VCS/ACVP Oncology-Pathology Working Group
Summary and Subgroup Recommendation for
Prognostic and Predictive Significance of KIT Protein Expression and *c-kit* Gene Mutation

Species/Tumor: CANINE CUTANEOUS MAST CELL TUMOR SUBGROUP

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Overall Summary / Recommendations:
Based on review of the literature, the ***Canine Cutaneous Mast Cell Tumor*** subgroup has concluded and recommends the following regarding *the prognostic and predictive value of c-kit mutation analysis and KIT protein localization* in ***canine cutaneous mast cell tumors.***

Introduction

Mast cell tumors (MCT) are the most common malignant cutaneous tumors in the dog, accounting for between 16 and 21% of all cutaneous tumors.[1,2] While a majority of canine MCT can be effectively treated with local therapy (surgery +/- radiation therapy), a subset of tumors can be associated with a high risk of metastasis and accordingly short overall survival times. Furthermore, locally recurrent, large or infiltrative tumors, and those in locations not amenable to wide surgical excision represent a therapeutic challenge. Histologic appearance remains one of the mainstays for determining likely biologic behavior;[3,4] however, a subset of tumors may behave aggressively despite an unremarkable histologic appearance. Additional tests, to better predict potential biological behavior and clinical outcome and identify patients that may benefit from adjuvant medical therapy, would be useful. Furthermore, given the recent expansion of medical options for canine MCT treatment, including a variety of cytotoxic agents as well as small-molecule tyrosine kinase inhibitors (TKIs), information that could aid in predicting drug response, and aid in the selection of effective treatment, would be likewise helpful.

Mutations in the *c-kit* gene have been identified in approximately 15% of canine cutaneous MCT, with an increased incidence up to 35% in higher grade MCT. Internal tandem duplications (ITD) in exon 11 are the most commonly characterized *c-kit* gene mutations in MCT,[5-7] but lower numbers of deletion mutations have been identified in exon 11, and ITDs and substitutions have been identified in exons 8 and 9. Rare mutations have been identified in exon 17.[5] To date, all mutations that have been characterized *in vitro* have been shown to result in constitutive autophosphorylation of the KIT protein in the absence of its ligand, stem cell factor (SCF).[5] Additionally, TKIs targeting KIT have been shown to inhibit phosphorylation of these mutated proteins, except for those resulting from exon 17 mutations. Furthermore, recent studies have demonstrated that a proportion of canine MCT may demonstrate aberrant subcellular localization of the KIT protein when assessed via immunohistochemistry (IHC), and that this aberrant localization may correlate with the presence of *c-kit* gene mutation as well as outcome.

Multiple studies have sought to evaluate the significance of the presence of activating *c-kit* mutations and/or aberrant KIT protein localization on postsurgical outcome in dogs with MCT. Furthermore, some studies have started to evaluate whether *c-kit* mutation status may affect response to therapy, specifically with TKIs. The goal of this review is to summarize the available data regarding the utility of *c-kit* gene mutation and KIT protein localization as prognostic and predictive tests for canine MCT.

Prognostic value of* c-kit *gene mutation

Most studies evaluating the association between *c-kit* gene mutations and prognosis have been focused on ITD mutations in exon 11. Zemke et al. originally found increased ITDs and deletions in higher histologic grade MCTs,[8] and Downing et al. found that 35% of grade II and grade III MCTs had ITD mutations, compared to only 8% of grade I tumors.[9] In Downing's study, MCT with ITD mutations were twice as likely to recur or develop metastases. These results were not statistically significant, but might be influenced by the relatively small numbers of low grade tumors included in this study.[9] Additionally, Webster et al. found that dogs whose MCTs possessed ITD mutations had significantly decreased overall survival times and an increased incidence of MCT-related death and recurrence when treated with surgery, and when treated with multimodality therapy.[10,11] In a subsequent study, this group found that mutations were associated with increased cellular proliferation indices.[12] Anecdotally, in a prospective study evaluating the TKI masitinib in dogs with measurable MCT, patients in the placebo arm of this study with *c-kit* gene mutations experienced shorter times to progression and overall survival times compared to patients without mutations (42 d vs 98 d and 182 d vs median not reached, respectively), although this was not evaluated statistically.[13] However, in a study by Giantin et al, only 1 of 5 patients with *c-kit* mutations had recurrent disease and MCT-related mortality.[14]

Given its documented strong association with both histologic grade and proliferation indices,[8,9,12] it is not clear whether the presence of a *c-kit* gene mutation represents an independent prognostic factor, or whether it may largely correlate with histologic grade / proliferation rate, which can be assessed more simply and inexpensively. However, mutation presence/absence is a considerably more objective measurement than either histologic grade or proliferation indices, both of which can be associated with considerable observer bias. Thus, this objective measure could still provide important objective information useful in decision-making.

This body of data suggests that patients with *c-kit* gene mutations are more likely to have more aggressive disease as measured by increased recurrence rates and decreased survival times. However, the presence of a *c-kit* mutation has not been definitively validated as an **independent** prognostic factor, when taking into account known factors such as histologic grade and proliferation rate.

Prognostic Value of KIT protein localization

Regeura et al. first described variations in KIT expression in canine MCT by IHC. In this study, it was noted that Patnaik grade I MCTs had weak KIT labeling scattered in the cytoplasm or on the membrane, while grade II and III tumors tended to have increased cytoplasmic labeling.[15] Evaluations of KIT localization and prognosis have produced variable results. In three studies, patients with focal or diffuse cytoplasmic KIT expression had a worse postsurgical prognosis, either in terms of recurrence and/or survival, compared to MCTs with peri-membrane labeling.[10,14,16] Although cytoplasmic KIT localization was associated with a worse prognosis in these studies, it had low positive predictive value, suggesting that membrane localization was fairly predictive of a good prognosis, but cytoplasmic KIT expression could not clearly delineate aggressive disease.

In contrast to the studies described above, Costa Casagrande et al. found no association with KIT staining pattern and histologic grade or survival measures,[17] and Preziosi et al. found that focal peri-nuclear labeling was associated with a worse prognosis than diffuse cytoplasmic labeling, but too few patients with membranous labeling were evaluated to comment on associations with survival.[18] A concern with the varying results of the last 2 studies is that the IHC labeling procedure can influence the interpretation of KIT protein localization. Specifically, over-developing the IHC reaction can result in increased cytoplasmic background in MCTs, so it appears as weak, diffuse cytoplasmic labeling. This is especially noteworthy in Preziosi's study where the image of diffuse cytoplasmic labeling demonstrates strong membrane labeling with weaker cytoplasmic labeling. Therefore, some membrane localizing tumors may be misclassified as diffuse cytoplasmic labeling. A potential way to control for this would be to include a tissue section with normal mast cells. These cells should have KIT restricted to the plasma membrane and therefore would serve as a positive control that the reaction was performed appropriately.

As above regarding *c-kit* gene mutation, given the potential correlation between KIT protein localization and other validated prognostic factors (grade, proliferation),[12,14,15] it is similarly unclear whether KIT localization represents an **independent** prognostic factor, when taking into account these other features.

This body of literature suggests that increased cytoplasmic KIT localization is associated with worse prognosis as measured by recurrence and survival, but it has a low positive predictive value and therefore should not be used alone. More power likely lies in using membrane localization to rule out potentially aggressive tumors.

Value of* c-kit *mutation status and KIT localization in predicting response to therapy

The potential predictive value of *c-kit* mutation status in predicting outcome following treatment with the TKIs toceranib phosphate and masitinib has been evaluated to some degree in the 2 published registration trials for these respective agents. In the toceranib registration study as well as in preliminary investigations, patients with *c-kit*

gene mutations had objective response rates twice as high as those without mutations (60% vs 30%), although effect on long-term outcome (progression free interval, overall survival) was not reported.[19,20] In the masitinib registration study, a significant difference in outcome between masitinib and placebo arms was observed only in the patients with *c-kit* mutations. Additionally, patients in the masitinib arm of this study with *c-kit* mutations appeared to have longer times to progression (230 d vs 83 d) and higher overall response rates at 6 months (20 vs 10%), although these differences were not evaluated statistically.[13] A similar observation was made in a small number of dogs treated with the KIT TKI imatinib; dogs with *c-kit* exon 11 ITDs were numerically more likely to experience objective responses to imatinib, although long-term outcomes were not reported.[21]

Interestingly, patients with *c-kit* mutations had significantly **decreased** progression free survival times compared to those without *c-kit* mutations in a single arm study of toceranib and hypofractionated radiation therapy, although this was evaluated in a small number of patients.[22] This suggests that single agent responses may be different than combination therapeutic responses in terms of their associations with mutation status, and therefore may need to be evaluated individually.

It is noteworthy that patient subsets without *c-kit* mutations have been demonstrated to respond to TKI therapy.[13,19,20] This may be due to non-mutational activation of KIT (e.g. autocrine or paracrine signaling, amplifications), due to the presence of activating mutations not screened for as part of testing, or due to inhibition of other tyrosine kinases (e.g. PDGFR, VEGFR2), as these drugs are not 100% selective for KIT.

Most importantly, the presence of *c-kit* gene mutations appears to be able to predict a subset of patients that will be more likely to respond to TKIs, although these initial responses may or may not translate into improved long-term outcomes and these observations may not translate to multi-modality therapy, e.g. when TKIs are combined with surgery, radiation therapy or traditional cytotoxic chemotherapy. Although a subset of patients without mutations will also respond, this additional information can be valuable in considering potential costs and therapeutic benefits.

No studies have evaluated the association between KIT localization and response to TKIs. Such a study could be of enormous value to better define the utility of KIT localization in the diagnostic setting.

Conclusions

In summary, no prognostic marker can be considered to have 100% positive and negative predictive values. Instead, all prognostic markers can provide varying levels of risk assessment or hazard ratios. Co-morbidities, disease heterogeneity, multigenic influences, variations in tolerance of adverse effects and ability to treat will all impact the clinical course of a given patient. Therefore, the greatest prognostic benefit will likely stem from using multiple prognostic markers in concert. *c-kit* gene mutation analysis and KIT localization may be most be informative in histologically "ambiguous" tumors. *c-kit* gene mutations may be most informative in the identification of tumors that are histologically low grade, but are likely to be biologically aggressive, while membrane KIT localization is most likely to identify tumors that are **not** likely to have progressive disease. The largest value of *c-kit* mutation assessment may lie in the ability to determine which patients are likely to most benefit from single agent TKIs, especially in those cases where "neoadjuvant" medical cytoreduction may facilitate surgical excision.

Future Directions / Additional Studies

Multiple unanswered questions remain. What is the incidence of *c-kit* gene mutation and/or aberrant KIT protein localization specifically in "low risk" (e.g. low-mitotic Patnaik grade I/II, 2-tier Grade I) tumors, and does the presence of a *c-kit* mutation and/or aberrant KIT localization have an effect on outcome in these "low risk" patients? Are *c-kit* mutations and KIT localization **independent** prognostic factors when taking into account known factors such as histologic grade and proliferation index? Do these same factors carry prognostic significance in patients treated with chemotherapy or radiation therapy?

Further investigations should be conducted to better determine if there are differences in the prognostic significance of mutations located in various exons, since most work to date has focused on exon 11.

The predictive value of KIT IHC in assessing TKI response remains to be evaluated, which would be best in a prospective study.

Additionally, a multi-center prospective study to evaluate histologic grade, mitotic index, proliferation markers, *c-kit* mutations, and KIT localization in concert on postsurgical outcome is greatly needed; not only to determine how these can be used in concert, but to also standardize criteria for these assessments. The OPWG would be an optimal forum to facilitate the organization of such a multi-center study, since it will require integration of pathologists, oncologists, surgeons and molecular biologists.

Literature Reviewed (list and provide subgroup summary for each individual paper)

1. Costa Casagrande, T. A., L. M. de Oliveira Barros, et al. (2013). "The value of molecular expression of KIT and KIT ligand analysed using real-time polymerase chain reaction and immunohistochemistry as a prognostic indicator for canine cutaneous mast cell tumours." Vet Comp Oncol.

Study Objective – *Evaluate the KIT protein (IHC) and c-kit mRNA (RT-PCR) expression in canine MCT and their correlation with tumor recurrence and tumor-related death.*

Study Design (e.g. retrospective, prospective, other) – *Retrospective*
Materials & Methods - *81 dogs with a cytologic diagnosis of MCT, 1 year post-surgical follow-up, histologic confirmation. KIT IHC was performed and evaluated for membrane, focal or stippled cytoplasmic, or diffuse cytoplasmic labeling. c-kit and SCF mRNA expression was evaluated by RT-PCR. Log rank and multivariate Cox regression was used for statistics, but variables included in the multivariate model were not listed.*

Conclusions Drawn – *KIT IHC patterns were not associated with relapse-free survival or histologic grade. scf mRNA expression was correlated with relapse-free survival, but c-kit mRNA expression was not. Tumor-related death was most common in patients with peri-membrane KIT expression, but statistics were not performed.*

Statistical Soundness – *Mulitvariable analysis, but confounders that were controlled for were not included in the study.*

SUBGROUP CONCLUSIONS: *This study contrasts other studies in the distribution of KIT IHC between histologic grades and the increased incidence of tumor-related mortality in patients with peri-membrane KIT localization. However, taken alone, this study does not support an association between KIT protein localization and prognosis.*

2. Downing S et al. (2002). Prevalence and importance of internal tandem duplications in exons 11 and 12 ogf c-kit in mast cell tumors of dogs. Am J Vet Res 63 (12):1718-23.

Objective – *To determine the prevalence of c-kit ITD mutations in exons 11 and 12 and their correlation with prognosis*.

Study design (e.g. retrospective, prospective, other) - *Retrospective*

Materials & Methods - *157 dogs; 12 grade 1, 119 grade 2, 26 grade 3 tumors. No control for treatment. PCR detection of exon 11 and 12 mutations. Logistic regression for associations with tumor grade, local recurrence, metastases, and overall survival.*

Conclusions drawn – *Grade 2 and 3 MCTs were 5x more likely to have an ITD mutation than Grade 1 tumors. MCTs with ITD mutations were twice as likely to result in metastasis and were twice as likely to recur, although these trends were not statistically significant.*

Statistical soundness – *Yes*

SUBGROUP CONCLUSIONS: *There appears to be a trend for increased mutations in higher grade tumors. Metastatic and recurrent disease appears to be more frequent in patients with ITD mutations. However, these trends were not statistically confirmed. The lack of statistical associations could be due to the lower number of grade I tumors included in this study, which could under-power the study.*

3. Giantin, M., M. Vascellari, et al. (2012). "c-KIT messenger RNA and protein expression and mutations in canine cutaneous mast cell tumors: correlations with post-surgical prognosis." J Vet Diagn Invest 24(1): 116-126.

Objective – *To evaluate correlations between c-kit mRNA expression with tumor grade, KIT IHC patterns, prognosis, and c-kit mutations in canine MCT.*

Study design (e.g. retrospective, prospective, other) - *Retrospective*

Materials & Methods - *60 MCTs with histologic confirmation. Quantitative reverse transcriptase PCR of total RNA isolated from the center and margins of tumors. KIT IHC with*

scoring based on the 2004 paper by Webster et al., PCR amplification of exons 8, 9, and 11 and sequencing.

Conclusions drawn – *Diffuse cytoplasmic KIT labeling was significantly associated with high histologic grade tumors. Peri-membrane localization was a good predictor of good prognosis, but increased cytoplasmic KIT did not necessarily indicate poor prognosis. However, KIT pattern III was associated with significantly decreased survival. c-kit mRNA was increased in MCTs, but this was not significantly associated with prognosis or survival.*

Only 5 c-kit mutations were identified and these were more common in low histologic grade tumors. Only 1 patient with mutant c-kit had recurrence or death, and this patient had diffuse cytoplasmic labeling.

Statistical soundness –*Multivariable analysis was not performed.*

SUBGROUP CONCLUSIONS: *KIT IHC patterns are correlated with histologic grade. KIT pattern I is a better predictor of good prognosis. KIT pattern III is associated with decreased survival, but it has relatively low predictive value. This study found no associations between c-kit mutations and survival. Although c-kit mRNA is increased in MCT patients compared to normal skin, it appears to have no prognostic value.*

4. Isotani M et al. (2008). Effect of tyrosine kinase inhibition by imatinib mesylate on mast cell tumors in dogs. J Vet Intern Med. 22 (4):985-88.

Objective – *To evaluate the response of canine MCT to imatinib, and correlate response with presence of absence of c-kit exon 11 ITD*

Study design (e.g. retrospective, prospective, other) – *Retrospective study (21 dogs)*

Materials & Methods - *Presence or absence of Exon 11 ITD was determined using standard PCR methods. Dogs were treated with imatinib and responses assessed using RECIST criteria.*

Conclusions drawn – *Dogs with c-kit exon 11 ITDs had higher response rates than dogs without ITDs (100% vs 31%), suggesting that responses were more likely in c-kit mutants, but not precluded in MCT without mutations.*

Statistical soundness – *No statistical analysis was performed.* ***Although the Authors state that "response could not be predicted based on presence or absence of a… mutation", post-hoc comparison of objective response rates in mutant vs non-mutant cases by one of the Subgroup members demonstrates a statistically significant difference using a 2-tailed Fisher's exact test.***

SUBGROUP CONCLUSIONS: *These results suggest that presence of an exon 11 ITD may be predictive of initial response to imatinib, but absence of an ITD does not preclude response. Shortcomings include the lack of long-term follow-up (many dogs had treatment discontinued*

after a few weeks even if they were responding), concurrent use of prednisone in some patients, and a lack of evaluation of other known activation mutations.

5. Kiupel, M., J. D. Webster, et al. (2004). "The use of KIT and tryptase expression patterns as prognostic tools for canine cutaneous mast cell tumors." Vet Pathol 41(4): 371-377.

Objective – *To evaluate the prognostic significance of KIT and tryptase expression patterns in canine cutaneous MCTs*

Study design (e.g. retrospective, prospective, other) – *Retrospective study – 100 dogs treated with surgery only.*

Materials & Methods - *KIT and tryptase staining patterns were identified using immunohistochemistry. Follow-up was obtained on all patients and disease-free and overall survival times calculated.*

Conclusions drawn – *Increased cytoplasmic KIT staining was significantly associated with an increased rate of local recurrence and a decreased survival rate. No information was provided regarding any correlation between KIT staining pattern and histologic grade.*

Statistical soundness – *Statistical analysis is appropriate, with the exception that histologic grade was not reported and incorporated into the multivariate model.*

SUBGROUP CONCLUSIONS: *KIT staining pattern II and III (cytoplasmic) correlated with a higher risk of local recurrence and death. However, relatively few dogs, even those with aberrant KIT localization, developed treatment failure or MCT-related death. Limitations include the fact that no information was provided regarding any correlation between KIT staining pattern and histologic grade.*

6. Letard S et al (2008). Gain-of-function mutations in the extracellular domain of KIT are common in canine mast cell tumors. Mol Cancer Res 6 (7):1137-45.

Objective – *Evaluate the type and frequency of c-kit mutations in canine MCTs*

Study design (e.g. retrospective, prospective, other) – *Descriptive laboratory*

Materials & Methods - *Sequencing c-kit cDNA from exons 8-13 and 17-19 from 191 dogs with grade 2 or 3 tumors that were recurrent post-surgery or non-resectable. Expression of wild-type and mutant KIT proteins in Ba/F3 cells to assess autophosphorylation, proliferation, and response to the tyrosine kinase inhibitor AG1296.*

Conclusions drawn – *50/191 MCTs (26.2%) had mutations. 32/50 (64%) exon 11 with 78% being ITDs and the remaining being deletions or in-frame mutations. 9/50 (18%) in exon 8, 8/50 (16%) in exon 9 and 1 mutation in exon 17. Two ITDs, 1 exon 11 deletion, and 1 exon 9*

point mutant proteins were expressed in Ba/F3 and all resulted in ligand independent phosphorylation and increased proliferation in the absence of ligand. These responses were sensitive to tyrosine kinase inhibitors. However, the exon 17 mutation was not responsive to tyrosine kinase inhibitors (supplemental material).

Statistical soundness – *N/A (not applicable)*

SUBGROUP CONCLUSIONS: *This study found a 26% mutation incidence in histologic grade 2 and 3 MCTs that were recurrent or non-resectable; this might suggest a lower incidence if grade 1 and non-recurrent grade 2 and 3 MCTs are considered. Mutations occur most commonly in exon 11, as previously reported, but mutations were also identified in exons 8, 9, and 17. All mutations that were characterized including exon 11 and 9 mutations resulted in KIT autophosphorylation and increased proliferation in absence of ligand. Exon 11 and 9 mutant proteins were inhibited by TKIs, but the exon 17 mutant protein was not, similar to what is seen in human patients.*

7. London CA et al. (1999). Spontaneous canine mast cell tumors express tandem duplications in the proto-oncogene c-kit. Exp Hematol. 27 (4): 689-697.

Objective – *To evaluate canine MCT samples for the presence of c-kit mutations*

Study design (e.g. retrospective, prospective, other) – *Descriptive laboratory*

Materials & Methods - *RT-PCR c-kit and scf genes with sequencing*

Conclusions drawn – *No kinase domain mutations similar to what is seen in human mastocytoma patients were observed. Tandem duplication mutations were identified in 5/11 tumors. Mutant KIT is autophosphorylated in the absence of ligand, suggesting constitutive activity.*

Statistical soundness – *N/A*

SUBGROUP CONCLUSIONS: *This is one the first studies identifying c-kit tandem duplications in canine MCT and demonstrates that these mutations result in an autophosphorylated, constitutively activated protein.*

8. Ma Y, et al. (1999). Clustering of activating mutations in c-kit's juxtamembrane coding region in canine mast cell neoplasms. J Invest Dermatol. 112 (2) 165-70.

Objective – *Determine if c-kit juxtamembrane domain mutations occur in canine MCTs*

Study design (e.g. retrospective, prospective, other) *Descriptive laboratory*

Materials & Methods - *cDNA sequencing of 7 canine MCTs and 3 MCT cell lines originally from 2 dogs. Evaluation of protein phosphorylation in human constructs with the identified canine c-kit mutations. Modeling of the kinase domains.*

Conclusions drawn – *Identification of deletions and point mutations in the juxtamembrane domain of c-kit from patient tumor samples and an ITD mutation in a cell line. All mutations resulted in constitutive phosphorylation in the absence of ligand.*

Statistical soundness – *N/A*

SUBGROUP CONCLUSIONS: *This study further demonstrates deletions, point mutations, and ITDs in the juxtamembrane domain of c-kit that result in autophosphorylation of KIT in canine MCTs. This suggests that not only do c-kit mutations occur in canine MCTs, but they also result in a constitutively active product.*

9. Preziosi R, Marini M et al (2004). Expression of the KIT protein (CD117) in primary cutaneous mast cell tumors of the dog. J Vet Diagn Invest 16: 554-561.

Objective – *To compare KIT IHC labeling patterns with histologic grade, histologic features of cellular differentiation and nuclear grade, and evaluate its predictive role in terms of survival.*

Study design (e.g. retrospective, prospective, other) - *Retrospective*

Materials & Methods - *31 dogs, with survival data on 28 dogs. Survival data captured as cancer-free interval and survival time. Patnaik histologic scoring. KIT IHC and scoring based on membrane, focal cytoplasmic or diffuse cytoplasmic labeling. Kaplan-Meier survival curves.*

Conclusions drawn – *Grade 3 MCTs were associated with focal cytoplasmic labeling, grade 2 tumors had membranous or membranous and peri-nuclear labeling, Grade 1 tumors had diffuse cytoplasmic or membranous and diffuse cytoplasmic. Peri-nuclear labeling was associated with decreased survival time compared to diffuse cytoplasmic labeling.*

Statistical soundness – *Low sample size and lack of multivariable analyses to control for confounders.*

SUBGROUP CONCLUSIONS: *This KIT labeling patterns were different than those reported in other studies. Specifically, diffuse cytoplasmic labeling was identified in most Grade 1 MCTs. This is challenging because most normal mast cells have the protein localized to the membrane, as this is a transmembrane protein. One potential issue is that over-development of an IHC assay can result in membrane labeling appearing as cytoplasmic labeling due to saturation of the signal. Overall, these results contradict most other studies, making the interpretation difficult.*

10. Regeura MJ, et al. (2000) Canine mast cell tumors express stem cell factor receptor. Am J Dermatopathol 22 (1):49-54.

Objective – *Characterize the expression of KIT on canine cutaneous MCTs by immunohistochemistry.*

Study design (e.g. retrospective, prospective, other) – *Descriptive laboratory*

Materials & Methods - *KIT IHC on 23 MCTs, 10 histiocytomas, and 5 melanomas. Correlate pattern of KIT expression with histologic grade.*

Conclusions drawn – *Normal mast cells: KIT is expressed weakly on the membrane. All MCTs express KIT. Grade 1 MCTs had weak KIT expression on the membrane or scattered in the cytoplasm. Most grade 2 MCTs had peri-nuclear KIT localization with fewer having membrane localization. Grade 3 MCTs had variable KIT expression including diffuse cytoplasmic or polarized cytoplasmic labeling.*

Statistical soundness – N/A

SUBGROUP CONCLUSIONS: *KIT is expressed in all MCTs evaluated. Normal mast cells and grade 1 MCTs have weak KIT expression, often at the cell membrane. Grade 2 and 3 MCTs have increased KIT expression, often with peri-nuclear localization in the cytoplasm. The results are only descriptive, but suggest KIT expression and cellular localization may be associated with histologic grade and therefore, possibly also survival.*

11. Riva, F, Brizzola S, et al (2005). A study of mutation in the c-kit gene in 32 dogs with mastocytoma. J Vet Diagn Invest 17: 385-388.

Objective – *Evaluate c-kit exons 9-13 for mutations by sequencing in 32 canine MCTs.*

Study design (e.g. retrospective, prospective, other) - *descriptive laboratory*

Materials & Methods - *Sequence exons 9-12 of c-kit cDNA from 32 canine MCTs. 11 Grade 1, 14 Grade 2, and 5 Grade 3 tumors.*

Conclusions drawn – *Identified a 6 bp deletion in exon 11 and a point mutation. The reported point mutation is actually a SNP described by Zemke et al. (Vet Pathol 2002;39:529) and results in no amino acid change.*

Statistical soundness – *N/A*

SUBGROUP CONCLUSIONS: *This manuscript offers minimal additional information since it only describes one deletion that is in a previously characterized region known to have deletions in MCTs.*

12. Thompson JJ, Yager JA, et al. (2011) Canine subcutaneous mast cell tumors: cellular proliferation and kit expression as prognostic indices. Vet Pathol 48(1): 156-168.

Objective – *Evaluate the prognostic value of cellular proliferation markers and KIT IHC patterns for canine subcutaneous MCTs.*

Study design (e.g. retrospective, prospective, other) – *Retrospective case-control study*

Materials & Methods - *24 dogs with local recurrence vs. 24 dogs without local recurrence. 12 dogs with metastases vs. 12 dogs without metastases. Metastases were confirmed by FNA, radiography, lymph node biopsy (histology), buffy coat, laparotomy, ultrasound, or MRI. Evaluation of mitotic index, AgNOR staining, Ki-67 immunolabeling, KIT IHC pattern, and c-kit mutations. Only exon 11 was sequenced.*

Conclusions drawn – *Diffuse cytoplasmic, or any cytoplasmic, KIT labeling was associated with an increased risk of local recurrence and metastases compared to membrane labeling. c-kit mutations were not identified in this study.*

Statistical soundness – *Univariable statistics only.*

SUBGROUP CONCLUSIONS: *No c-kit mutations were identified in subcutaneous MCTs; however, only exon 11 was evaluated in this study. Cytoplasmic KIT labeling has an increased risk of local recurrence and metastasis compared to peri-membrane KIT labeling. KIT localization was more sensitive for survival, but less sensitive for local recurrence or metastases than mitotic index. Therefore, these different indices/ prognostic markers may provide unique, but complementary information regarding clinical outcome.*

13. Webster JD et al (2008). Evaluation of prognostic markers for canine mast cell tumors treated with vinblastine and prednisone. BMC Vet Res Aug 13 (4); 32.

Objective – *To determine whether c-kit mutation or KIT localization correlated with outcome in dogs with MCT treated with surgery (+/- RT) and prednisone/vinblastine chemotherapy*

Study design (e.g. retrospective, prospective, other) – *Retrospective study*

Materials & Methods - *Exon 11 ITDs were assessed using PCR. KIT localization was assessed using IHC.*

Conclusions drawn – *26 of 28 studied dogs had aberrant KIT localization. KIT staining pattern (Pattern 2 vs 3) and presence of c-kit ITD correlated with reduced DFI and OS in dogs treated with surgery (+/- RT) and chemotherapy. All ITDs were present in dogs with Patnaik grade 3 tumors. Histologic grade retained statistical significance upon multivariable analysis.*

Statistical soundness – *Appropriate statistics were performed.*

SUBGROUP CONCLUSIONS: *Both c-kit ITD and KIT Pattern 3 correlate with reduced disease-free and overall survival following multimodality therapy in dogs with MCT. These are independent prognostic factors that complement traditional histologic grading. Although there were only 2 dogs with KIT IHC pattern 1, both of these dogs were long-term survivors.*

14. Webster JD, Yuzbasiyan-Gurkan V, et al (2006). The role of c-kit in tumorogenesis: Evaluation in canine cutaneous mast cell tumors. Neoplasia 8 (2): 104-111.

Objective – *To define the prognostic significance of c-kit mutations identified in canine MCTs and the associations between c-kit mutations, KIT localization, and KIT expression levels.*

Study design (e.g. retrospective, prospective, other) – *Retrospective study*

Materials & Methods - *Microdissection and PCR were performed on 60 MCTs to identify c-kit Exon 11 ITD mutations. Anti-KIT antibodies were used for IHC evaluation of KIT localization. Forty-two MCTs were included in a tissue microarray, and KIT expression was quantified using immunofluorescence.*

Conclusions drawn – *c-kit mutations were significantly associated with an increased incidence of local and distant recurrence and death. c-kit mutations were also significantly associated with aberrant protein localization, although many dogs with aberrant protein localization did not have c-kit ITDs.*

Statistical soundness – *Univariate and multivariate statistics performed appropriately.*

SUBGROUP CONCLUSIONS: *The methods in this paper are sufficiently rigorous to allow for the conclusion that the presence of an exon 11 ITD is a predictor of poorer survival in dogs treated with surgery alone. This study could be used as a basis of clinical decision making.*

15. Webster, J. D., V. Yuzbasiyan-Gurkan, et al. (2007). "Cellular proliferation in canine cutaneous mast cell tumors: associations with c-KIT and its role in prognostication." Vet Pathol 44(3): 298-308.

Objective – *To evaluate the associations between cellular proliferation and c-kit mutations and between cellular proliferation and aberrant KIT protein localization in canine MCTs.*

Study design (e.g. retrospective, prospective, other) - *Retrospective*

Materials & Methods - *Proliferation was assessed via IHC staining for AgNOR, PCNA and Ki-67. c-kit ITD presence was assessed using PCR, and KIT localization was assessed using IHC.*

Conclusions drawn – *Aberrant KIT protein localization or c-kit ITDs are associated with increased cellular proliferation.*

Statistical soundness – *Both univariable and multivariable correlation and survival analyses were performed appropriately. Impact of KIT localization / c-kit mutation on outcome was not assessed nor incorporated into the multivariable model.*

SUBGROUP CONCLUSIONS: *Aberrant KIT protein localization and c-kit ITDs are associated with increased cellular proliferation. Multiple aspects of cellular proliferation correlate with clinical outcome, but effect of KIT localization / c-kit mutation status was not assessed.*

16. Zemke D, Yamini B et al (2002). Mutations in the Juxtamembrane Domain of c-kit are associated with higher grade mast cell tumors in dogs. Vet Pathol 39: 529-535.

Objective – *To evaluate correlations between c-kit mutation and histologic grade. To attempt to correlate mutation frequency and breed.*

Study design (e.g. retrospective, prospective, other) – *Laboratory study*

Materials & Methods - *PCR amplification for Exon 11 ITDs only. Grade established via Patnaik criteria.*

Conclusions drawn – *Mutations were found in 12 of 88 tumors. An association was found between presence of mutation and higher tumor grade. No associations found between breed and grade or breed and presence of mutation: however, there was minimal statistical power to draw any meaningful correlations regarding breed.*

Statistical soundness – *Rudimentary statistics performed. Differences in mutation frequencies between grades assessed via chi-square. No power calculations performed to determine minimum detectable differences between breeds.*

SUBGROUP CONCLUSIONS: *c-kit ITDs appear to be more common in higher-grade MCT. No meaningful association between mutation status and breed can be assessed in this study, due to low power.*

17. Carlsten KS, London CA, et al. Multicenter prospective trial of hypofractionated radiation treatment, toceranib, and prednisone for measurable canine mast cell tumors. J Vet Intern Med 26: 135-141, 2012.

Objective – *Evaluate toceranib, prednisone, and hypofractionated radiation treatment for canine MCTs*

Study design (e.g. retrospective, prospective, other) – *Single-arm, multi-institutional prospective study.*

Materials & Methods - *17 dogs treated with hypofractionated radiation, prednisone, and toceranib. Evaluation for toxicity and response (RECIST criteria). Evaluate association with mutations in exons 8 and 11.*

Conclusions drawn – *Dogs with c-kit mutations had a lower progression-free survival compared to dogs without mutations. Lower 1-year survival in dogs with c-kit mutations.*

Statistical soundness – *Single arm study with low patient numbers. Univariable analysis only.*

SUBGROUP CONCLUSIONS: *Patients with c-kit mutations tended to have a worse prognosis with combined therapy in this study. Although other studies suggest that c-kit mutations may predict improved response to tyrosine kinase inhibitors, these results suggest that the association between c-kit mutations, combination therapies, and response to treatment should be evaluated independently because associations may not mimic responses observed in single agent protocols.*

18. London CA, Hannah AL, et al. Phase I dose-escalating study of SU11654, a small molecule receptor tyrosine kinase inhibitor, in dogs with spontaneous malignancies. Clin Cancer Res 9: 2755-2768, 2003.

Objective – *Determine the maximum tolerated dose and dose limiting toxicities of SU11654 (toceranib) in dogs with malignancies. Preliminary correlation of c-kit mutation status and toceranib response.*

Study design (e.g. retrospective, prospective, other) – *Single-arm, open-label prospective phase-I study.*

Materials & Methods - *Standard phase-I clinical trial. c-kit Exon 11 ITD presence assessed using PCR and correlated with short-term outcome in MCT patients.*

Conclusions drawn – *Objective responses were noted in 9/11 patients with ITDs, versus 2/11 patients without ITDs. The median time to progression in dogs with MCTs possessing an ITD was 21.0 weeks, compared with 3.9 weeks for those dogs without an ITD ($P < 0.05$). The difference in overall survival between groups was not statistically significant.*

Statistical soundness – *Correlation between ITD status and response made by chi-square analysis. Effect of ITD on outcome assessed via univariate Kaplan-Meier statistics.*

SUBGROUP CONCLUSIONS: *The presence of a c-kit exon 11 ITD appears to predict toceranib response in dogs with MCT. Dogs with ITD also experienced longer progression-free intervals than dogs without ITD.*

19. London CA, Malpes PB, et al. Multi-center, Placebo-controlled, Double-blind, Randomized Study of Oral Toceranib Phosphate (SU11654), a Receptor Tyrosine Kinase Inhibitor, for the Treatment of Dogs with Recurrent (Either Local or Distant) Mast Cell Tumor Following Surgical Excision. Clin Cancer Res 15: 3856-3865, 2009.

Objective – *Determine the ORR following treatment of MCT with toceranib or placebo.*

Study design (e.g. retrospective, prospective, other) – *Multicenter, randomized, placebo-controlled trial.*

Materials & Methods - *Dogs were randomized to receive either Palladia (toceranib) or placebo in a 4:3 ratio. Dogs were stratified based on regional lymph node metastasis (yes or no) and tumor grade (2 or 3). Secondary efficacy end points included biological response, response after escape, duration of response (DOR), and time to tumor progression (TTP) or death. The primary study end point was the objective response rate at the end of the 6-week blinded phase. Genomic DNA was evaluated for internal tandem duplication detection in Exons 11 and 12.*

Conclusions drawn – *Within the Palladia-treated group, dogs with ITD–positive tumors were more likely to respond than those with ITD–negative tumors (60.0%, 12/ 20 versus 31.3%, 20/64, respectively; P = 0.0099; odds ratio, 4.41). Effect of ITD on secondary endpoints was not assessed (e.g. TTP, RD).*

Statistical soundness – *Statistical analysis was appropriate and the sample size was comparatively large for a veterinary clinical trial.*

SUBGROUP CONCLUSIONS: *c-kit mutation status appears to be able to predict likelihood of initial response to toceranib. However, this initial response does not necessarily translate into improved progression free interval or duration of response. Additionally, a substantial percentage of c-kit wild-type patients may still respond. This could be as a result of the presence of unsurveyed mutations, autocrine/paracrine KIT protein activation, or anti-angiogenic mechanisms.*

20. Hahn KA, Ogilvie G, et al. Masitinib is safe and effective for the treatment of canine mast cell tumors. J Vet Intern Med 22: 1301-1309, 2008.

Objective – *To evaluate the efficacy of masitinib, a potent and selective inhibitor of KIT, in the treatment of canine MCT.*

Study design (e.g. retrospective, prospective, other) - *Double-blind, randomized, placebo-controlled, multicenter phase III clinical trial.*

Materials & Methods - *Dogs were administered masitinib (12.5 mg/kg/d PO) or a placebo. Time-to-tumor progression (TTP), overall survival, objective response at 6 months, and toxicity were assessed. c-kit mutation status was assessed in exons 8-13 and 17-19.*

Conclusions drawn – *Mutations were found in exons 8, 9, 11, 17. All of the mutations appeared to cause constitutive activation of KIT. Treatment with masitinib significantly prolonged TTP in all dogs compared with placebo. When subdivided by mutation status, all of the clinical benefit appeared to be as a result of the improvement in the c-kit mutant patients (mutant, TTP 230 vs 42 days, WT, TTP 83 vs 98 days). Masitinib appeared to increase the OS compared with placebo, although the difference was significant only when considering only*

those dogs with tumors expressing a mutant form of KIT (417 versus 182 days). Although a direct comparison of mutant vs WT dogs in the masitinib arm was not performed, there were substantial numeric improvements in the mutant patients with regard to TTP (230 vs 83 days) but not OS (417 d vs median not reached for mutant and WT respectively).

Statistical soundness – *Statistical methods seem appropriate and sample size is very good for a veterinary oncology trial.*

SUBGROUP CONCLUSIONS: Masitinib demonstrated clinical benefit (prolonged TTP, ST) only in *c-kit* mutant patients, and there was a trend toward improved outcome in *c-kit* mutants treated with Masitinib compared with *c-kit* WT (not statistically compared).

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