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Title: Prognostic value of somatic focal amplifications on chromosome 30 in canine oral melanoma

Running Title: Genetic prognostic factor in canine oral melanoma

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The authors declare no conflict of interest.

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Abstract

Canine oral melanoma is the first malignancy of the oral cavity in dogs and is characterized by a local invasiveness and a high metastatic propensity. A better knowledge of genetic alterations is expected to improve management of this tumor. Copy number alterations are known characteristics of mucosal melanomas both in dogs and humans. The goal of this study was to explore the prognostic value of somatic focal amplifications on chromosomes (CFA) 10 and 30 in canine oral melanoma. The cohort included 73 dogs with oral melanoma confirmed by histology, removed surgically without adjuvant therapy and with a minimal follow-up of 6 months. Epidemiological, clinical and histological data were collected and quantitative PCR were performed on FFPE samples to identify specific focal amplifications. The 73 dogs included in the study had a median survival time of 220 days. Focal amplifications on CFA10 and 30 were recurrent (49,3% and 50.7% of cases respectively) and CFA30 amplification was significantly associated with the amelanotic phenotype ($p=0.046$) and high mitotic index (MI) ($p=0.0039$). CFA30 amplification was also linked to poor prognosis ($p=0.0005$). Other negative prognostic factors included gingiva location ($p=0.003$), lymphadenomegaly ($p=0.026$), tumor ulceration at diagnosis ($p=0.003$), MI superior to 6 mitoses over ten fields ($p=0.001$) and amelanotic tumor ($p=0.029$). In multivariate analyses using Cox proportional hazards regression, CFA30 amplification (Hazard Ratio-HR=2.08; $p=0.011$), tumor location (HR=2.20; $p=0.005$) and histological pigmentation (HR=1.87; $p=0.036$) were significantly associated with shorter survival time. Focal

amplification of CFA30 is linked to an aggressive subset and constitutes a new prognostic factor.

Key-words

dog, chromosome 30, focal amplification, oral melanoma, prognosis

Introduction

Malignant melanoma is a relatively common tumor in dogs and is the first oral malignancy, accounting for 14.4% to 45.5% of oral tumors.^{1,2} It arises in individuals older than 10 years^{3,4} and some breeds are over-represented in several studies including breeds such as cocker spaniel, golden and Labrador Retrievers, Scottish terrier, poodle, daschund, chow-chow and Boston terrier³⁻⁷. Oral malignant melanoma (OMM) is an aggressive tumor type with a rapid growth and local invasiveness. The metastatic propensity is high with invasion of regional lymph nodes, lung and abdominal organs, with reported metastatic rates between 59-74% and 17-51% for lymph node^{8,9} and lungs^{2,6,10} respectively. The prognosis of affected dogs is often poor and survival time after surgery varies from 3 to 24 months, in particular depending on the clinical stage at diagnosis⁴.

The first line treatment is wide surgical resection of the primary tumor sometimes associated with radiation therapy. Aggressive surgical excision with at least 1-cm-margins or hemimaxillectomy/mandibulectomy (if necessary) result in survival times between 7-10 months, but recurrence rate is still high (between 22-48%)^{2,11,12}. Radiation therapy can also be a primary treatment when surgery is not feasible, and can induce partial or complete clinical response in 82-94,4% of cases^{4,6,13}. Canine melanoma is a chemoresistant tumor and adjuvant chemotherapy

using mostly platinum agents failed to show any clinical benefits^{4,5,14,15}. In the last decade, the development of a xenogeneic melanoma DNA vaccine has shown some promising results in terms of safety, clinical response and survival, but needs further investigations with randomized controlled trials¹⁶⁻²⁰. Effective systemic therapies, including targeted therapies and immunotherapy are strongly needed to treat this cancer.

The knowledge of somatic genetic alterations is crucial to better understand tumor biology and to identify valuable therapeutic targets. Canine OMM genetics is characterized by an abundance of chromosome or chromosomal regions gains or losses, also called copy number alterations (CNA)²¹⁻²³. These alterations include whole chromosome gains of the *Canis FAmiliaris* (CFA) 13, 17, 20, 29, 36, losses of CFA 2, 22, 27, as well as focal losses and gains on CFA 10, encompassing *MDM2* and *CDK4*, and on CFA 30²¹⁻²³. Particularly, the focal amplification of a 600 kb region (16,1-16,7 Mb canFam3) of CFA 30 has been found to be highly recurrent in canine OMM^{22,24}. Single nucleotides variations (SNV) have also been recently identified in some genes such as *NRAS*, *TP53*, *PTEN*, *KIT* and *PTPRJ*, but were absent in *BRAF* gene, which is concordant with the non-UV etiology of this cancer^{7,21-28}.

The objective of this study was to evaluate the frequency of CFA 10 and CFA 30 focal amplifications in a cohort of dogs with OMM, to define if these alterations were associated to clinical or histopathological features and to investigate their potential prognostic significance.

Material and methods

Cases selection

Cases recruitment was performed thanks to Cani-DNA biological resource center and 3 french veterinary histopathology laboratories, and included dogs with OMM removed surgically and diagnosed between June 2008 and January 2015. A questionnaire was sent to referring veterinarians to gather epidemiological and survival data such as age at diagnosis, sex, breed, tumor characteristics (size, pigmentation, ulceration, location), regional lymphadenomegaly at diagnosis, surgical characteristics (type of surgery and macroscopic evaluation of margins status) and follow-up (development of metastasis, tumor recurrence, date and cause of death). We excluded dogs that had other malignancies, received an adjuvant treatment to surgery and dogs with a follow-up of less than 6 months. The measured outcome was melanoma related death to get the specific survival time (SST).

Histological data

After surgical removal, melanomas were fixed in formalin 10% and embedded in paraffin (FFPE). Histological examination was performed on 3- μ m-thick hematoxylin-eosin-saffron (HES) stained sections by a board-certified pathologist. For each case, evaluation included architectural features (nests, sheets, bundles, mixed architecture), percentage of necrosis, lymphocytic infiltration, ulceration, pathological margin status, lymphovascular invasion and junctional activity (nest of tumoral cells within the epithelium). Cellular tumoral morphology was also specified with shape (epithelioid, spindle cell, mixed), pigmentation, size, degree of nuclear atypia (% of cells presenting atypias) and mitotic index (number of mitotic figures by 10 most proliferative High-Power Fields [\times 400, diameter of the field of view 0.55 mm]). Prognostic metrics were evaluated in relation with specificity,

sensitivity, positive and negative predictive value in terms of 6-month survival rate²⁹.

Genetic study

For DNA isolation, ten 6- μ M-thick sections were cut from each FFPE tissue and were collected in DNase-free sterile microcentrifuge tubes. Genomic DNA was extracted using a FFPE Tissue DNA Kit (Macherey-Nagel®) according to the manufacturer's protocol. The quality and the quantity of the isolated DNA were determined using our routine laboratory protocols (dosage with Nanodrop®).

Quantitative-PCR (q-PCR) was performed to detect focal amplifications on CFA 10 and CFA 30. We used primer pairs targeting two genes on CFA 10 (*MDM2* and *CDK4*) and two distinct regions on CFA 30: a recurrent lost region, and the recurrent 600kb amplified region as previously shown²¹⁻²⁴, by targeting *BUB-1* (7.3Mb) and *TRPM7* (16.5Mb), respectively, since they are strong candidate driver genes in these regions²¹⁻²⁴ (**Table 1**). A primer pair targeting a region of CFA 9 was used as internal control as it was shown to have the higher stability of copy number in previous data,^{23,24} and each experiment was done with DNA of an unaffected dog as an external control. q-PCR was performed on tumor DNA samples after pre-amplification with the SYBR green PCR master mix (Thermo Fisher Scientific) on a 7900HT Fast Real-Time PCR System (Applied Biosystems) using standard procedures. Each sample was measured in triplicate, and relative amounts of the sequence were determined using the $\Delta\Delta C_t$ method (relative amount of target= $2^{-\Delta\Delta C_t}$). A gene was considered amplified in the tumor when it was present 5 times more than in the control sample. This threshold was chosen with the aim to detect

high number of amplifications, and after performing q-PCR with the same probes on healthy oral mucosa FFPE samples.

In order to confirm the q-PCR results, Fluorescence in Situ Hybridization (FISH) was performed on 4 μm sections of FFPE tissue blocs using 199H02 and 1E17 BAC (bacterial artificial chromosome) clones ordered at <http://bacpacresources.org/library.php?id=253>. BAC 199H02 overlapped *MDM2* and BAC 1E17 overlapped CFA 30: 16,5Mb region. These BAC clones were labeled with green-dUTP (Abbott Molecular) and Cy3-dCTP (Amersham Biosciences) respectively. Slides were analyzed by an experienced cytogeneticist (FC), using a fluorescence microscope (Axioskop2, Axio Imager Z2, Zeiss, Göttingen, Germany) and Isis imaging software (Metasystems, Altussheim, Germany). At least 100 non-overlapping tumor nuclei were examined for each case.

Statistical analyses

The R® statistical software (R Core Team 2018, <https://www.R-project.org/>) was used for statistical analyses. Continuous variables were expressed as median [range], mean \pm standard deviation. Correlations between categorical variables were analyzed using the Pearson χ^2 test or Fisher exact test. Correlations between categorical and numerical variables were analyzed using Student's t-test. Specific survival time (SST) was defined as the time between histopathological diagnosis and death attributable to melanoma. Dogs that were lost to follow-up or that have died due to unrelated cause were censored. The Kaplan–Meier method and log-rank tests were used for univariate survival analyses, and Cox proportional hazards models for multivariate survival analyses, whose results are reported using the Hazard Ratio (HR), its confidence interval (95%-CI), and the p-value of each covariate. The

statistical evaluation of the prognostic value of a factor was based on the following strategy: all the factors (clinical, histological and genetic) were tested by an univariate analysis to test its significance in terms of specific survival. Then all the prognostic factors which were significant in univariate analysis were tested using bivariate models with the variable « CFA 30 amplification ». This allowed us to test if the « CFA 30 amplification » prognostic value was complementary to the other variables. We then determined the best multivariate models in terms of AIC including significant variables using a stepwise selection (glmulti function of R glmulti library).

Results

The characteristics of the cohort are summarized in **Table 2** and in the **supplementary Table 1**.

Descriptive analysis

The cohort comprised 73 dogs diagnosed with oral melanoma, including 18 spayed females (24.7%), 18 intact females (24.7%), 1 spayed males (1.4%) and 36 intact males (49.3%). The mean age at diagnosis was 12.2 ± 1.9 years [range (7.5-17.2), median 12.3 years]. The most common breeds were poodle (19.2%), golden retriever (9.6%), Labrador retriever (8.2%) and Brie shepherd (5.5%). In most cases, melanoma developed on the oral mucosa of the lips or cheeks (47.8%), then on the gingiva (40.6%), and other sites including the tongue (7.2%), the pharynx (1.4%) and the hard palate (1.4%). Tumor size was superior to 3 cm at time of diagnosis for 30 dogs (45.5%) and 34 tumors were ulcerated (46.6%). The most common clinical sign was dysorexia (16%), and 21.7% of dogs showed lymphadenomegaly of draining lymph nodes by palpation.

Macroscopic surgical margins status was available for 46 dogs (63%) and 14 of these (30.4%) did not show tumor infiltration.

Tumor recurrence on the primary site was observed in 40 dogs (54.8%) after surgery, and pulmonary metastases were diagnosed by radiography in 10 dogs during follow-up (13.7%).

Regarding histopathology, the predominant tumor architectures were sheets on 28 tumors (38.4%) and bundles on 22 tumors (36%) whereas only 7 tumors (9.6%) showed nest organisation (**Figure 1**). Lymphocytic infiltration was marked in 22 tumors (30.2%) and mucosal ulceration was present in 59 tumors (80.8%). Visible tumor emboli were found in 5 melanomas (6.8%) and junctional activity was observed in 36 tumors (49.3%). Concerning cell morphology, 40 melanomas (54.8%) showed mainly an epithelioid shape, 26 cases (35.6%) a mainly spindle shape and 7 cases (9.6%) had a mixed morphology. Fifty-nine tumors (80.6%) were mainly composed of large cells with a diameter higher than 20 μm , 21 (28.8%) were characterized by the absence of melanic pigments in the cytoplasm (amelanotic melanomas) and 57 (78.1%) showed nuclear atypia on more than 40% of tumor cells. The mean mitotic index (MI) was 17 ± 17 mitoses over ten high power fields (HPF) [range (1-80), median 10 mitoses] and the threshold of 6 mitoses over 10 HPF had the best predictive value in terms of 6 months survival probability (**Table 3**). Fifty-one dogs (69.9%) had tumor with a MI higher than this threshold. Amelanotic melanomas were associated with a MI higher than 6 over 10 HPF compared to tumors with melanic pigments in the cytoplasm ($p=0.0065$). Histopathological evaluation of surgical margins was available for 46 dogs, and 30/46 dogs had infiltrated margins. OMM located on the gingiva were associated with an incomplete resection of the tumor ($p=0.035$).

Regarding CNA detected through quantitative PCR, the CFA 10 or CFA 30 analyzed regions were amplified in 52/73 tumors (72%). Regarding CFA 10, the *MDM2* gene was amplified in 36/73 dogs (49.3%), and *CDK4* was amplified in 30/73 dogs (41.1%). Although *MDM2* and *CDK4* are not so close along the CFA 10 (spaced 9 Mb apart), their amplification was often associated ($p=0.0002$).

Focal amplification on CFA 30 was detected in 50.7% of cases. Interestingly, amplifications were always detected with the same probe (16.5 Mb), whereas the region targeted by the second probe (7.3 Mb) was never amplified (**supplementary Table 1**). This finding is concordant with the fact that this last region is recurrently found lost²¹⁻²⁴ alongside with the focal gain of CFA 30: 16.5 Mb, and with the fact that the amplification involves a focal region and not the whole chromosome. Moreover, there was an enrichment of cases presenting the amplification of *CDK4* gene when these cases also had amplification of CFA 30: 16.5 Mb ($p=0.041$), and 27.4% of dogs had both alterations. Melanomas with a focal amplification on CFA 30 showed higher mitotic index ($p=0.0039$) and were significantly associated to achromia ($p=0.0461$). To support the q-PCR results, FISH was performed on 12 cases (3 cases with no amplifications, 3 cases with only *MDM2* amplification, 3 cases with only CFA 30: 16.5 Mb amplification and 3 cases with both *MDM2* and CFA 30 amplifications). Over the 24 FISH experiments performed (12 cases x 2 regions), only 2 had discordant results between q-PCR and FISH. With a p-value of 0.0001 (Fisher exact test), we concluded that the FISH results validated the q-PCR results (Supplementary Table 2, Figure 2).

Survival analysis

The median time to death attributable to melanoma was 220 days [range (14-1147)] and the mean time to death was 236 days \pm 201 days. By univariate analysis (**Figure 3**), reduced specific survival time (SST) was observed for dogs with gingival melanoma (median survival time-MST=169 days for gingiva location vs. 309 for other locations, HR= 2.05; p=0.0033), with macroscopic ulceration (HR= 2.06; p=0.0033) and with locoregional lymphadenomegaly (HR= 1.89; p=0.0259). Regarding histological criteria, the two main prognostic parameters were the pigmentation and the MI. Dogs with an amelanotic melanoma had a significantly reduced survival time compared to others (HR= 1.80; p=0.0296), and dogs with a MI > 6 mitosis figures over 10 HPF had also a poorer outcome (HR= 2.49; p=0.0011). Concerning genetic features, the amplification detected on CFA 30: 16.5 Mb in the tumor was associated with reduced SST (MST= 159 days for dogs with the amplification vs. 317 days for dogs without the amplification, HR= 2.34; p=0.0005). However, the analyzed somatic alterations on CFA 10 were not linked to prognosis in this cohort.

In bivariate analysis, Cox proportional hazards models showed that the amplification of CFA 30: 16.5 Mb was still a significant prognostic factor in association with other variables that were significant in univariate analysis, particularly with MI, a well known and used prognostic parameter (**Table 4, model 1**). These results strongly suggest that CFA 30: 16.5 Mb amplification is a novel prognostic factor that brings information complementary to known prognostic factors in canine oral melanoma.

On this cohort, the best predictive models are presented in Table 4 (models 2 and 3). The first one is a bivariate model containing tumor location and the mitotic index (HR: 2.67; p= 0.00045 and HR: 3,16; p= 0.00088 respectively). The second one is a trivariate model including the focal amplification on CFA 30: 16.5 Mb (HR= 2.08; p=0.011),

pigmentation (HR= 1.87; p=0.036) and tumor location (HR= 2.20; p=0.005), with a reduced SST for dogs carrying the amplification, amelanotic and gingival tumor.

Discussion

The epidemiological characteristics of our cohort of 73 dogs are similar to data presented in other studies about canine OMM, with a mean age at diagnosis of 12.2 years^{1,3,7,30}. Our study also confirms the over-representation of particular breeds like poodle, Labrador and golden Retriever. The median survival time of the whole cohort is comparable to those described in the literature, ranging from 3 months to 24 months depending on disease stage and treatment^{2,10,13-16,19,31-33}. Recently, Sarowitz et al. specified a median specific survival time of 206 days for dogs with OMM treated by surgery as a unique treatment, very similar to the MST of 220 days described here⁹.

In our study, the first anatomic location of the tumor is the labial and buccal mucosae whereas most studies showed higher prevalence of gingival melanocytic tumors^{9,29}. Moreover, gingival location appears as a significant negative prognostic parameter in univariate and multivariate analyses in our results. This can be explained by the difficulty to perform a complete surgical removal of the tumor due to the proximity of bone and tooth. Indeed, there was a significant positive correlation between gingival tumor location and incomplete resection. To avoid this, many authors recommend performing wide complete resection with partial mandibulectomy/maxillectomy when needed^{2,10-12}. To our knowledge, no study confirmed this poor outcome of dogs with gingival melanomas but a significant difference was established between tumor in rostral and caudal parts of the oral cavity, showing a better prognosis for dogs with rostral melanomas due to earlier detection⁶. It should be noted that higher prevalence of labial and buccal mucosae melanomas in this

cohort could have influenced other variables such as median survival time, mitotic index and copy number alterations rate, even if the two last parameters are not associated with tumor location. Nevertheless, bivariate and trivariate models showed that CFA 30:16.5 Mb amplification has a prognostic value complementary to tumor anatomical location.

In the present study, locoregional lymphadenomegaly constitutes a significant negative prognostic factor. This is in agreement with previous studies which assessed the prognostic value of clinical staging including lymph node infiltration^{11,31}. However, in the absence of systematic microscopic evaluation, the increased size of the lymph nodes may not reflect a true tumor infiltration but may correspond to reactive hyperplasia due to tumor ulceration or periodontal disease. This is confirmed with a recent study that compared lymph nodes evaluation in case of melanocytic tumor by palpation, cytology and histology with a high percentage of false positive and negative results⁴⁰.

After clinical staging and complete surgical excision, a histological analysis is recommended to confirm the malignancy of melanocytic tumor and to precise prognostic histological factors. Among these markers, our study shows that MI is an objective parameter with a strong prognostic value both in univariate and multivariate analysis. These findings highlight the poor outcome of dogs with higher MI, confirming previous results^{29,34}. Bergin et al., in 2011, thus suggested thus a threshold of 4 mitoses over 10 HPF predicting a pejorative outcome; however, in their study, the cohort included dogs with melanocytic tumors (comprising oral melanocytomas) and this threshold helps more to differentiate malignant from benign tumors. In our cohort, we excluded melanocytomas and proposed the cut-off of 6 mitoses over 10 HPF because it

showed better sensitivity and specificity than the cut-off of 4 mitoses in terms of 6-month survival rate.

Another prognostic factor in our cohort is the degree of pigmentation of the tumor, with amelanotic melanomas (absence of cytoplasmic pigment in all tumor cells) showing a poorer prognosis. This result confirms those of Bergin et al. who showed that high pigmentation (more than 50% of tumor cells with pigments) is correlated with a better outcome compared with other categories (0%, 1-10% and 11-50% tumors cells with pigments). However, they did not find any significant correlation between survival and achromia³⁴.

In the last decade, it has been suggested that canine cancers can constitute relevant spontaneous models for their human counterparts and that comparative oncology approaches may be valuable for human³⁵⁻³⁷. In particular, canine oral melanomas share many similar features with human mucosal melanomas, considering epidemiology, clinical behavior and pathology^{7,27,38-40}. The need to better understand the underlying genetic characteristics of this rare and devastating human cancer has led to the emergence of comparative genetic studies. As human mucosal melanomas, canine OMM harbor extensive somatic copy number alterations and structural variants, and the genetic features of these tumors have been highly studied these years²¹⁻²⁴. In both species, the mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K) pathways are frequently activated^{22,41}. Other common genetic features include the mutational landscape of human and canine mucosal melanoma, with mutations on the *RAS* family members genes, *TP53* gene and the absence of *BRAF* mutation^{21,23,25,27}. In the present study, we only focused on focal amplifications on CFA 10 and CFA 30, and found that these alterations were highly recurrent in canine OMM, with 72% of dogs having at least one of these. Although we did not evaluate the whole

CFA 10 and 30 chromosomes, previous studies described those amplifications as focal²¹⁻²³. Moreover, the high copy numbers observed in our cohort as well as the fact that the CFA 30: 7.3 Mb region was never found amplified are in favor of focal amplifications. As it was suggested by Hendricks *et. al*, this could be the results of telomere crisis or chromothripsis, in which one or a few chromosomes are shattered into tens to hundreds of pieces and reassembled incorrectly with the consequence of defined copy number changes²¹. Such events have been associated to poor prognosis in human cutaneous melanoma⁴². We found here that focal amplification on CFA 30:16.5 Mb is associated with an aggressive behavior of canine OMM (higher mitotic index and an amelanotic phenotype) and is linked to a poor outcome. In our study, bivariate analyses showed that this amplification is an informative prognostic factor complementary to other known factors and is partly responsible for canine oral melanoma aggressiveness. Nevertheless, taking into account the MI and the tumor location, the CFA 30 amplification was not anymore significant, probably due to its strong association with the MI. Indeed, genes lying on this recurrent amplified region probably bring an advantage for the proliferation and progression of the tumor, such as *TRPM7* involved in the MAPK pathway^{21,43-45}. Focal amplifications along CFA 10 were often found in two distinct regions covering the targeted genes *MDM2* and the cycline-dependent-kinase *CDK4*, and this association was demonstrated in a previous study²¹. Both are known oncogenes that could constitute therapeutic targets. *CDK4* is involved in the early phase of the cell cycle, and is frequently amplified in human oral melanoma⁴⁶. *MDM2* is able to block the tumor suppressor P53 and promotes its degradation, and its gene is also focally amplified in human mucosal melanomas²³. Our results confirm those of Poorman *et al.*, who studied the genetics of 44 canine OMM, 5 cutaneous melanomas and 18 melanocytomas, and found that molecular aberrations correlated with cell phenotype

and histology. Particularly, malignant melanomas that clustered with melanocytomas according to their copy number profile had a higher pigmentation level and a lower MI²². It has to be noted that canine OMM carries a lot of other alterations, other than those studied here, that can be important for tumor initiation and progression. For example, the recurrent lost region on the CFA 30 described by Wong *et al.* is located at 2-12 Mb and contains our targeted gene *BUB1* (7.3 Mb), as well as *KNSTRN* and *B2M*. Those genes are involved in chromosome segregation and immune evasion²³. This region also contains the mucosal melanoma driver gene *SPRED1*²³. Moreover, this deletion has been recently identified in the orthologous chromosomal region in human mucosal melanomas, reinforcing the interest of the dog model in comparative oncology studies²³.

Conclusion

This study highlights the presence, in canine oral malignant melanoma, of a highly recurrent focal amplification on CFA 30 that is associated to a poor outcome and to pejorative factors such as a high mitotic index and an amelanotic phenotype. To our knowledge, this is the first time that genetic features of canine oral melanoma are confronted to clinical and histopathological data, providing a significant prognostic marker in this canine cancer. Further prospective studies are warranted to confirm these results and to determine if this chromosomal region indeed contains interesting genes that could be further used as therapeutic targets both in dogs and humans.

Conflict of interest : no conflict of interest to declare

Data availability statement: the table containing epidemiological, histopathological, genetic and survival information is available on request.

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Table 1- Characteristics of the primers used for CNA detection on CFA 10 and CFA 30

Targeted region on the canine genome	Forward/Reverse	primer sequence	primer size (pb)	amplicon size (pb)
CFA9: 43651120-43730748 (Control)	F	GCCCAACTCACTGGACTTTG	20	95
	R	CAACTCCATCTGGGAGCATT	20	
CFA10: 10936607-10962527 (MDM2 gene)	F	TTGGAGTGCCAAGCTTCTCT	20	73
	R	CCCAGCTGGCTTTTACAAC	20	
CFA10: 1814134-1814208 (CDK4 gene)	F	GATACAGCCGACACTCCACA	20	73
	R	TGGTATCGTGCTCCAGAAGTT	21	
CFA30: 16466910-16556480	F	TGGTATAATCCTCACATTACCTGTGT	26	63
	R	GTTACAACCGGAGCCTGGAT	20	
CFA30: 7361964-7425992	F	GTCCACACTTCAGGGAGCAT	20	98
	R	ACAGGTTGACATCCCACCAT	20	

Table 2- Main descriptive features of the cohort (n=73). HPF: High power fields

	Total	CFA 30 amplification	P-Value
Age at diagnosis (years)	12.2 ± 2.0	12.5 ± 1.8	0.09 ^a
Sex			
Female	36/73 (49%)	20/36 (56%)	0.57 ^b
Male	37/73 (51%)	16/37 (44%)	
Breed			
Poodles	14/73 (19%)	8/14 (57%)	0.40 ^c
Golden Retriever	7/73 (10%)	3/7 (43%)	
Other	52/73 (71%)	26/52 (50%)	
Location			
Lips/Cheeks	33/69 (48%)	11/33 (33%)	0.014 ^{c*}
Gingiva	28/69 (41%)	18/28 (64%)	
Other	8/69 (11%)	4/8 (50%)	
Tumor size			
≥3 cm	30/66 (45%)	18/30 (60%)	0.15 ^b
< 3 cm	36/66 (55%)	14/36 (39%)	
Margins status			
Complete removal	32/58 (55%)	15/32 (47%)	0.73 ^b
Incomplete removal	26/58 (45%)	17/26 (65%)	
Recurrence			
Yes	40/73 (55%)	22/40 (55%)	0.56 ^b
No	33/73 (45%)	15/33 (45%)	
Metastasis			
Yes	10/73 (14%)	4/10 (40%)	0.69 ^c
No	63/73 (86%)	33/63 (60%)	
Tumour architecture			
Sheets	28/73 (38%)	13/28 (46%)	0.80 ^c
Bundles	27/73 (37%)	15/27 (56%)	
Nests	7/73 (10%)	5/7 (71%)	
Mixt	11/73 (15%)	4/11 (36%)	
Junctional activity			
Yes	36/66 (55%)	18/36 (50%)	0.49 ^b
No	30/66 (45%)	14/30 (47%)	
Pigmentation cell			
Yes	52/73 (71%)	22/52 (42%)	0.0461 ^{b*}
No	21/73 (29%)	15/21 (71%)	
Mitotic index			
> 6 mitoses over ten HPF	51/73 (70%)	32/51 (63%)	0.0039 ^{b*}
≤ 6 mitoses over ten HPF	22/73 (30%)	5/22 (23%)	

^a Student's t-test; ^b Pearson χ^2 test; ^c Fisher exact test; * p-value < 0.05 was considered significant

Table 3– Characteristics of thresholds for mitotic index (MI) in terms of 6-month survival rate

Performance metrics	MI > 6 mitoses	MI > 4 mitoses
Sensitivity	80.0%	83.3%
Specificity	37.2%	27.9%
Positive predictive value	47.1%	44.6%
Negative predictive value	72.7%	70.6%
Overall correct classification	54.8%	50.7%

Table 4- Multivariate Cox models with variables identified to have significant association with survival time after surgical resection of oral melanoma in 73 dogs.

	HR	lower 95%	upper 95%	p-value
Model 1				
CFA 30 amplification in the tumor versus no CFA 30 amplification	1.98	1.12	3.52	0.019
MI > 6 versus MI ≤ 6	2.23	1.12	4.47	0.023
Model 2				
Gingival tumor versus othe location	2.67	1.54	4.63	0.00045
MI > 6 versus MI ≤ 6	3.16	1.60	6.22	0.00088
Model 3				
CFA 30 amplification in the tumor versus no CFA 30 amplification	2.09	1.18	3.68	0.011
Amelanotic tumor versus pigmented tumor	1.88	1.04	3.37	0.036
Gingival tumor versus othe location	2.20	1.27	3.82	0.005

MI: Mitotic Index ; CFA: Canine Chromosome; HR: Hazard Ratio

Figure legends

Figure 1. Light microscopic images of canine oral melanomas. Hematoxylin-Eosin-Saffron. Objective 40x. Bar = 100 μm. **(A)** Non-pigmented large epithelioid cells organized in sheets with numerous mitoses (arrowheads). **(B)** Pigmented spindle cells organized in bundles. **(C)** Pigmented large epithelioid cells organized in nests. **(D)** Mucosal epithelium showing junctional activity (arrow)

Figure 2. Light microscopic image of FISH on 4 canine oral melanomas x1000. Green (dUTP) probe targets MDM2 region on CFA 10 and red (Cy3-dCTP) probe targets CFA

30: 16.5Mb. **(A)** Canine oral melanoma case with normal copy numbers of MDM2 (CFA 10) and CFA 30: 16.5Mb. **(B)** Canine oral melanoma case with MDM2 (CFA 10) amplification and normal copy number of CFA 30: 16.5Mb. **(C)** Canine oral melanoma case with CFA 30: 16.5Mb amplification and normal copy number of MDM2 (CFA 10). **(D)** Canine oral melanoma case with MDM2 (CFA 10) and CFA 30: 16.5Mb amplification.

Figure 3. Cancer-specific survival times in dogs with oral melanoma. **(A)** Cancer-specific survival of all dogs of the cohort with a median survival time of 220 days **(B)** Dogs with gingival melanoma displayed significantly shorter survival (HR=2.05 [1.17-3.60], Logrank test, p=0.0033, Kaplan–Meier curves) than dogs with melanoma in other locations. **(C)** Dogs with high mitotic melanoma (cut-off of 6) displayed significantly shorter survival (HR=2.49 [1.49-4.14], Logrank test, p=0.0011, Kaplan–Meier curves) than dogs with low mitotic melanoma. **(D)** Dogs with amplification on CFA 30 displayed significantly shorter survival (HR=2.34 [1.38-3.96], Logrank test, p=0.0005, Kaplan–Meier curves) than dogs without.





