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Abstract

Feline oral squamous cell carcinoma (FOSCC) is a frequent and progressively invasive tumour. Early lesions are difficult to recognize based on the sole clinical examination and may be misinterpreted as non-neoplastic. Mutations of *TP53* and epigenetic alterations of specific genes are present in FOSCC and may be early detected. Aim of this prospective study was to investigate the DNA methylation pattern of a 17-gene panel and *TP53* mutational status of FOSCC cytological samples obtained by oral brushing. Results were compared with a control group, in order to validate this non-invasive procedure for the screening of FOSCC. In FOSCC, the same analyses were carried out on the corresponding histological sample, if available. Thirty-five FOSCC and 60 controls were included. Mutations of *TP53* were detected in 17 FOSCC brushings (48%) and in none of the controls ($P < 0.001$). Six genes (*ZAP70*, *FLI1*, *MiR124-1*, *KIF1A*, *MAGEC2*, *MiR363*) were differentially methylated in FOSCC and were included in a methylation score. An algorithm based on *TP53* mutational status and methylation score allowed to differentiate FOSCC from controls with a 69% sensitivity and a 97% specificity (accuracy, 86%).

In 19 FOSCC histological samples, *TP53* mutational status was fully concordant with brushings, and a positive methylation score was observed in all cases. These results are promising for the identification of FOSCC by oral brushing, although some factors may limit the accuracy of this technique, and further studies are required to assess its reproducibility in clinical practice.

Keywords: oral squamous cell carcinoma; feline; oral brushing; early diagnosis; p53; DNA methylation; bisulfite next generation sequencing.

Introduction

Feline oral squamous cell carcinoma (FOSCC) is a frequent tumour, characterized by high local invasiveness and rapid progression. Histological biopsy currently provides the best means of diagnosis, but early lesions can pass unnoticed or be misdiagnosed as a dental complaint, which is commonly encountered in aged cats. Thus, in most cases, FOSCC are not identified until the lesion has progressed significantly, with associated oral pain, anorexia and halitosis due to bacterial infection. At this time, the prognosis is usually poor, regardless of treatment. A timely diagnosis is therefore a cornerstone to improve the chances of survival of affected cats.[1,2]

Aberrant DNA methylation involving cytosine-phosphate-guanine (CpG) islands is an early epigenetic change in carcinogenesis and has long been considered as a promising biomarker for the diagnosis of cancer.[3-5] Several studies have explored the possibility of detecting aberrant DNA methylation in cells obtained by non-invasive techniques, allowing the identification of early-stage human oral squamous cell carcinoma (HOSCC) and preneoplastic lesions.[6-8]

Recently, a step-wise algorithm including the methylation profile of 4 genes (*KIF1A, FLI1*, *MiR124-1* and *MAGEC2*) and the mutational status of *TP53* allowed to differentiate histological biopsies of FOSCC from non-neoplastic samples with a 94% sensitivity and a 100% specificity.[9]

The aim of the present study was to investigate the methylation profile of a larger panel of genes and the mutational status of *TP53* on cytological samples obtained by oral brushing. FOSCC were compared with a control group including normal oral mucosa and other oral lesions, in order to validate the diagnostic utility of this non-invasive procedure for the screening of feline oral cancer.

Materials and Methods

Study design and cytobrush procedure

A prospective study was carried out on feline patients presented with oral squamous cell carcinoma at the Veterinary Hospital of XXX and at different private veterinary clinics in XXX. In addition, cats of at least 5 years of age referred for other oral lesions (control group) were enrolled from a dental facility center (XXX). Finally, healthy control samples represented by cats without dental problems, were recruited upon owner compliance on a voluntary basis. Written informed consent was required for inclusion.

Demographic information and clinical presentation were collected for each case.

Cytological or histological diagnosis was a mandatory requirement for all FOSCC cases.

For the other oral lesions, diagnoses were obtained either histologically or clinically by a single specialist (XX). Healthy oral mucosa was judged as such by the same specialist.

A cytobrush was used to collect exfoliated cells from oral mucosa. For the cats requiring general anesthesia for a medical procedure, cell sampling was performed during anesthesia.

For FOSCC and other oral lesions, the surface of the lesion was gently brushed repeatedly for at least five seconds. For FOSCC, whenever possible, a second brush from the lesion and a third one from the clinically normal oral mucosa distant to the tumour were also obtained. For healthy control samples, brushes were obtained by gently rubbing all the oral mucosa, including gingiva, vestibule, palate and tongue. After sampling, each cytobrush was placed in a 1.8-mL tube containing DNA/RNA Shield (Zymo Research Europe, Freiberg, Germany) for cell preservation.

For FOSCC, genetic analyses were carried out in parallel on the corresponding histological samples, if available.

Ethics statement

All the examined FOSCC samples (cytobrush and biopsies) were collected with a primary diagnostic intent as part of routine standard care. For control cases, brushings were obtained either under general anesthesia for other medical procedures or on alert cats, in the case of healthy and good-natured subjects. Since no invasive procedures have been performed and the results of the present research did not influence any therapeutic decision, approval by an ethics committee was not required. However, all cats' owners were informed of the study purposes and methods and participated on a voluntary basis, by signing a written informed consent.

Genetic and Epigenetic Analyses

Analyses were performed at XXX.

For DNA methylation analysis, DNA from cytobrush specimens were purified using the MasterPure Complete DNA extraction kit (Lucigen, code MC85200, Madison, WI, USA). DNA from formalin-fixed and paraffin-embedded (FFPE) tissues (five consecutive 10 µm section for each sample) were purified using the QuickExtract FFPE DNA Extraction kit (Lucigen, code QEF81050)

Bisulfite treatment of genomic DNA (200-500 ng) was carried out with the EZ DNA Methylation-Lightning Kit (Zymo Research Europe, Freiberg, Germany), according to the manufacturer's protocol.

The panel included 17 target genes (*RB1, TERT, MiR296, CELSR3, EGFR, ZAP70, LRRTM1, KIF1A, PDPN, PARP15, FLI1, GP1BB, CDKN2A, CDH1, MiR124, MiR363, MAGEC2*), selected because their human orthologs were previously identified with altered methylation pattern in HOSCC and/or because an altered protein function had been previously documented in FOSCC (Table 1).[8-22]

In order to identify putative CpG island on promoter regions or early transcriptional regions of genes, genomic sequence stored on Ensembl genome (http://www.ensembl.org/index.html) were employed as query sequence. MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) designing was applied to identify CpGs and the primers of

choice.[23] Primer sequences used in this study are available in Table 1. The interrogated CpG sites for each gene have been numbered consecutively.

Locus-specific amplicon libraries were generated with tagged primers in two steps: a first PCR amplification for target enrichment, and a second shorter amplification session (eight cycles) to allow the barcoding of the template-specific amplicons obtained from the first amplification step. The DNA barcoding was performed using the Nextera Index Kit (Illumina, San Diego, CA, USA) as previously described.[8,20,24] The sequencing was conducted on MiSeq sequencer (Illumina, San Diego, CA, USA), according to the manufacturer's protocol. Each next-generation sequencing (NGS) experiment was designed to allocate at least one-thousand reads/region, in order to have a depth of coverage of 1000×.

FASTQ files were processed in a Galaxy Project environment by the tool Filter by Quality for the quality control (>Q 30) and Filter FASTQ reads for read lengths (>80 bp). FASTQ files were then mapped by BWAmeth, generating bam files which were in turn processed by MethylDackel using Felis_catus_9.0 as reference genome. This tool created a file for each case, assigning the exact methylation level for each investigated CpG position.[25]

TP53 mutations analysis of exons 5-8 was performed as previously described.[26] Cases were classified as mutated when presenting one or more alterations in the nucleotide sequence of the amplified exons of feline *TP53*, resulting in amino acid changes with negative impact on the protein function according to PolyPhen-2 (missense mutations) or PROVEAN (indels mutations) and showing a variant allele frequency (VAF) >5%.[27,28]

Statistical analysis

Continuous data were tested for normality with the D'Agostino and Pearson omnibus normality test. Variables were summarized as mean ± standard deviation in case of normal distribution, or as

median and range in case of non-normal distribution; categorical variables were summarized as frequencies and percentages.

For methylation analysis, the total number of reads of brushings obtained under general anesthesia was compared with those obtained from alert cats and the number of reads of brushings was compared with biopsies with Mann-Whitney U test. The VAF of *TP53* mutations in brushings was compared with that of the corresponding biopsy by Wilcoxon's signed rank sum test for paired data.

An algorithm to differentiate FOSCC cytobrushes from controls was developed by integration of previously published criteria with the results of new epigenetic analyses, if appropriate.[9]

The frequency of *TP53* mutations and alterations in the methylation profile in FOSCCs was compared with that found in controls using the chi-square test. The ~~sensibility~~sensitivity, specificity and accuracy of this algorithm was calculated.

The proportion of FOSCC correctly identified by the algorithm was compared between cats undergoing anesthesia and alert cats using Fisher's exact test.

In the cases where a second cytobrush was obtained from the lesion, the agreement of results obtained from the two samples was calculated. Finally, in FOSCC with available histological biopsy, the accuracy of the algorithm was compared between cytobrush and biopsy.

Analyses were carried out with a commercial software program (SPSS Statistics v19, IBM, Armonk, NY, USA) and the significance level was set at 0.05.

Cell Line Validation Statement

No cell lines were used in the current study.

Results

FOSCC

Thirty-five samples of FOSCC were included in the analysis. Tumours belonged to 29 Domestic Shorthair (DSH) cats, two domestic longhair (DLH) cats and one each of the following breeds: Siamese, Maine Coon, Thai and Chartreux. There were 15 castrated males (43%) and 20 spayed females (57%). The median age was 12 years (range, 5-19).

Tumours were located on mandibular gingiva (*n* = 11; 31%), maxillary gingiva (*n* = 10; 29%), tongue (*n* = 9; 26%), caudal oral mucosa (*n* = 4; 11%) and vestibular mucosa (*n* = 1; 3%).

Cytobrushes were performed under general anesthesia in 26 cases (74%), whereas the remaining 9 cats were awake (26%). Eleven cats (31%) with FOSCC received a second cytobrush of the tumour, while in 19 cats (54%), clinically healthy oral mucosa distant from the tumour was also sampled.

For 19 FOSCC (54%), the corresponding histological sample was submitted for genetic/epigenetic analyses.

Controls

Sixty cats were enrolled in the control group, including 51 DSH, 3 DLH, 3 Persians and one each of the following breeds: Siamese, Norwegian Forest Cat and Sphynx. There were 30 males (50%; 1 intact and 29 castrated) and 30 females (50%; 1 intact and 29 spayed). The median age was 10 years (range, 5-18). Lesions were grouped according to the clinical or histological diagnosis in periodontal disease with mucositis (*n* = 20), feline chronic gingivostomatitis (FCGS; *n* = 15), pyogenic granuloma (*n* = 4), eosinophilic granuloma (*n* = 3) and oral sarcoma (*n* = 3). Fifteen cytobrush of healthy oral mucosa were also included in the analysis.

Cytobrushes were performed under general anesthesia in 42 cases (70%), whereas the remaining 18 cats (30%) were alert.

TP53 mutational status

Mutations in the DNA-binding domain of *TP53* were detected in 17 tumours (48%). There were 12 missense mutations, 1 nonsense mutation, 1 in frame deletion, 2 frameshift deletions and 1 frameshift insertion; all the examined exons were involved (exon 5, *n* = 3; exon 6, *n* = 6; exon 7, *n* = 5; exon 8, *n* = 3).

In the 11 mutated FOSCC where the corresponding histological sample was tested, the mutational status was confirmed and the same mutation was detected; however, the median VAF of histological samples was significantly higher compared with the brush samples (35% vs. 13%; *P* = 0.05). In all 8 wild type FOSCC cytobrushes with the corresponding histological sample being tested, no *TP53* mutation was detected, resulting in a 100% agreement between brushing and biopsies.

Of the 11 FOSCC in which a double brushing of the tumour was performed, 8 (73%) were in agreement and 3 (27%) returned discordant results. Of the 19 cases of FOSCC in which a cytobrush of the clinically normal oral mucosa distant from the tumour was performed, none had *TP53* mutations.

Mutations in the DNA-binding domain of *TP53* were not detected in any control case ($P < 0.001$).

Genetic analysis and diagnostic algorithm

A total of 209 CpGs from the genes *RB1, TERT, MiR296, CELSR3, EGFR, ZAP70, LRRTM1, KIF1A, PDPN, PARP15, FLI1, GP1BB, CDKN2A, CDH1, MiR124, MiR363, MAGEC2* were investigated by bisulfite-NGS.

Of the previously tested genes, significant differences between FOSCC and control cases were obtained for *ZAP70*, *KIF1A*, *FLI1*, *MiR124* and *MAGEC2*, confirming earlier results.[9] Of the newly tested genes, only *MIR363* showed relevant differential methylation.

Based on these results, a methylation score was formulated, resulting from the sum of the points attributed to alterations in the methylation profile of the above-mentioned genes at specific CpG sites, including hypermethylation of *ZAP70*, *FLI1* and *MiR124* and hypomethylation of *KIF1A*,

MIR363 and *MAGEC2*. Any combination of the detailed alterations totaling ~~A~~ a methylation score ≥3 was classified as suggestive of FOSCC (Table 2).

Seventeen FOSCC cytobrushes (48%) received a methylation score ≥3, versus 2 brushings in the control group (3%, both diagnosed with periodontal disease; P <0.001). The methylation score was not associated with *TP53* mutations (P = 0.238).

Of the 11 FOSCC in which a double brushing of the tumour was performed, only 3 cats (27%) obtained a positive score in both brushes; 4 cats (36%) obtained a positive result in only one case and 4 (36%) in none. Of the 19 cases of FOSCC in which a cytobrush of the clinically normal oral mucosa distant from the tumour was performed, 7 (37%) had an altered methylation profile similar to the tumour (Table 3).

Of the 19 FOSCC cases in which the analyses were carried out in parallel on the corresponding biopsy sample, all 19 biopsies (100%) had a methylation score ≥3, compared with 11 cytobrushes (58%; *P* = 0.003). However, the overall number of reads was not significantly higher compared with brushings (*P* = 0.851; Table 3). Similarly, in FOSCC, the total number of reads of the cytobrushes obtained under general anesthesia was not significantly different compared with those obtained from alert cats (*P* = 0.393).

Based on these results, a step-wise algorithm was proposed. According to this algorithm, a diagnosis of FOSCC is highly probable in case of at least one of the following:

- *TP53* mutations;
- methylation score ≥3

This algorithm allowed to differentiate FOSCC cytobrushes with a 69% sensitivity and 97% specificity (overall accuracy, 86%).

The proportion of FOSCCs positive to the algorithm was significantly higher in cats in which the sample was obtained under general anesthesia (21 out of 26; 81%) compared to alert cats (3 out of 9; 33%; $P = 0.014$).

Discussion

In a previous study, an algorithm based on the mutational analysis of *TP53* and the methylation pattern of 4 genes allowed to differentiate histological biopsies of FOSCC from healthy and inflammatory controls with a 97% accuracy.[9]

In ~~this~~ the present study, genetic and epigenetic alterations were detected in samples obtained by a non-invasive approach: oral brushing. This method has already provided promising results for the early diagnosis of HOSCC and, if applicable in cats, could lead to a significant advantage in identifying suspected neoplastic lesions before they reach an advanced and incurable stage.

~~In the present study,~~ *TP53* mutational status was confirmed as a reliable marker, with a 100% concordance between histological biopsies and brushings, albeit with a lower VAF value in the latter, suggesting a greater dilution of neoplastic cells, probably due to the presence of non-neoplastic exfoliated cells. However, in this study, as in the previous ones, *TP53* mutations were only found in a proportion of cases, meaning that there is a percentage of FOSCC ranging from 30 to 50% that does not harbor mutations in the sequenced exons. The correct identification of these cases is therefore entirely dependent on the detection of abnormal methylation patterns, which has returned more variable results in this study.

In addition to the 10 previously investigated genes, we tested the diagnostic potential of 7 other genes whose molecular pathways showed significant dysregulations in previous studies on FOSCC and/or the human counterpart, including *RB1*, *CELSR3*, *EGFR*, *PDPN*, *p16*, *CDH1*, *MIR363*.[12,13,18,19,22,29]

Of these genes however, only *MIR363* was differentially methylated in FOSCC compared with controls. Of the previously investigated genes, the 4 already included in the formerly developed algorithm (*FLI1*, *MIR124-1*, *KIF1A* and *MAGEC2*) confirmed their diagnostic relevance, and *ZAP70* was included as well.

However, compared with biopsies, the amplitude of differential methylation between FOSCC and controls was significantly lower in brushings and extremely variable among cases. As a result, an altered methylation pattern was found in all FOSCC biopsies, while only 48% of FOSCC cytobrushes had a methylation score ≥3. Indeed, oral brushing allows a lower selectivity on the lesion when compared with biopsy and, being superficial, runs the risk of sampling cellular debris and necrotic material, whereas the microscopic examination of histological biopsies ensures that a significant amount of neoplastic cells is being tested. Furthermore, during brushing execution, saliva and exfoliated cells may dilute samples, thereby contaminating neoplastic cell DNA with that of normal keratinocytes, other cell types and, potentially, microbial DNA. This hypothesis is supported by the evidence that the number of reads of the analyzed genes in brushing samples was not significantly lower than that found in the corresponding biopsy samples, meaning that a similar amount of DNA was collected. Furthermore, the percentage of cases with altered methylation pattern was significantly higher in samples obtained under general anesthesia, suggesting that a more targeted and probably more prolonged sampling can improve diagnostic accuracy.

Importantly, in only 27% of FOSCC receiving a double sampling the test returned a positive result in both. According to a possible explanation, the first sampling may only collect necrotic material and cellular debris; once the outer crust is removed, the second sampling could successfully collect neoplastic cells. Alternatively, the second sampling may have been inadvertently performed too hastily, especially if the cat was awake.

Finally, an altered methylation pattern was observed in 37% of cases in which normal oral mucosa distant from the tumour was sampled. This may be due to neoplastic cell exfoliation in saliva, but the evidence of this finding only in a part of cases could also be explained with the theory of field cancerization. According to this theory, large tissue areas exposed to the same insult for a prolonged period of time can be initiated to the process of carcinogenesis; this predisposes to the onset of multiple independent tumours following a promoter event.[30]

According to several studies, the alteration of the epigenetic pattern is a very early event in the carcinogenetic~~sis~~ process, potentially earlier than *TP53* mutations, which in fact were not detected in any brushing of oral mucosa distant from tumours. This leads to the assumption that it is precisely on methylation that we must focus to develop an early screening test. Another possibility is that epigenetic alterations occur in senescent cells even if not neoplastic and therefore may represent non-specific event linked to the advanced age of patients, but the fact that they have not been found significantly in cats of the control group (which only had a mildly lower median age) leads us to consider this hypothesis less likely.[31]

Further encouraging data is that very rare control cases showed alterations, indicating a high specificity of the test. This let us hypothesize that, in most cases, chronic inflammation does not significantly alter the methylome, suggesting the possibility of identifying neoplastic lesions at their initial stage, and to effectively differentiate them from morphologically similar lesions of different nature, such as a pyogenic granuloma or ulcerative-hyperplastic gingival lesions in the context of chronic inflammatory diseases. Not even sarcomas, the second most frequent oral cancer in cats, showed alterations in the examined genes, further confirming the selectivity of these changes in FOSCC.

Nevertheless, it must be acknowledged that the great majority of FOSCC tested in this study were at an advanced stage. Hence, the actual sensitivity of the test in identifying the subclinical lesions

(i.e. not obvious lesions which would be biopsied anyway) needs to be addressed to ultimately confirm the clinical utility of this procedure. Only the cases detected early can be treated effectively and get an improvement in terms of survival and quality of life. In this scenario, a sensitivity of 70%, even if not excellent, could bring significant benefits to a number of patients. Reasoning in terms of technical applicability and cost analysis, the availability of NGS platforms is increasing in veterinary medicine; and evidence suggests the value of these methods applied in clinical fields, beyond research purposes, as demonstrated for infectious diseases.[32,33] In human oncology, NGS technologies have revolutionized the approach to molecular diagnostics, thanks to their ability to investigate hundreds of targets simultaneously instead of performing numerous single-gene biomarker assays. This can result in improved tissue utilization, efficiency and cost-effectiveness.[34] Although the initial investment cost remains high, the price per information unit (nucleotide) is lower compared with first generation sequencing, and the analysis of larger numbers of samples may make the cost for the single sample comparable to other ancillary molecular tests performed in veterinary laboratories. The time commitment is also comparable to other molecular diagnostic techniques, although the execution of NGS experiments requires specific competences for the instrument and the bioinformatic analysis of data.

Despite these promising data, the detection of the methylation score in FOSCC remains a critical point, especially in the subgroup without *TP53* mutations, whose identification by oral brushing seems less problematic. The effectiveness of the methylation score in histological biopsies leads to assume that the limits of the method are mainly related to the execution of sampling. Namely, the cooperation of patients appears to be critical. The fact that the technique is less effective in alert cats can be considered a limitation for a screening test, but only during general anesthesia cat's mouth can be carefully inspected, allowing the identification of potentially neoplastic lesions. Sampling could therefore be obtained during scaling sessions, which are frequently performed in

aged cats. Additionally, earlier lesions could be less painful and therefore easier to sample in alert patients. Further measures to enhance method's sensitivity could be to systematically carry out a double sampling, compare the performances of different collection devices, provide the clinicians with detailed instructions on how to perform the procedure in order to reduce operator variability and ensure an adequate restraining of animals in the absence of general anesthesia.

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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Table 1. List of genes interrogated in this study with their relative primers.

Gene	Description	Forward Primer	Reverse Primer	Number of interrogated CpG	Percentage of the feline sequence* matching the ~~sequence of the~~ human orthologue
RB1 *- Retinoblastoma*	Tumor suppressor gene whose protein pRb is dysregulated in FOSCC;[18] the human *RB1* gene was found hypermethylated in HOSCC.[13]	GGGGGAGTTAT GTYGTTTAAAAT	CRCTCRCTCACC TAAACAA	21	85%
TERT *- Telomerase reverse transcriptase*	Up-regulation of *TERT* can increase proliferative activity in cancers; *TERT* hypomethylation was reported both in HOSCC and FOSCC.[9,20]	GGTTTGGGATTT GGGATTAG	AAACCAAACAA AAGCCTACTCTA ACT	6	66%
MiR296 *-MicroRNA 296/KMT2C*	MicroRNA gene found hypomethylated in HOSCC and hypermethylated in FOSCC.[9,20]	TGATTTTTGGTT ATTTTAGTTTTG	CACTCTAAAAAT TTACACTAAACA CC	8	87%
CELSR3 *- Cadherin EGF LAG seven-pass G-type receptor 3*	Considered a promising biomarker in HOSCC based on the "triple evidence" of alterations (protein expression, somatic mutations and DNA methylation).[22]	ATTTGTAAATAG AAGAAAGAGTA AGAGATG	CATAACAAATCT AACTACTAAAAC C	9	90%
EGFR *- Epidermal growth factor receptor*	Dysregulation of this tyrosine kinase receptor can promote neoplastic transformation and its overexpression has been reported in FOSCC.[12]	GGTTAGTTTTTG ATTTTTATTAGG GTTT	AATTTATACCAA AATTCCCATTTC C	8	91%

ZAP70 *- Zeta chain of T cell receptor associated protein kinase 70*	Tyrosine kinase normally expressed by natural killer cells and T cells. Hypermethylation of ZAP70 was found in both FOSCC and HOSCC.[8,9,20]	GATTTYGAYGG GTTGTTTTG	CTCTCACCTCCA ACTTCCAC	19	94%
LRRTM1 *- Leucine-rich repeat transmembrane neuronal 1*	Encodes a type I transmembrane protein involved in nerve development and regeneration; hypermethylation of *LRRTM1* was found in both HOSCC and FOSCC.[9,20]	TATAGTTTGGYG GAGGGAAG	CCACTAACCRAT ACCACCTTTT	20	77%
KIF1A *- Kinesin family member 1A*	Encodes a microtubule-dependent molecular motor involved in important intracellular functions as cell division; *KIF1A* was found hypermethylated HOSCC and hypomethylated in FOSCC.[8,9,20]	GGGTAGGGTTG TAGGAGTTTAG	AACTCCAACCTC TTCAAAACAAAT	10	97%
PDPN *- Podoplanin*	Transmembrane glycoprotein whose overexpression can favor neoplastic transformation and metastatic invasion in HOSCC;[29] expression of podoplanin was also reported in FOSCC.[19]	TTTTTAATTGTA AAGTTTGTTTTT T	AATTAACTAATC CTCTTTAAAACC C	7	64%
PARP15 *- Poly (ADP-ribose) polymerase family member 15*	Nuclear enzyme involved in DNA repair, cell proliferation, and differentiation; *PARP15* was found hypermethylated in HOSCC and hypomethylated in FOSCC.[9,20]	AATTTTAAGATT ATAAGGAAGGT AGAAGTG	CCACAAATTACT ATACAAATTCTT C	10	70%

FLI1 *- Fli-1 proto-oncogene, ETS transcription factor*	Encodes a transcription factor that regulates genes involved in proliferation and differentiation; *FLI1* was reported to be hypermethylated both in HOSCC and FOSCC.[9,20]	AGGGTTTAGGGYGTTAGGG	CCACTACCCAATCRCTTACC	10	95%
GP1BB *- Glycoprotein 1b platelet beta subunit*	Encodes a transmembrane protein that normally mediates platelet adhesion; it was supposed to act as tumor suppressor in HOSCC, showing aberrant DNA methylation levels.[8,10,20]	GTTGTTGTTGTTGTTGTTGTTGT	AAACTCCTTAAAACAAAATCCTATC	26	89%
p16/CDKN2A *- Cyclin dependent kinase inhibitor 2A*	Tumor suppressor protein that can be dysregulated in FOSCC;[18] the silencing of this gene by hypermethylation can occur both in HOSCC and in human preneoplastic oral lesions.[13]	GAGYGAGTAGGATTGGAAG	CTTCCCCCACRACTTCTTTC	19	80%
CDH1 *- Cadherin 1*	Encodes E-Cadherin, which regulates intercellular adhesion and polarity of epithelial cells. The under-expression of E-Cadherin may favor the invasiveness of HOSCC; hypermethylation of *CDH1* was reported in HOSCC.[13]	GGAGGGAATTTGGTGGAAAT	AACCRCAACCAATAAACRAC	15	82%
MiR124-1 *- (ENSFCAG00000016467.6) MicroRNA 124-1*	Acts as tumor suppressor by regulating target genes and is down-regulated in several human cancers including HOSCC; *Mir124* was found hypermethylated in both HOSCC and ~~FOSCC~~.[9,20]	GGATTAAGATTAGATTTTGTTTTT	AAATTTATTCTACTACCCCTCAAACC	7	100%

MIR363 *- Micro-RNA 363*	Encodes the MicroRNA 363, which targets the *PDPN* gene and inhibits its expression. Hypermethylation of *MIR363* was associated with podoplanin overexpression in HOSCC.[15]	TTTGGTTTTATTTTATTGTAGTGTGGGTAT	AAAACATATATAAAATCCCAAAACC	4	97%
MAGEC2 *- Melanoma-associated Antigen C2*	Expressed in several cancer types but not in somatic cells; demethylation of this gene was associated to resisting cell death. Hypomethylation of *MAGEC2* was reported in FOSCC.[9]	AGTAGTTTGGGGAAGTTTGTTTTTT	CAATTTAACTACCATCTTATCTAAAACATC	10	37%

* Reference genome: *Felis_catus_9.0/felCat9.* FOSCC: feline oral squamous cell carcinoma. HOSCC: human oral squamous cell carcinoma.

Table 2. Methylation score assigned to 95 bushings from feline oral mucosa.

Gene	**Criteria** (% methylation)
ZAP70	>20% in at least one CpG (#3-19): 1 point >40%: 2 points
KIF1A	<70% in at least one CpG (#1-3, 5-7) or <30% in CpG #4: 1 point
FLI1	>20% in at least one CpG (#2-5): 1 point >40%: 2 points
MiR124	>40% in at least one CpG (#2, 3): 1 point
MiR363	<70% in CpG #1 or <80% in at least one CpG (#2-4): 1 point
MAGEC2	<70% in one CpG (#7-10): 1 point
Methylation score: total score	
Positive (suggestive of carcinoma)	≥ 3 points

~~Positive methylation score (suggestive of carcinoma)~~ ~~**≥ 3 points.**~~

Table 3. Oral squamous cell carcinom samples: mutation analysis of *TP53*, *methylation score* and algorithm applied on oral brushings and on their corresponding biopsy samples.

ID	Brushing sample						Biopsy sample			
	TP53	**VAF**	**MS**	**Algorithm**	**Algorithm on additional brushing**	**Algorithm on normal oral mucosa**	***TP53***	**VAF**	**MS**	**Algorithm**
01	WT	-	0	Neg	-	-	-	-	-	-
02	p.Y227C	46%	5	Pos	-	-	p.Y227C	25%	5	Pos
03	p.S234F	6%	1	Pos	-	-	-	-	-	-
04	C231_N232 del *in frame*	13%	5	Pos	-	-	-	-	-	-
05	WT	-	5	Pos	-	Pos	WT	-	5	Pos
06	WT	-	2	Neg	-	-	WT	-	5	Pos
07	WT	-	0	Neg	-	-	-	-	-	-
08	WT	-	4	Pos	-	-	WT	-	7	Pos
09	WT	-	0	Neg	Neg	Neg	WT	-	3	Pos
10	p.R150H	18%	3	Pos	-	-	p.R150H	35%	6	Pos
11	p.R167H	13%	3	Pos	-	-	p.R167H	17%	5	Pos
12	WT	-	4	Pos	-	-	WT	-	4	Pos
13	p.R206*	24%	4	Pos	-	Pos	p.R206*	73%	6	Pos
14	p.V209A	7%	0	Pos	-	-	p.V209A	13%	4	Pos
15	p.V209A	13%	5	Pos	-	Pos	p.V209A	52%	8	Pos
16	WT	-	0	Neg	-	-	-	-	-	-
17	WT	-	0	Neg	-	-	-	-	-	-

18	WT	-	0	Neg	-	-	-	-	-	-
19	p.G272E	26%	5	Pos	-	-	-	-	-	-
20	WT	-	0	Neg	-	Neg	-	-	-	-
21	WT	-	4	Pos	Neg	Pos	-	-	-	-
22	p.R242K	70%	4	Pos	Pos	Pos	-	-	-	-
23	p.E172K	38%	4	Pos	-	Neg	-	-	-	-
24	p.R206Q	12%	2	Pos	-	Neg	p.R206Q	27%	6	Pos
25	p.E264K	12%	1	Pos	Neg	Neg	p.E264K	49%	5	Pos
26	WT	-	1	Neg	Neg	Neg	WT	-	4	Pos
27	T277del *frameshift*	16%	2	Pos	-	Neg	T277del *frameshift*	48%	5	Pos
28	WT	-	0	Neg	Neg	Neg	-	-	-	-
29	WT	-	1	Neg	Neg	-	-	-	-	-
30	WT	-	3	Pos	Neg	Pos	-	-	-	-
31	S253 ins *frameshift*	60%	7	Pos	Pos	Pos	S253ins *frameshift*	34%	6	Pos
32	P183_L187 del *frameshift*	8%	2	Pos	Neg	Neg	P183_L187del *frameshift*	47%	4	Pos
33	p.R206Q	7%	2	Pos	-	Neg	-	-	-	-
34	WT	-	3	Pos	Pos	Neg	WT	-	6	Pos
35	WT	-	3	Pos	-	Neg	WT	-	6	Pos

WT: *wild type*; VAF: *Variant allele frequency*; MS: *Methylation score*; Pos: positive; Neg: negative.