of the paper.

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