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ORIGINAL ARTICLE

Detection of *Felis catus* papillomavirus type-2 DNA and viral gene expression suggest active infection in feline oral squamous cell carcinoma

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Abstract

Papillomavirus (PV) infection is associated with development of epithelial cancer in different species, including domestic cat (*Felis catus*). *Felis catus* PV type-2 (FcaPV-2) is considered the causative agent of a proportion of feline cutaneous squamous cell carcinoma (SCC), through the transforming properties of its E6 and E7 oncogenes. However, the possible role of FcaPVs in the aetiology of feline oral SCC (FOSCC) is still unclear. The aim of this study was to assess the presence and gene expression of FcaPV-2 in FOSCC samples. We detected FcaPV-2 DNA in 10/32 (31%) of the analysed FOSCC by the use of PCR methods. Importantly, viral mRNA was detected by RT-PCR in 7/10 (70%) of DNA positive samples. In particular, FcaPV-2 L1, E2 and E6E7 genes were found to be expressed in 5/10 (50%), 3/10 (33%) and 5/10 (50%) samples, respectively. Viral DNA was also detected in non neoplastic oral ulcerative lesions (ULs) (4/11, 36%); qPCR suggested a difference in viral load between ULs and FOSCCs, particularly in those expressing E6E7, although it was not statistically significant. These data suggest, but do not definitively prove, a possible role of FcaPV-2 in the development of a proportion of FOSCC. Moreover, L1 and E2 gene expression results indicate that FcaPV-2 infection associated with these tumours may possibly be productive.

KEYWORDS

cat, infection, mucosal, oncogenes, squamous cell carcinoma, viral tropism

1 | INTRODUCTION

Papillomaviruses (PVs) are small, non-enveloped viruses harbouring a circular, double-stranded DNA genome, with a preferential tropism for skin and mucosal epithelia in human and animal species.[1] PVs infection is associated with development of benign hyperproliferative lesions which, in several circumstances (eg, immunosuppression), may evolve to malignancy such as cutaneous and oral squamous cell carcinoma (SCC).[1]

In domestic cat (*Felis catus*), five specie-specific PV types (FcaPV types −1 to −5) have been fully sequenced and classified.[2] These viruses are associated with a variety of skin neoplastic and pre-neoplastic conditions, however, for the majority of them, the possible pathogenetic role is still unclear.[1,3] FcaPV-2 is the best characterized feline PV: it is frequently detectable and biologically active in feline cutaneous viral plaques, Bowenoid in situ carcinoma and SCC and the transforming properties of its E6 and E7 oncogenes have been clearly demonstrated in vitro, suggesting

that the virus is an etiological factor in the development of these tumours.[4-6]

In humans, a distinct subcategory of head and neck SCC (HNSCC) consists of oral SCC associated with infection by alpha high-risk (HR) human PVs (HPVs). This particular group of oral cancers shows different biological features compared to HPV-negative counterpart, such as molecular markers, prognosis and response to therapies.[7] In veterinary oncology, recent evidences suggest that few oral SCCs in dogs may be triggered by canine PVs, although they represent a minority with respect to the vast majority showing no signs of PV involvement.[8-10] Instead, concerns regarding the possible involvement of PVs infection in the pathogenesis of feline oral SCC (FOSCC) still exist. A series of studies have attempted to look for PVs DNA in FOSCC by polymerase chain reaction (PCR). In these works, different sets of consensus oligos were employed, since they are able to amplify conserved regions of mucosal and cutaneous PVs, including FcaPV-2 from feline skin SCC.[11-16] However, Munday et al detected PV DNA in 1/20 FOSCC (mostly similar to HPV-76) vs 0/20 non-neoplastic oral lesions, and in 0/30 FOSCC by the use of FAP59/64 and MY09/11 primers in two different reports.[12,13] By similar approach, O'Neill et al found two PVs sequences in 2/30 FOSCC, however, one was not sequenced and the other was identified as HPV-38.[14] The use of alternative degenerated oligos such as CP4/5 by Munday et al had the same low rate of success, detecting only FcaPV-1 in 1/31 FOSCC and 0/16 non-neoplastic oral lesions.[11] In a very recent work on a larger cohort of samples (52 FOSCC), neither FAP59/64 nor CP4/5 amplified any PVs sequence.[15] Summing up, only FcaPV-1 and FcaPV-4 have been shown to exert putative oral tropism in cats, as the former was associated also with oral papillomas and the latter was isolated from the oral cavity.[17,18] However, we have recently reported the presence and gene expression of FcaPV-2 in one FOSCC and two FOSCC-derived cell lines by type-specific PCR, hence the need for further investigations to specifically analyse the presence of FcaPV-2 in a larger series of samples.[4,6]

The aim of this study was to analyse FOSCC samples by validated type-specific PCR and reverse transcription (RT)-PCR methods to detect the presence and gene expression of FcaPV-2 DNA, in order to clarify its possible role in the development of these tumours.

2 | METHODS

2.1 | Tissue samples and histological analysis

For this study, 32 (T1-T15, T17-T26, T28-T34) formalin fixed-paraffin embedded (FFPE) FOSCC, 2 SCC samples of nasal cavities (T16, T27), 1 oral papilloma (T35) and 12 oral non-SCC ulcerative lesions (UL1-UL12) were retrieved from the archives of the 'Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "M. Aleandri" ' (samples T1-T26, T35) and "Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, National Reference Center of Veterinary and Comparative Oncology (CEROVEC)" (samples T27-T34). Details on animals' breed, age, sex, anatomical sites of sampling and diagnosis of tumours are summarized in Table S1. For each tissue sample, 5/10 μm thick sections were cut with separate microtome blades and stored in sterile tubes for molecular biology tests or onto slides for histopathological analysis. Samples were routinely stained with Haematoxylin and Eosin for light microscopy observation. Histologic review was performed by two veterinary pathologists (GB and CE) for diagnostic confirmation.

2.2 | DNA and RNA extraction

DNA was extracted from 10 μm thick FFPE sections by using the QIAamp DNA Blood Mini Kit (Qiagen) following the manufacturer's protocol. RNA was obtained from 2/3 FFPE sections of 10 μm thickness for each sample and subjected to DNase digestion by using RNeasy FFPE Kit (Qiagen) according to the manufacturer's recommendations. The obtained DNA and RNA samples were quantified by a NanoVue Plus spectrophotometer (GE Healthcare).

2.3 | PCR for detection of FcaPV-2 DNA

For each tumour sample, 100 to 150 ng of DNA were subjected to PCR using the AmpliTaq Gold DNA Polymerase kit (Applied Biosystems). The JMPF/R primers were employed to amplify a fragment of FcaPV-2 L1 gene (177 bp) by using the amplification protocol described elsewhere.[16] Samples with no DNA template were run as negative control, the specificity of PCR was ensured by sequencing. The quality of DNA samples was checked by amplifying a segment of feline GAPDH (163 bp) as previously reported.[4] PCR products were run through electrophoresis along with a 100-bp DNA ladder in a 2% agarose gel with TBE (Tris-Borate-EDTA) buffer, stained with ethidium bromide and visualized under UV using the ChemiDoc gel scanner (Bio-Rad Laboratories).

2.4 | RT-PCR for detection of FcaPV-2 L1, E2 and E6E7 gene expression

cDNA was obtained by RT from RNA by using the iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer's protocol. RT with no reverse transcriptase enzyme was also performed on each RNA sample as control (No-RT control) to exclude genomic DNA contamination. PCR amplifying specific fragments of the FcaPV-2 L1 (150 bp), E2 (126 bp) and E6E7 genes (111 bp) was carried out on cDNA as previously described.[4,19] The presence of amplifiable cDNA in the samples was ensured by RT-PCR for feline β2-microglobulin (β2MG) by using the primers described elsewhere.[20] PCR on FcaPV-2 genome was run along with cDNA samples as positive control; one sample with no template was used as negative control. The amplicons were visualized as mentioned above.

2.5 | qPCR for relative quantization of FcaPV-2 DNA

DNA samples were also analysed by Real-time qPCR by using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories). Viral DNA

was specifically amplifed as previously reported, along with feline β-globin as reference gene, in order to normalize the amount of DNA used for each sample.[19,21] Relative quantization (RQ) of FcaPV-2 viral load was achieved by employing the $2^{-\Delta\Delta Cq}$ method as previously described.[3,22] Fold change in viral DNA amount was calculated for each individual SCC sample against FcaPV-2 positive ULs considered as the control group. Additionally, mean relative FcaPV-2 DNA amounts for the two groups were also calculated as follows: one UL sample was set as control, then fold change values with respect to this control sample were obtained by $2^{-\Delta\Delta Cq}$ method for all ULs and FOSCC samples and these values were used to calculate mean and SD for the two groups. Data were run through statistical analysis as described below.

2.6 | p16 immunohistochemistry (IHC)

Sections were dewaxed in xylene, dehydrated in graded alcohols and washed in 0.01 M phosphate buffered saline (PBS), pH 7.2 to 7.4. Endogenous peroxidase was blocked with hydrogen peroxide 0.3% in absolute methanol for 30 minutes. The streptavidin-biotin-peroxidase method was used. Antigen enhancement was performed by pretreating with microwave heating in citrate buffer, pH 6.00, twice for 5 minutes at 750 W. As primary antibody, a mouse anti-human p16 (clone G175-405, BD Biosciences) diluted 1:50 in PBS was used and applied overnight at 4°C. After two washes in PBS, MACH 1 mouse probe (Biocare Medical) was applied for 20 minutes at room temperature (rt). After, MACH-1 Universal HPR-Polymer (Biocare Medical) was applied for 30 minutes at rt. To reveal immunolabelling, diaminobenzidine tetrahydrochloride was used as a chromogen, and haematoxylin was used as counterstain.

The immunolabelling procedure included negative control sections incubated with PBS instead of the primary antibody and one FcaPV-2 cutaneous SCC used as p16 antibody positive control.[6,23]

2.7 | Sequencing

PCR amplicons were purified with the QIAquick PCR Purification kit (Qiagen) and sequenced using the respective primers with the BigDye Terminator Cycle Sequencing Ready Reaction kit, version 3.1 (PerkinElmer, Applied Biosystems) in an automated sequencer (ABI Prism 310 DNA sequencer, Applied Biosystems). Sequences were obtained using the software Geneious R8 v. 8.1.7 and aligned to the viral gene sequences (Genbank EU796884.1) using the Basic Local Alignment Search Tool (NCBI/BLAST).

2.8 | Statistical analysis

For statistical analysis, *t*-test was performed by using the SPSS 17.0 software (SPSS Inc.) and differences were considered statistically significant for $P < .05$.

3 | RESULTS

3.1 | Histological diagnosis

By histological examination, disruption of normal tissue stratification was observed. Cords and islands of squamous epithelial cells supported by fibrovascular stroma, extending from mucosa into the sub-mucosa, were evident. Diagnostic revaluation was consistent with SCC (Figure 1).[24] In sample T24, cells with dark shrunken peripheralised nuclei surrounded by a clear halo, suggestive of koilocytes, were observed. (Figure 1A).[25]

In sample T35, markedly thickened folded epithelium projecting in the underlying sub-mucosa with a partial retention of normal epithelial stratification was observed (Figure 1B). Diagnosis was consistent with

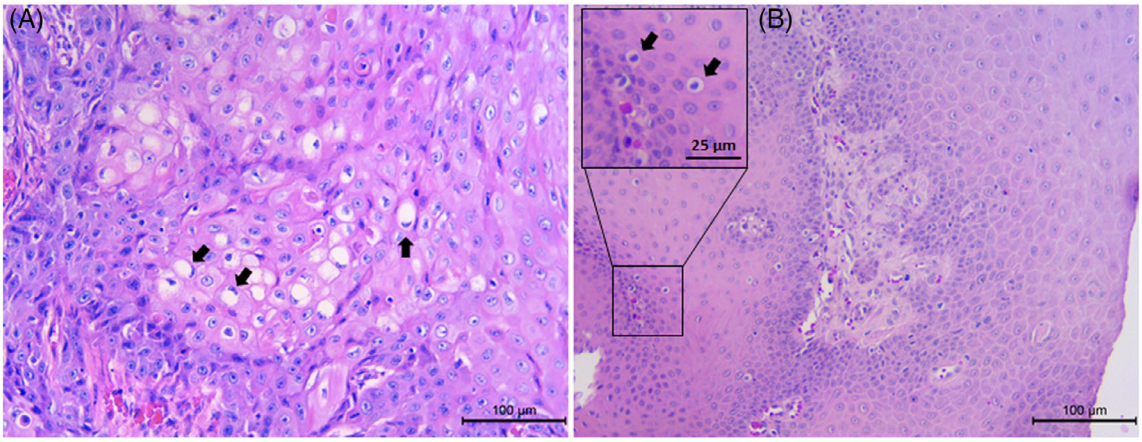

FIGURE 1 A, Squamous cell carcinoma, hard palate, cat, T24. Proliferation of cords and islands of squamous epithelial cells supported by fibrovascular stroma. Proliferating cells frequently have dark shrunken peripheralised nuclei, surrounded by a clear halo, being suggestive of koilocytes. Haematoxilin and Eosin, ×20. Scale bar: 100 µm. B, Papilloma, tongue/palate, cat, T35. Markedly thickened folded epithelium projecting in the underlying sub-mucosa, with a partial retention of normal epithelial stratification. Haematoxilin and Eosin, ×20. Scale bar: 100 µm. Arrows in the inset at higher magnification indicate koilocytes-like cells (scale bar 25 µm) [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 The presence of FcaPV-2 DNA and mRNA in feline oral squamous cell carcinoma (FOSCC), papilloma and ulcerative lesions (ULs)

Sample type	FcaPV-2 DNA	FcaPV-2 mRNA
FOSCC	10/32 (31%)	7/10 (70%)
Nasal cavities SCC	2/2 (100%)	2/2 (100%)
Oral papilloma	1/1 (100%)	0/1 (0%)
Total	13/35 (37%)	9/13 (69%)
ULs	4/11 (36%)	N/A

Abbreviation: N/A, not assessed.

papilloma. Koilocytes-like cells were visible in basal, supra-basal and corneum stratum also in this sample (Figure 1B).

3.2 | Detection of FcaPV-2 DNA in FOSCC and one papilloma

FcaPV-2 DNA was amplified by JMPF/R PCR in 10 out of 32 FOSCC samples (31%) (Table S1 and Table 1). Among the additional SCC samples of nasal cavities, 2/2 (100%) harboured viral DNA, the papilloma sample also tested positive (Table S1 and Table 1). For a comprehensive view of PCR results see Table 1. Sequencing analysis of nine randomly chosen PCR products confirmed the identity of the amplicons.

3.3 | FcaPV-2 gene expression

To further investigate the possible biological actvity of FcaPV-2 in the analysed tumours, all viral DNA positive FOSCC, SCC of nasal cavities and papilloma samples were run through RT-PCR assays for L1, E2 and E6E7 gene expression analysis. FcaPV-2 was found to be transcriptionally active in 7/10 (70%) FOSCC and 2/2 (100%) SCC samples of nasal cavities (Table 1). Papilloma sample tested negative for gene expression. L1, E2 and E6E7 were found to be expressed in 5/10 (50%), 3/10 (33%) and 5/10 (50%) FOSCC samples, respectively; both SCC of nasal cavities expressed L1 (100%), whilst E6E7 were expressed in 1/2 (50%). Considering all the analysed tumours, one sample expressed all the investigated viral genes (T10), two samples expressed only L1 (T16, T24), one sample exclusively E6E7 (T26), whilst E2 was never expressed alone. At least two genes were expressed in the remaining samples (T13: L1, E2; T15: L1, E6E7; T21: E2, E6E7; T27: L1, E6E7; T28: L1, E6E7). No-RT controls consisting of RNA samples subjected to reverse transcription without the addition of reverse transcriptase did not yield any amplification by PCR, excluding that RT-PCR results were affected by contamination of genomic DNA. A detailed description of viral mRNAs detection pattern in each sample is reported in Table 2. Representative RT-PCR gels are shown in (Figure S2).

TABLE 2 Detailed mRNA detection pattern of FcaPV-2 L1, E2 and E6E7 in viral DNA positive feline oral squamous cell carcinoma samples

Sample	FcaPV-2 mRNA		
	L1	E2	E6E7
T10	+	+	+
T13	+	+	–
T14	–	–	–
T15	+	–	+
T16	+	–	–
T21	–	+	+
T23	–	–	–
T24	+	–	–
T25	–	–	–
T26	–	–	+
T27	+	–	+
T28	+	–	+
T35	–	–	–

3.4 | RQ of viral load

To gain insights on the possible causality of viral infection in FOSCC, tumour samples were further investigated by qPCR for FcaPV-2 DNA along with ULs. Seven out of 13 DNA positive neoplastic lesions (T10, T13, T16, T21, T24, T25, T26) and 11 out of 12 ULs samples yielded succesfull and reproducible Cq of reference gene and were further included in the analysis, the remaining samples were excluded. Of ULs, 4/11 (36%) harboured amplifiable FcaPV-2 DNA (UL3, UL4, UL7, UL9). RQ analysis according to $2^{-\Delta\Delta Cq}$ method suggested that the viral load was higher in 6/6 FOSCCs (100%) but not in SCC of nasal cavities (T16) compared to the UL group, however, the difference between mean FcaPV-2 DNA amounts of FOSCC and ULs groups was not statistically significant ($P > .05$) (Figure 2). Interestingly, among FOSCC samples, higher viral load was associated with E6E7 expression (T10, T21, T26).

3.5 | Detection of p16 protein

To further investigate whether FcaPV-2 might contribute to cell transformation in FOSCC, the presence of p16 as surrogate marker of PVs infection was investigated by IHC.[26] Among FcaPV-2 positive samples, 11 were available for IHC analysis and were examined for the presence of p16 along with one FcaPV-2 positive feline cutaneous SCC, used as positive control.[6,23] Of these, 2/11 were not evaluable because of technical hampering (T13, T23). Six out of 9 (67%) samples showed positive p16 immunosignal (T10, T14, T15, T16, T21, T35). Four out of 9 (44%) showed an intense, diffused cytoplasmic signal in 60% to 90% of neoplastic squamous cells (T10, T14, T15, T16) (Figure 3A); 2/9 samples (22%) showed a moderate cytoplasmic

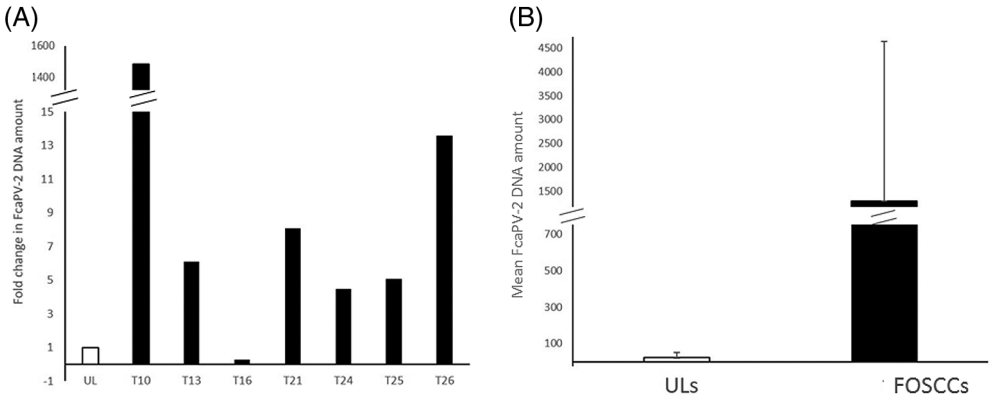

FIGURE 2 Analysis of viral load in feline oral squamous cell carcinoma (FOSCC) samples compared with oral ulcerative lesions (ULs). A, Relative quantization data obtained by Real-time qPCR are expressed as fold change with respect to a pool of FcaPV-2 positive UL samples (n = 4), which were set equal to 1, according to the $2^{-\Delta\Delta Cq}$ method. B, Mean relative DNA amounts ± standard deviations in FOSCCs vs ULs groups. The difference between the two groups was not statistically significant

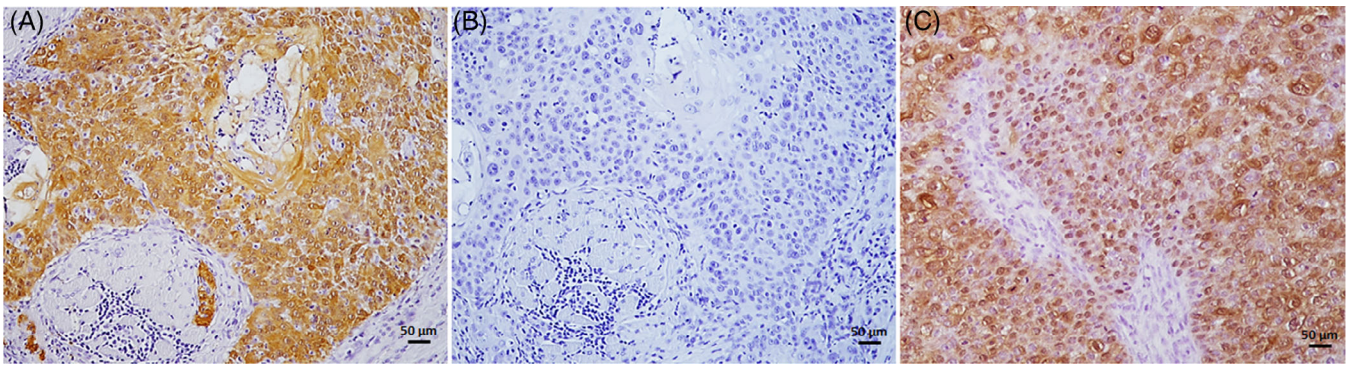

FIGURE 3 p16 expression in FcaPV-2 positive FOSCC. A, Intense cytoplasmic p16 expression in almost all neoplastic squamous cells of sample T10 (Streptavidin-biotin-peroxidase stain). Magnification ×20, scale bar: 50 µm. B, Secondary-only negative control for p16 on a serial section of sample T10 (Streptavidin-biotin-peroxidase stain). Magnification ×20, scale bar: 50 µm. C, FcaPV-2 positive feline cutaneous SCC run as positive control showed cytoplasmic and nuclear signal for p16 in almost all neoplastic cells (Streptavidin-biotin-peroxidase stain). Magnification ×20, scale bar: 50 µm [Colour figure can be viewed at wileyonlinelibrary.com]

labelling in 20% to 30% of neoplastic squamous cells (T21, T35), whilst 3/9 (33%) were negative for p16 expression (T24, T25, T26). Cytoplasmic/nuclear signal was revealed in the positive control, whilst the negative control did show any labelling (Figure 3B,C).

4 | DISCUSSION

In the current study, we detected FcaPV-2 DNA in 10/32 (31%) FOSCC: this is in disagreement with literature data, considering that even studies with comparable amount of samples failed in finding viral DNA.[11-15] In these previous works, PCR have been conducted by employing consensus oligos, since they are supposed to amplify sequences from most of PVs; however, studies on feline and bovine PVs (BPVs) indicate that they are less sensitive than type-specific primers as those used here, therefore this might explain the appearent discrepancy with our results.[11-13,23,27] Moreover, amplification of larger DNA sequences by the use of consensus primers attempted in the aforementioned reports, might have been hampered also by technical difficulties, such as DNA fragmentation because of the use of archival material, as well as by issues during sample preparation, for instance inappropriate formalin fixation time.[10] In recent studies, FcaPV-3, -4 and -5 have been found in cutaneous SCC in Japan, whilst FcaPV-2 has not been detected neither in skin nor in oral SCC: in this case, a lower prevalence of this virus in that geographical area or less extensive sampling might be a possible explanation for the discrepancy with our results.[28,29]

In situ hybridization (ISH) is considered the standard tool to detect FcaPV-2 DNA within neoplastic cells.[3,22] We attempted at performing ISH procedures on our samples, however, results were not conclusive.

As far as the natural history of oral SCC is concerned, it is known to possibly occurr through malignant progression of pre-neoplastic conditions such as papillomas, for instance in the case of bovine or canine PVs-triggered oral SCC.[9,10,30] In wild felids, oral papillomas are widely associated with specie-specific PVs infection, whilst in the

domestic cat they have been shown to be rarely harbouring only FcaPV-1 DNA.[17,31,32] This is the first time, to the best of our knowledge, that FcaPV-2 DNA is found in this type of lesion, therefore, despite no viral gene expression being detected, the hypothesis that FcaPV-2-associated papillomas may evolve to FOSCC is also conceivable. Additional hints of PV infection came from the observation of koilocytes-like cells in the papilloma, consistently with what is reported in FcaPV-1 associated papillomas, and in one SCC sample.[17]

In agreement with previous works on animal and human PVs, viral DNA was detected in normal oral mucosa (unpublished data) and non neoplastic oral UL samples, confirming that FcaPV-2 exerts oral tropism other than cutaneous.[33-35]

Recent studies validated quantitative measure of FcaPV-2 viral load by relative qPCR as a useful tool which may be more suggestive of a possible role in oncogenesis with respect to conventional PCR alone.[3,22] Consistently, despite a similar percentage of infection (31% vs 36%), qPCR data suggested a difference in viral load between FOSCCs and ULs, however, analysis of additional samples will be needed to confirm the significance of this difference. Whether confirmed, this may suggest that FcaPV-2 infects oral cavity with a certain frequence but may trigger FOSCC development when yielding high viral load.

Beyond viral DNA detection, we conducted gene expression analysis to further investigate whether the virus was biologically active in FOSCC. Strong evidences indicate that FcaPV-2 E6 and E7 exert potent transforming properties in vitro by corrupting p53 and pRb pathways, therefore the presence of viral mRNA encoding for E6E7 oncogenes in FOSCCs suggests that FcaPV-2 might contribute to cell trasformation within oral mucosa.[4-6] Among FOSCCs, E6E7 expression was detected in samples harbouring higher amount of viral DNA, similarly to what is described in feline cutaneous SCC, where FcaPV-2 infection is believed to be causative when sustained by high viral load associated with oncogenes expression.[3,19,22] Yet, oncogenes expression is proportionally related with viral load also in BPV-positive sarcoids, thus our data suggest that the presence of the FcaPV-2 may influence growth and replication of tumour cells, at least in a subset of FOSCCs.[36]

p16 is considered a surrogate marker of PVs infection in human cancer and feline cutaneous SCC because its increased expression is related to impairing of pRb pathway by E7 oncogene, whilst the possible significance of p16 in FOSCC is not fully understood. [15,23,26,37] In this study, part of analysed FcaPV-2 positive FOSCC samples showed the presence of p16, thus providing additional clues of possible viral pathogenesis. Interestingly, a recent report on a large number of samples shows that a proportion of FOSCCs displays p16 expression and this is associated with a longer survival time, similarly to human PVs-related oral SCC.[15] However, in the aforementioned work, no association with PVs sequences was found and this might be still due, as stated above, to the use of less sensitive PCR tecniques.[15] Then, further investigations are needed to clarify the significance of p16 expression in FOSCC and whether it might be considered a marker of PVs infection in these tumours.

The product of PVs E2 gene finely regulates transcription of viral genes, therefore disruption of its open reading frame (ORF) by viral genome integration leads to unscheduled expression of E6E7.[4] In partial agreement, most of our samples with detectable E6E7 mRNA did not express E2. However, concomitant detection of E2 and E6E7 transcripts in two samples suggest a scenario similar to that reported in feline cutaneous SCC, HPV-driven SCC and BPV-induced urothelial cancer, where their expression may be simoultaneous, independently from the physical status of viral genome.[4,38-40]

Generally, PVs oncogenic infections do not support viral replication, since expression of capsidic protein L1 is lost along with E2 through viral integration.[40] However, the data reported here in FOSCCs and previous studies on feline cutaneous SCC demonstrate that L1 is often expressed in concomitance with E6E7, suggesting that FcaPV-2 may successfully be replicating during infection associated with cancer development.[19] It should be noticed that this is plausible in other species, as L1 expression and viral replication have been reported also in PV-induced bovine urothelial carcinoma and human cutaneous SCC.[19,40] It is worthwile noting that L1 is responsible for immunogenicity and stimulation of the adaptive immune responses, therefore it might contribute to the chronic inflammation commonly observed in FOSCC, as suggested for other PV-induced tumours.[40,41]

FOSCC is considered a spontaneous animal model of human HNSCC, since a series of histopathological and biological similarities exist between feline and human disease.[41,42] A subgroup of human oropharyngeal SCC, accouting for 25% of all HNSCC, is associated with infection by mucosal alpha HR-HPVs.[7] Our study points out that FcaPV-2 infection may be an additional risk factor for the development of FOSCC as HPVs in human counterpart; moreover, FcaPV-2 E6 and E7 oncoproteins impair p53 and pRb pathways with molecular mechanisms comparable to those from mucosal HR-alpha HPVs, suggesting that FcaPV-2 induced FOSCC might be assumed as a naturally occurring animal model also for HPV-related HNSCC. [4,6,43] HR-HPVs have also been implicated as etiologic agents in nasopharyngeal cancer, thus the data obtained here in feline SCC of the nasal cavities may indicate an additional similarity with the human disease.[44] However, PV-induced human HNSCC mostly affects the oropharynx, whilst here we found FcaPV-2 in non oropharingeal sites.[7] SCC from this specific oral region were not enrolled in this study, therefore this discrepancy cannot be fully confirmed and further studies are warranted.

Generally, cutaneous and mucosal tropism are believed to be mutually exclusive for PVs, therefore the cross-over between skin and oral epithelium by FcaPV-2 could appear as unusual.[45] However, the association of BPV-1/-2 with both cutaneous and mucosal lesions suggests that mixed tropism is likely for some animal PVs types and recent studies indicate that this possibility should be further investigated in humans as well.[30,46]

The most probable hypothesis is that FcaPV-2 reaches the oral cavity through skin licking, given that FcaPV-2 appears as mainly cutaneous. This discordance with mucosal, sexually transmitted HR-HPVs may explain the difference in the age of infection onset, which is very early in cats but later and related to puberty in humans.[47] Moreover, we have recently demonstrated active viral infection in blood cells, thus the possibility that infected leucocytes may carry FcaPV-2

particles to the oral mucosa cannot be excluded.[21] In HR-HPV-driven oropharyngeal cancer, oral inflammation is a known co-factor, therefore, given that oral chronic inflammatory conditions are very common in cats, a similar interplay between FcaPV-2 and local flogistic co-factors might be conceived.[48,49]

In conclusion, the data obtained in this study indicate that a proportion of FOSCC is infected by FcaPV-2 and suggest, but do not definitively prove, that the virus may contribute to cell transformation in a subset of these tumours. A safe and immunogenic vaccine based on FcaPV-2 virus like particles has been recently developed, however, it failed in decreasing viral load in adult cats formerly infected.[50] Nevertheless, given the recent success in vaccination strategy for prevention of HR-HPVs-associated cancer in pre-pubertal age, it is tempting to speculate that a similar approach might be hypothesized in the future for the prophilaxys of FOSCC in young cats prior to FcaPV-2 infection.[51]

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CONFLICT OF INTEREST

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper neither in study design nor in the collection, analysis and interpretation of data, nor in the decision to submit the manuscript for publication.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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