Cutaneous Melanocytic Neoplasms - Canine
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Introduction

This protocol is intended for use with cutaneous melanocytic neoplasms inn dogs, including those arising from the haired skin side of the lip - the tumor can extend from the haired skin side to the mucosal side but must appear to arise from the haired skin side; eyelid and digit tumors are included here. This protocol is not intended for the following tumor types: Oral mucosal melanocytic neoplasms and lip mucosal melanocytic neoplasms see Protocol Melanoma, oral, canine.

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. This protocol is a “living” document which will be modified as new information becomes available.

See also Protocol 3: Melanoma, oral, canine

Histological Type (Note A)

_____Cutaneous malignant melanoma

_____Melanocytoma

_____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

_____Amputation (e.g. digit)

_____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

_____Digit

_____ Subungual

_____ NOT subungual

_____Eyelid

_____Upper

_____Lower

_____Haired skin side

_____Conjunctival side

_____Lip (haired skin side)

_____Other cutaneous site (specify location on body)

_____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 epidermis and measuring the greatest thickness of the tumor (Note C)

Additional dimensions: _____

Macroscopic Assessment of Pigmentation
_____ Present

____ ≥50% of the mass

____ <50% of the mass

_____ Absent

Histologic Assessment of Pigmentation
_____ ≥50% of cells pigmented

_____Diffuse heavy pigmentation – visualization of cellular morphology is histologically difficult

_____ <50% of cells pigmented

Histologic Microsatellite(s)
___ Not identified

___ Present

___ Cannot be determined (e.g. punch biopsy, margins dirty or too narrow, etc.)

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

_____ Superficial dermis (superficial dermal vascular plexus)

_____ Middle dermis (mid dermal vascular plexus)

_____Deep dermis (deep dermal vascular plexus)

_____Subcutis

_____Skeletal muscle

_____Cutaneous trunci

_____Regional muscle

_____ Bone

_____Other (e.g. cartilage if ear, etc.)_____

Deepest Tissue Layer Infiltrated: (Histologic assessment)

_____ Epidermis only

_____ Superficial dermis (superficial dermal vascular plexus)

_____ Middle dermis (mid dermal vascular plexus)

_____Deep dermis (deep dermal vascular plexus)

_____ Subcutis

_____ Skeletal muscle

_____Cutaneous trunci

_____Regional muscle

_____ Bone

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

Mitotic Count (per 2.37 mm^2)

(Note D) (Guidelines 1 and 2)

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 E) Assess in epithelioid predominant neoplasms and in spindloid neoplasms with sufficiently observable nuclear detail. Bleach if necessary. Count 100-200 cells at 400X

magnification and estimate the percentage of neoplastic cells with nuclear atypia using a threshold of 20%. Consult references for definitions of “typical” and “atypical” nuclei.[1-4]

____ <20% atypical nuclei

____ ≥20% atypical nuclei

Ki67

Determine as a percentage by counting the number of positively labeled melanocyte nuclei among 500 cells in the highest labeling area at 400x magnification. Avoid areas of ulceration and inflammation. (Note F)

____ % positive melanocyte nuclei

____<15%

____ ≥ 15%

Necrosis

(Estimate percent of tumor which is necrotic.) (Note G) (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 H)

_____ Present

_____ Absent

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

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

_____ 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 H)

_____ Present

_____ Absent

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

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

_____ Present

_____ Absent

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

Margins
(Note I) (See 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 (HTFM)

_____ 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
(see 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 J)

_____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 – brief explanation e.g. = not searched for

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)

Notes:

A. Tumor type should be designated as precisely as possible using stated or referenced criteria which can be duplicated in subsequent studies. Features of a cutaneous melanocytoma include: intact surface epidermis, forms a raised mass, small size(<2 cm diameter and ≤0.45 cm thickness), restricted to the dermis, abundant pigment, <20% atypical nuclei, low MC [<3/2.37mm^2], and low Ki67 index (<15%).[5,6,2,4] Features of a cutaneous malignant melanoma include one or more of the following: ulceration, low or no pigmentation, ≥20% atypical nuclei, high MC [≥3/2.37mm^2], high Ki67 index (≥15%), a thickness of >0.95 cm, invasion beyond the dermis, and vascular invasion.[5,2,4] Neoplasms often 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. Neoplasms with mixed features should be designated as *melanocytic neoplasms* and each parameter reported and discussed.[3] Ki67 index is the most predictive and objective marker to date.[3]

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. Finding neoplastic cells in a re-excision is problematic due multiple factors: number of sections evaluated; number of neoplastic cells; etc. Therefore, consider reporting if the re-excisional biopsy extended to normal tissue, indicating the entire lesion was removed. Also evaluate to determine if any neoplastic cells can be found in the material submitted (see Guideline 3).

C. Tumor thickness is measured for routine diagnostics by applying a ruler to the surface of a glass slide perpendicularly to the epidermis and measuring the largest thickness of the tumor.[2] A threshold of 0.95 cm thickness has been shown to be able to predict favorable and unfavorable (tumor-related death) clinical outcomes. [2] A threshold of 0.75 cm has been shown to predict the possibility of recurrence and metastasis. [2]

D. While MC is subjective, it still has shown statistical relevance in several canine cutaneous melanocytic neoplasm studies.[3] In one study, cutaneous MC threshold was established by counting mitoses in 10 random 400x fields (400x magnification),[6] but this may result in an underestimation of malignant neoplasms by not assessing “hot spots” and the area in mm^2 was not defined. Assessing MC in the area of highest mitotic activity may increase the identification of potentially malignant neoplasms and is recommended.[3] It is recommended the MC be

determined at 400x magnification in the most mitotically active area of the tumor in a 2.37 mm^2 field (see Guidelines 1 and 2) 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. A threshold of ≥3 mitoses in 10 hpf (ocular FN and area were not provided in the study) has been associated with more aggressive behavior and shorter survival times.[6,3] Neoplasms with an MC of < 3 generally exhibit benign behavior. This parameter has prognostic relevance but, like any parameter, it should not be used as a standalone parameter. On multivariate analysis both mitotic index (count) and Ki67 index were significantly correlated with survival.[6] Authors commented that future studies should count mitotic figures in areas of high proliferative activity. MC, histologic features (nuclear atypia, differentiation) and KI67 index were all correlated with survival.[6]

E. In order to decrease interobserver variation, nuclear atypia should be assessed according to the criteria outlined by Spangler and Kass (2006).[4] 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*.[4] 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.[4] Count 100-200 neoplastic cells and report the % using 20% as a threshold. Statistical significance for this parameter and for the

threshold of 20% atypical nuclei has been demonstrated in multiple studies.[2,4] This parameter should not be used as a standalone parameter.

F. While avoiding areas of ulceration and inflammation, the region of highest nuclear labeling for Ki67* should be determined at a low magnification (e.g. 100X magnification). 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. When this region has been identified, the number of melanocyte nuclei with positive labeling for Ki67 can be counted among 500 cells at 400X magnification. A $1cm^2$ grid reticle can aid with counting. Positively labeled nuclei include those with a range of weak to strong detectable antigen throughout the nucleus as well as those with labeling only in the nucleolus. If needed, bleach should be applied after immunohistochemical labeling in order to facilitate visualization of nuclei in heavily pigmented neoplasms; however, when red chromagen detection is used bleaching is generally not necessary. In contrast to counting mitotic figures, the area does not need to be standardized, as the region for counting is determined by the numbers of cells. A threshold value of 15% has been empirically determined and has been evaluated in regard to survival.[5,6] The percentage of correctly classified neoplasms using the Ki67 index (97%) was higher than that of MC (91%) and histological criteria (93%) in one study.[6] On multivariate analysis Ki67 index and mitotic index (count) were both statistically significant regarding survival. Authors recommended the MIB-1 antibody be used by other investigators.[1,6,7]

* The antibody used to detect Ki67 (MIB-1) in referenced manuscripts was of the same clone but sold by different vendors: Mouse monoclonal anti-Ki67 antibody (MIB-1; Dako Cytomation, Carpinteria, CA)[1] and Monoclonal antibody (MIB-1, code 0505, Immunotech, Marseille, France)[6]

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

G. 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).[4] 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).[3] For these reasons, this parameter is not typically used to predict prognosis of melanocytic neoplasms and future studies should determine its utility.(See Guideline 5, Necrosis)

H. 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.[3] Nonetheless, these parameters should be recorded and further investigated for prognostic significance.

I. Complete margin evaluation, using both the radial method as well as tangential margin sections, should optimize 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.[8] For melanocytic neoplasms, evaluation of the lateral flanking epidermis for lentiginous spread is especially important for margin evaluation. It is important to evaluate the tangential proximal margin for tissues such as digits. 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 digits. Owners need to be informed of approximate costs. (See Guideline 3, Margins)

J. 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

1. Precise designation of cutaneous malignant melanoma or melanocytoma 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 parameters 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.

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.

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

4. Develop more objective assessment of nuclear pleomorphism; compare results by counting different numbers of nuclei (e.g. 100 cells versus 200 cells versus other); consider quantifying in defined area in mm^2; consider enumeration with AIA, CPATH.

5. Standardize the methodology for Ki67; compare to standardized PHH3; if molecular proliferation indices are available consider PAM50.

6. Determine Ki67 index for cutaneous melanocytic neoplasms using the grid method for oral and lip melanocytic neoplasms.[1]

a. Compare this method with percentage method[6]

b. Determine the threshold value by Receiver Operator Curve (ROC) analysis using the grid method.

7. 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.

c. Correlate Ki67 labeling in different areas and different sized areas with recurrence, disease free interval and survival times.

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

8. Genetic analysis of canine cutaneous melanocytic neoplasms

a. Identify genetic mutations associated with canine melanocytic neoplasms similar to Simpson et al.[9]

b. Further exploration of targeted therapies and predicted responses to therapeutic modalities.

9. Determine if necrosis of tumors is prognostic as reported previously,[4] use more objective methods such as AIA, CPATH.

References:

1. 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.
2. Silvestri S, Porcellato I, Mechelli L, Menchetti L, Rapastella S, Brachelente C. Tumor Thickness and Modified Clark Level in Canine Cutaneous Melanocytic Tumors. *Vet Pathol*. 2019;56**:** 180-188.
3. 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.
4. Spangler WL, Kass PH. The histologic and epidemiologic bases for prognostic considerations in canine melanocytic neoplasia. *Vet Pathol*. 2006;43**:** 136-149.
5. Lacroux C, Raymond-Letron I, Bourges-Abella N, et al. Study of canine cutaneous melanocytic tumours: Evaluation of histological and immunohistochemical prognostic criteria in 65 cases. *Revue de Medecine Veterinaire*. 2012;163**:** 393.
6. 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.
7. Smedley RC, Lamoureux J, Sledge DG, Kiupel M. Immunohistochemical diagnosis of canine oral amelanotic melanocytic neoplasms. *Vet Pathol*. 2011;48**:** 32-40.
8. Kamstock D, Ehrhart E, Getzy D, et al. Recommended guidelines for submission, trimming, margin evaluation, and reporting of tumor biopsy specimens in veterinary surgical pathology. *Veterinary Pathology*. 2011;48**:** 19-31.
9. 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.