Oral Melanoma – Canine
Version: Canine Oral Melanoma 1.0
Protocol date: May 2021

Authors: Rebecca Smedley*, Cynthia Bacmeister, Laura Bongiovanni, Craig A. Clifford, Taryn A. Donovan*, D. Glen Esplin, Michael Goldschmidt, Matti Kiupel, Frances M. Moore*, Mark Simpson, Paul Woods

**Denotes communication author. All other contributing authors are listed alphabetically;* contact the *communication* author to suggest updates, provide edits and comments: Rebecca Smedley (smedley1@msu.edu), Taryn Donovan (Taryn.Donovan@amcny.org), Frances Moore (fmoore1977@centurytel.net)

Recommended Citation: Smedley R et al. Canine Oral Melanoma Guideline, version 1.0. Veterinary Cancer Guidelines and Protocols. http://vetcancerprotocols.org

Accessed on (date).

Introduction

This protocol is intended for use with oral melanocytic tumors of the mucous membranes in dogs, including those arising from the mucosal surface of the lip; the tumor can extend from the lip to the skin. This protocol is not intended for the following tumor types: Cutaneous melanocytic neoplasms including those restricted to the haired skin portion of the lip; melanocytic neoplasms of the eyelids; cutaneous melanocytic neoplasms of the digit. Some of these parameters have not been associated with prognosis previously or have not been evaluated previously. The purpose of this protocol is to provide standards for accruing data so that, over time, large data sets with comparable information can be evaluated to enable meaningful conclusions and accurate prognostic information. Ultimately this will enable meaningful conclusions and accurate prognostic information that will improve patient care. This protocol is a “living” document which will be modified as new information becomes available.

See also Protocol 2: melanoma, cutaneous, canine

Histological Type (Note A)

_____ Oral/lip malignant melanoma

_____ Histologically well-differentiated melanocytic neoplasm of the mucous membranes of the lips and oral cavity (HWDM) (aka melanocytic tumor of low malignant potential)

_____Melanocytic neoplasm

Predominant Cell Subtype

_____Epithelioid/polygonal

_____Spindloid/fibromatous

_____Mixed epithelioid and spindloid

_____Round cell

_____Small cell

_____Balloon/clear/signet-ring cell

_____Whorled/dendritic

_____Adenomatous/papillary

_____Osteocartilaginous differentiation

_____Angiotropic or angiomatoid (pseudovascular)

_____Giant cell

_____Rhabdoid

_____Myxoid

Surgical Procedure

_____ Biopsy (Note B)

- _____ Wedge
- _____ Needle

_____Excision

_____Jaw resection

_____Re-excisional biopsy (Note B)

Mode of Tissue Assessment
____ Light microscopy with glass slide evaluation

____ Whole slide digital image assessment

Tumor Site:
Describe and/or indicate below:

_____Oral

_____Gingiva (indicate maxillary or mandibular and adjacent tooth)

_____Tongue

_____Buccal mucosa

_____ Tonsil

_____ Palate

_____Lip

_____Upper

_____Lower

_____Combination (state which sites)

Ulceration Macroscopic
___ Not present

___ Present

Extent of ulceration (millimeters): ____ mm

___ Cannot be determined (e.g. sample too small to evaluate, trimmer did not note, etc.)

Ulceration Microscopic
(will depend on sectioning)

___ Not present

___ Present

Pedunculated?
____Yes
____ No

Tumor Size: Macroscopic
Greatest dimension: _____

Additional dimensions: _____

Tumor Size: Microscopic
Greatest thickness: _____ Measured by applying a ruler perpendicularly to the epithelium and measuring the greatest thickness of the tumor

Additional dimensions: _____

Macroscopic Assessment of Pigmentation
_____ Present

____ ≥50% of the mass

____ <50% of the mass

_____ Absent

Histologic Assessment of Pigmentation
_____Present

_____ ≥50% of cells pigmented

_____Diffuse heavy pigmentation – visualization of cellular morphology is histologically difficult

_____ <50% of cells pigmented

_____ Absent

Histologic Microsatellite(s)
___ Not identified

___ Present

___ Cannot be determined

Deepest Tissue Layer Present in the Sections Examined: (Histologic assessment)
_____ Mucosal epithelium only

_____ Submucosa

_____ Regional skeletal muscle

_____ Salivary gland

_____ Bone

_____Other (e.g. nasal cavity, etc.)_____

Deepest Tissue Layer Infiltrated: (Histologic assessment)

_____ Mucosal epithelium only

_____ Submucosa

_____ Skeletal muscle

_____ Salivary gland

_____ Bone

_____ Cannot be determined (e.g. neoplastic cells extend to the deep margin)

Mitotic Count (per 2.37 mm^2)
(Guideline 1and 2) (Note C)

Mitotic Count (MC) w/o bleach; if bleached, report both MCs

___ None identified

___ Specify mitoses/2.37 mm^2

___ Cannot be determined, explain

Nuclear Atypia

(Note D) Assess in epithelioid predominant neoplasms and in spindloid neoplasms with sufficiently observable nuclear detail. Bleach if necessary. Count 100 cells at 400X magnification and estimate the percentage of neoplastic cells with nuclear atypia using a threshold of 30%. Consult Note D and references for definitions of “typical” and “atypical” nuclei.[1,3,5,6]

____ <30% atypical nuclei

____ ≥30% atypical nuclei

Ki67

Average number of positively labeled neoplastic cell nuclei per area of a $1cm^2$ optical grid reticle at 400x magnification (5 grid areas counted) in the highest labeling area avoiding areas of ulceration and inflammation (Note E)

____ Average number of positively labeled nuclei per grid

____<19.5 positive nuclei per grid reticle

___ ≥19.5 positive nuclei per grid reticle

Necrosis
(Estimated percent of tumor which is necrotic.) (Note F) (See Guideline 5)

_____ <50%

_____ > 50%

_____ Necrosis estimated by microscopic assessment only

_____ Necrosis estimated by gross and microscopic assessment

Neoplastic Cells at Epidermal-Dermal Junction (Note G)
_____ Present

_____ Absent

_____ Cannot determine (e.g. due to diffuse ulceration)

Junctional Activity
(nests of neoplastic cells within the basal layer of the epidermis) (Note G)

_____ Present

_____ Absent

_____ Cannot determine (e.g. due to diffuse ulceration)

Intraepithelial Nests
(nests of neoplastic cells in the layers of the epidermis superficial to the basal layer) (Note G)

_____ Present

_____ Absent

_____ Cannot determine (e.g. due to diffuse ulceration)

Lentiginous Spread
(lateral spread within the epidermis) (Note G)

_____ Present

_____ Absent

_____ Cannot determine (e.g. due to diffuse ulceration)

Margins
(Note H) (Guideline 3)

Histologic tumor free distance (HTFD) is the shortest distance between tumor and the inked margin. Measure margins in mm (no decimals) as accurately as possible. Report

"focal" if only a few foci of tumor cells are present at the margin. Report diffuse if large numbers of tumor cells are at the margin. Indicate if imaging or other technology was used to determine tumor infiltration of surrounding tissues.

Method of margin assessment

_____ Radial

_____ Tangential (since HTFD cannot be assessed in tangentially sectioned margins, indicate:

_____ Tumor cells present at margin

_____ Tumor cells not present at margin

_____ Combination (specify)

Peripheral (Lateral) Margin

____ mm tumor to margins (HTFD)

_____ Tumor is at margin

____ Focal

____ Diffuse

____Satellite foci

Were margins inked at the time of surgery?

_____Yes

_____No

If no, were margins inked by lab personnel?

____ Yes

____ No

_____ Margins not assessed (Explain)

Deep Margin

____ mm tumor to margins (HTFD)

_____ Tumor is at margin

____ Focal

____ Diffuse

____Satellite foci

Lateral tangential margins
____Tumor is present in lateral tangential margin sections

____Tumor is NOT present in lateral tangential margin sections

Deep tangential margins
____Tumor is present in deep tangential margin sections

____Tumor is NOT present in deep tangential margin sections

Is there a fascial plane deep to the tumor?

_____Yes

_____No

Were margins inked at the time of surgery?
_____Yes
_____No
If no, were margins inked by lab personnel?
____ Yes
____ No
_____ Margins not assessed (Explain)

Lymphovascular Invasion (Guideline 4)
Lymphovascular Invasion (report format below)

_____Not identified

_____ Equivocal

____ Present

Criteria used to determine lymphovascular invasion

____Thrombus adherent to intravascular tumor

_____Tumor cells invading through a vessel wall and endothelium

_____Neoplastic cells within a space lined by lymphatic or blood vascular endothelium

_____Neoplastic cells in a structure that has been confirmed to be a lymphatic or blood vessel using immunohistochemistry

Number of LVI foci (within a minimum of one representative section of tumor and peritumoral tissue. Report the number of foci of LVI within all sections examined.)

_____ Few (< 5 foci)

_____ Moderate (5 – 10 foci)

_____ Many (> 10 foci)

Type of vessels invaded

_____ Muscular wall evident

_____ No muscular wall evident

Site of lymphovascular invasion

_____ Intratumoral (number of LVI foci)

_____ Peritumoral (number of LVI foci)

Metastasis (Note I)

_____Not present

_____Suspected on the basis of image studies, but not confirmed. Indicate if presence of metastasis was based upon imaging studies with no confirmatory tissue sampling.

_____ State mode of imaging

_____ Present. Indicate means to confirm metastatic tumor (i.e., histopathology from biopsy/autopsy tissue; fine needle aspirate cytology, etc.)_____

_____ Lymph nodes (Indicate sites)

_____ Lungs

_____ Other (Indicate)

_____Not determined

Regional Lymph Nodes

Note: If multiple nodes included, each node should be reported separately.

___ No lymph nodes submitted

___ No evidence of metastasis

Total Number of Lymph Nodes Examined: ____

Total Number of Lymph Nodes Involved: ____

_____ Tributary node

_____ Sentinel node

Size of node: _____ mm

Sectioning of node: _____ single cross section

_____ parallel sectioning at 2 mm intervals

Other sectioning method: _____

Size of Largest Metastatic Deposit: ____mm

Gross or histologic measurement: _____

Extranodal Extension

___ Not identified

___ Present

___ Cannot be determined

Patient Outcome
See Guideline 7

_____Alive

_____Dead

____Length of time between diagnosis and death

____Was death tumor related?

If death was tumor related, how was this determined? (e.g. by clinician opinion, autopsy exam, some other way)

_____

_____Natural Death

_____Euthanized

Other

_____Immunohistochemical markers assessed and results (amelanotic neoplasms should be immunohistochemically labeled with melanocytic specific markers that have been validated and published in dogs, specifically: Melan-A, PNL2, TRP-1 or TRP-2)

_____Molecular tests performed and results (genetic tests)[1]

Notes:

A. Tumor type should be designated as precisely as possible using stated or referenced criteria which can be duplicated on subsequent studies. Histologically well-differentiated melanocytic neoplasms of the mucous membranes of the lips and oral cavity (HWDM) have the following features: typically raised, non-ulcerated, <2cm diameter*, heavily pigmented, lack cellular atypia, do not invade bone, often lack junctional activity and lateral (lentiginous) spread, and have rare mitoses*, a very low Ki67 index, and abundant collagenous stroma.[2,3] Dogs with HWDMs had mean survival time of approximately 2 years, median survival of almost 3 years with only local excision in one study.[3] Typical features of oral malignant melanomas include: junctional activity, lateral epithelial spread, poor pigmentation, bone invasion, marked nuclear atypia, and often have a very high MC and Ki67 index.[2,4-6] Some neoplasms may have mixed features, or parameters that are at or near the threshold values. It may be difficult to classify these as definitively benign or malignant. Thus, these neoplasms should be designated simply as melanocytic neoplasms and each parameter should be reported and discussed.[2,4] Bergin et al reported the percentages of tumors correctly classified with respect to death by 1 year postdiagnosis were comparable for Ki67 (86.1%, 68/79), the nuclear atypia score (86.3%, 63/73), and the mitotic index (count) (86.8%, 66/76).[2] They also indicated that nuclear atypia was the only parameter that was statistically significant with multivariate analysis, but that all 3 parameters evaluated performed similarly and a panel of all 3 was recommended.[2] Nuclear atypia is subject to interobserver variation, even when the guidelines by Spangler and Kass[5] are followed, and it can be cumbersome to enumerate since 100 cells must be evaluated. It is easier to identify areas of high proliferation with Ki67 labeling than it is to find high mitotic activity in sections stained with H&E. Therefore, some authors feel that all 3 parameters should be evaluated[2] but when there are mixed parameter results, Ki67 index should be used for final interpretation.[4] Colleagues can read the full manuscripts to decide which parameters may be most useful. Oncology would benefit by additional studies using standardized detailed guidelines for Ki67, nuclear atypia and MC.

*Esplin[2]: <2cm diameter (55/71 lesions had diameter <1.0 cm as measured on glass slide and only 1 tumor > 2 cm in diameter); rare mitoses (56 of 71 had 0 mitotic figures; all 71 <3 MF; area not defined beyond 10 HPF)

B. It may not be possible to assess all parameters in biopsy specimens. If the specimen is from a re-excisional biopsy procedure, the original diagnosis and the evaluated parameters should be reported together with the diagnosis and parameters evaluated for the re-excised tumor. It is often not possible to assess all parameters in a re-excisional biopsy sample, as often there are only a few neoplastic cells present.

C. While MC is subjective, it has shown statistical relevance in several studies of canine oral/lip melanocytic neoplasms.[2-5] For canine oral and lip melanocytic neoplasms, MC should be determined in the area of highest mitotic activity.[2,4] The MC should be determined at 400x magnification in the most mitotically active area of the tumor in a 2.37 mm^2 area that is free of ulceration, necrosis and inflammation. Bleaching may be needed if heavy pigmentation obscures nuclear detail. If the specimen is bleached, the MC with and w/o bleaching should be reported. Greater than or equal to 4 mitoses is a statistically determined threshold value for MC for canine oral/lip melanocytic neoplasms (ocular FN and area were not provided in the study) and higher counts have been associated with marked reduction in survival time.[2] This parameter, like others should not be used as a standalone parameter. (See Guideline 1, MC).

D. In order to decrease interobserver variation, nuclear atypia should be assessed according to the criteria outlined by Spangler and Kass.[5] These criteria are: *Well-differentiated nuclei are small with a single centrally located nucleolus and minimal clumping of chromatin. They may have condensed strands of nuclear chromatin extending from the nucleolus to the nuclear membrane or condensation of chromatin along the inner surface of the membrane. Cells that lack a nucleolus have fine and evenly dispersed chromatin at the periphery of the nucleus. Poorly differentiated nuclei have larger nucleoli of less regular shape that are eccentrically located within the nucleus. There are often multiple nucleoli that, in some cases, may be haphazardly connected to the inner surface of the nuclear membrane by thin strands of chromatin*

and give the appearance of a coarsely vacuolated nucleus. It may be difficult, or not possible, to assess nuclear atypia in some spindloid, whorled/dendritic, or balloon/clear/signet ring cell variants of melanocytic neoplasms and bleaching may be needed for neoplasms with abundant pigment. A threshold value of 30% has shown statistical significance for predicting survival times for canine oral/lip melanocytic neoplasms when the above criteria are used.[2,4,5] One method to do this accurately is to count the number of atypical nuclei among 100 cells.[2,5] Nuclear atypia should not be used as a standalone parameter.

E. The Ki67 index for canine oral/lip melanocytic neoplasms is defined as the average number of positively labeled neoplastic cell nuclei per area of a $1cm^2$ optical grid reticle* at 400x magnification (5 grid areas counted) in the highest labeling** area.[2] Nuclei with weak to strong diffuse labeling and nuclei with only nucleolar labeling are counted while avoiding areas of ulceration and inflammation. Bleaching the sections AFTER immunohistochemical labeling may be needed to better assess the Ki67 index in neoplasms with abundant pigment; however, when red chromagen detection is used bleaching is generally not necessary. The grid method allows for standardization of the area assessed regardless of the type of optical used. Nuclear labeling is easier to recognize at low magnification compared to mitotic figures, thus, it is easier to identify areas with the highest proliferation activity. Receiver operator curve analysis, rather than empirical determination, identified a statistically significant threshold value of 19.5 for canine oral/lip melanocytic neoplasms and Kaplan-Meier survival analysis showed that dogs with a Ki67 index < 19.5 and dogs with a Ki67 index ≥ 19.5 have a statistically significant difference in survival (low being better for survival) based on a one-year survival period.[2] Ki67 index, nuclear atypia, and MC had similarly high positive and negative predictive values in one study.[2] Colleagues are encouraged to read Table 7 in Bergin et al[2] and note that each parameter had a few cases in which the outcome was different from what would have been predicted by the results of a parameter. This is to be expected, as prognostic parameters (markers) are statistical estimates meant to predict an outcome metric on a population basis, not on an individual patient basis.

* The grid reticle is reported as 1 mm^2 in two references[2,4] but this should be 1cm^2 ocular reticle [2,4] with published corrections in progress. A 1cm^2 ocular reticle is placed into the eyepiece. With 40x magnification, 1 cm/40 = 0.25 mm (0.0625 mm^2). Five fields = 0.3125 mm^2 and 10 fields = 0.625 mm^2

Link to this reticle: https://bolioptics.com/microscope-eyepiece-reticle-net-grid-micrometer-10x10mm-100-squares-dia-22mm-1-5mm/

** MIB antibodies used in referenced manuscripts were of the same clone but sold by different vendors: Mouse monoclonal anti-Ki67 antibody (MIB-1; Dako Cytomation, Carpinteria, CA)[2] and Monoclonal antibody (MIB-1, code 0505, Immunotech, Marseille, France)[7]

F. In a study set of 389 benign and malignant melanocytic neoplasms from various locations, necrosis was negatively associated with survival for all sites (oral, feet and lip, skin).[5] While this study suggests that necrosis has prognostic significance, it is not an objectively measured parameter with no specific threshold values and it can be difficult to determine the cause of the necrosis (e.g. surface necrosis due to ulceration versus ischemia).[4,5] For these reasons, this parameter is not typically used to help predict prognosis of melanocytic neoplasms. (See Guideline 5, Necrosis).

G. Neoplastic cells at the epidermal-dermal junction, junctional activity (nests of neoplastic cells within the basal layer of the epidermis), intraepithelial nests (nests of neoplastic cells in the layers of the epidermis superficial to the basal layer) and lentiginous spread (lateral spread within the epidermis) have been investigated as prognostic parameters. Currently, there is not enough evidence in the published literature to support using these as prognostic parameters.[5] Nonetheless, these parameters should be recorded and further investigated for prognostic significance.

H. Complete margin evaluation, using both the radial method as well as tangential margin sections, may provide the most accurate assessment of complete excision. This allows for reporting of numerical margins in two planes via the radial method and assessment of the entire surgical margin surfaces for residual tumor cells via tangential sections. For melanocytic neoplasms, evaluation of the lateral flanking epithelium for

lentiginous spread is especially important for margin evaluation. The number of tangential sections for each specimen will vary greatly depending on the size of the tissue and decalcification may be needed for bony specimens such as jaws. For specimens such as a jaw, it is important to evaluate the tangential proximal margins as well as the tangential medial margins if a hemi-mandibulectomy is performed. Margin evaluation for complicated specimens, such as a jaw, is made easier when the clinician/surgeon inks the actual surgical margins. Owners need to be informed of approximate costs. (See Guideline 3, Margin).

I. Metastatic sites should be confirmed by histological evaluation. Imaging results suggestive of metastasis but not confirmed histologically should be reported as suspected metastases.

Future Considerations

Prognostic markers are approximately 85% correct for oral melanomas and hopefully can be improved further. Research on predictive markers is needed to extend quality of life of pets – see 5 below.

1. Precise designation of oral malignant melanoma or histologically well-differentiated melanocytic neoplasm of the mucous membranes of the lips and oral cavity (HWDM) with supporting criteria used for diagnosis available in materials and methods.

    a. Each evaluated element must be clearly defined.

    b. Melanocytic neoplasm can be used for a tumor which lacks characteristics for definitive diagnosis.

2. Additional examination of existing or additional prognostic markers with large case series, complete outcome data and prospective study designs.

    a. Correlate prognostication systems with accurate clinical outcomes for individual tumor types or groups of tumor types.

        i. Recommend use of autopsy data in 10 – 20% of study cases if possible.

        ii. Compare new parameters to previously established/validated prognostic parameters.

iii. Evaluate previously established parameters using the same methods as studies that established them.

iv. Evaluate previously established parameters with new methods (automated image analysis of MC and Ki67).

v. Compare new methods for labeling, enumeration or interpretation with parameters that can be determined with H&E stained sections.

b. Evaluate prognostic parameters in a population of dogs that receives a specific adjunct treatment and compare to a population of dogs that receives no additional therapy beyond surgical excision or a different type of adjunct therapy (potential predictive marker).

3. Further evaluation of MC

a. Enumerate MF in a defined area in mm^2 and compare different size areas and different regions of the tumor, determine if there is heterogeneity in hot spots; correlate each to outcomes.

b. Evaluate MC in regions other than the area of highest mitotic activity.

i. Examine the tumor periphery (invasive front).

c. Examine different sized areas for MC; (see Guideline 1, MC).

d. Correlate MC in different areas and different sized areas with recurrence, metastasis, disease free interval and survival metrics; see Outcomes Guideline 7.

e. Compare MC determined digitally with published manual methods for sensitivity, specificity, positive and negative predictive values for prognostic capabilities.

4. Evaluate Ki67 in regions other than the area of highest nuclear labeling.

a. Examine the tumor periphery (invasive front).

b. Examine different sized areas for Ki67; if grid is used detail how the area size was determined; compare Ki67 labelling in a defined area vs Ki67 index/100-500 cells.

c. Correlate Ki67 labeling in different areas of the tumors with recurrence, metastasis, disease free interval and survival metrics; see Outcomes Guideline 7

d. Compare Ki67 labeling determined digitally with published manual methods for sensitivity, specificity, positive and negative predictive values for prognostic capabilities.

e. Determine predictive MC thresholds; avoid single number thresholds (<#>); evaluate 3-tiered MC thresholds.

5. Genetic analysis of canine oral/lip melanocytic neoplasms

a. Identify genetic mutations associated with canine melanocytic neoplasms similar to Simpson et al.[1]

b. Further exploration of targeted therapies and predicted responses to therapeutic modalities is needed. Attempt to identify predictive markers.

6. Determine if necrosis of tumors is prognostic as reported previously,[5] use more objective methods such as AIA, CPATH.

References:

1. Simpson RM, Bastian BC, Michael HT, et al. Sporadic naturally occurring melanoma in dogs as a preclinical model for human melanoma. *Pigment cell & melanoma research*. 2014;27**:** 37-47.
2. Bergin I, Smedley R, Esplin D, Spangler W, Kiupel M. Prognostic evaluation of Ki67 threshold value in canine oral melanoma. *Veterinary pathology*. 2011;48**:** 41-53.
3. Esplin D. Survival of dogs following surgical excision of histologically well-differentiated melanocytic neoplasms of the mucous membranes of the lips and oral cavity. *Veterinary pathology*. 2008;45**:** 889-896.
4. Smedley RC, Spangler WL, Esplin DG, et al. Prognostic markers for canine melanocytic neoplasms: a comparative review of the literature and goals for future investigation. *Vet Pathol*. 2011;48**:** 54-72.
5. Spangler WL, Kass PH. The histologic and epidemiologic bases for prognostic considerations in canine melanocytic neoplasia. *Vet Pathol*. 2006;43**:** 136-149.
6. Vargas T, Pulz L, Ferro D, et al. Galectin-3 Expression Correlates with Post-surgical Survival in Canine Oral Melanomas. *Journal of comparative pathology*. 2019;173**:** 49-57.
7. Laprie C, Abadie J, Amardeilh MF, Net JL, Lagadic M, Delverdier M. MIB-1 immunoreactivity correlates with biologic behaviour in canine cutaneous melanoma. *Vet Dermatol*. 2001;12**:** 139-147.