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Joshua D. Webster

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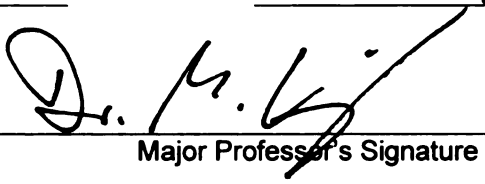

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PROGNOSTIC CLASSIFICATION OF CANINE CUTANEOUS MAST CELL TUMORS AND THE CHARACTERIZATION OF THE ROLE OF THE c-KIT PROTO-ONCOGENE IN CANINE CUTANEOUS MAST CELL TUMORS

By

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ABSTRACT

PROGNOSTIC CLASSIFICATION OF CANINE CUTANEOUS MAST CELL TUMORS AND THE CHARACTERIZATION OF THE ROLE OF THE c-KIT PROTO-ONCOGENE IN CANINE CUTANEOUS MAST CELL TUMORS

By

Joshua D. Webster

Canine cutaneous mast cell tumors (MCTs) are one of the most common neoplastic diseases in dogs, and have an extremely variable biologic behavior ranging from a benign, solitary mass to a potentially fatal metastatic disease. Due to the high prevalence of canine MCTs, their variable biologic behavior, the physical, emotional, and financial costs associated with various treatment protocols, accurate prognostication of canine MCTs is critical in order to identify patients that will benefit most from adjunct radiation and chemotherapy. Currently, histologic grading is the major prognostic and therapeutic determinant for canine MCTs as several studies have shown a significant association between histologic grades and survival. However, the marked degree of inter-observer variation associated with histologic grading, and the predominance of intermediate grade MCTs, has led many to question the relevance of the current system. An additional concern with the treatment and prognostication of canine cutaneous MCTs

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is the current lack of knowledge in terms of the biology of these tumors. Therefore, in light of our current knowledge gap and the need for improved prognostication of canine MCTs, the goals of this dissertation were: 1. to identify novel markers and characterize previously described markers for the prognostication of canine MCTs; and 2. to characterize the role of the c-KIT proto-oncogene in the pathogenesis of canine cutaneous MCTs. The studies described in this dissertation elucidate the utility of KIT staining patterns, c-KIT mutations, and proliferation markers such as Ki67 and AgNORs in the prognostication of canine cutaneous MCTs, and demonstrate the inadequacy of tumor depth, tumor location, tryptase staining patterns, and PCNA immunostaining for prognostication. Additionally, the studies described in this dissertation clarify the role of the c-KIT proto-oncogene in the pathogenesis of canine MCTs, demonstrating *c-KIT*'s importance in the progression of this disease. In summary, the results of these studies strengthen the current body of knowledge of canine MCTs both in terms of diagnostics and basic biology, and these results should serve as building blocks for further hypotheses and future studies.

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INTRODUCTION

MCT Biology

Canine cutaneous mast cell tumors (MCTs) accounting for 7-21% of all cutaneous neoplasms[1-4]. Clinically, canine MCTs often present as solitary neoplastic masses in the skin or subcutaneous tissue in older dogs, however a small proportion of canine MCT patients may have multiple synchronous masses at the time of diagnosis[5-7]. The mean age of onset is approximately 9 years of age, but MCTs have been reported in dogs as young as 2 weeks of age and in dogs as old as 19 years of age[5,7]. All breeds are affected by MCTs, although several breeds such as the boxer, bulldog, Boston terrier, Weimaraner, and Labrador retriever have been reported to have an increased incidence of mast cell disease[2,3,5]. Canine MCTs occur in males and females at an approximately equal frequency[5,7].

Human mast cell diseases are rare, primarily affect infants and juveniles, and commonly have a favorable prognosis[9-11]. In contrast, canine MCTs are common and have a variable biologic behavior[12-14]. Early studies based on necropsy findings reported that up to 96% of MCTs metastasize; however, these reports were grossly biased towards animals that died as a direct result of their mast cell disease due to the inclusion of only necropsy

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findings[7,15]. Several more recent studies have reported a much lower rate of metastasis and mast cell related mortality[8,16,17]. In the event of metastases, MCTs most commonly metastasize to the regional lymph nodes, followed by the spleen, the liver, and the bone marrow[5,7]. Solitary metastases in other viscera occur at much lower rates. In some cases, cutaneous MCTs may be associated with systemic mastocytosis, involving multi-organ metastases and mast cell infiltration. Systemic mastocytosis carries an extremely poor prognosis. In one retrospective evaluation of 16 dogs with systemic mastocytosis, 14 of the dogs evaluated had a primary cutaneous MCT and 88% of these animals died as a result of their MCT[18]. Primary gastrointestinal MCTs appear to be clinically distinct from cutaneous MCTs, as they are most commonly seen in toy breed dogs, and are associated with an extremely poor prognosis with a 39.1% 30-day survival rate and 8.7% 180-day survival rate. Gastrointestinal MCTs also occur at a much lower incidence than their cutaneous counterparts[19].

Due to the unique physiology of mast cells and their ability to release vasoactive and inflammatory mediators, such as histamine, serotonin, and heparin, both canine and human MCT patients may develop either a local or systemic para-neoplastic syndrome as a result of mast cell

degranulation. Most commonly this para-neoplastic syndrome will occur locally and consist of pain, swelling, ulceration, local bleeding, and wound dehiscence. In some cases, especially in the presence of systemic mast cell disease, the para-neoplastic syndrome may be remarkably more severe, characterized by gastric ulcerations, coagulopathies, and systemic hypotension, which may be fatal[5,7,10,11].

Canine MCT Prognostication

Histologic Grading

Due to the variable biologic behavior of canine MCTs and the potentially fatal outcome associated with this disease, an accurate diagnosis and prognostication is critical in order to determine the most appropriate therapeutic strategy for a given tumor. Cytology and histopathology can be routinely used to diagnose canine MCTs without much difficulty, but accurate prognostication of these tumors can be more challenging. Currently, histologic grading is the most commonly used prognostic and therapeutic determinant for canine MCTs, as several studies have found a significant association between histologic grade and survival[12,14]. The two most commonly used histologic classification systems for canine cutaneous MCTs, described by Bostock in 1973 and Patnaik in 1984,

classify MCTs into three histologic grades as being well-differentiated tumors (Patnaik grade I, Bostock grade III), moderately differentiated tumors (Patnaik grade II, Bostock grade II), and poorly differentiated tumors (Patnaik grade III, Bostock grade I)[12,14]. The Patnaik classification system is the most widely used system for histologically grading canine MCTs and defines grade I MCTs as being well-differentiated tumors located in the superficial dermis, grade II MCTs as intermediately differentiated tumors located in the superficial and/or the deep dermis, and grade III MCTs as being of poor differentiation[14]. In this study 93% of dogs with grade I MCTs, 47% of dogs with grade II MCTs, and 6% of dogs with grade III MCTs survived greater than 1,500 days, and this association was found to be statistically significant[14]. In his earlier study, Bostock used similar morphologic criteria to classify canine MCTs, also finding a significant correlation between histologic grade and patient survival[12]. A major point of contention between the Patnaik and Bostock classification systems, however, is the role of tumor depth in the classification of these tumors. In the Patnaik system, tumor depth is a primary criterion used to differentiate low grade and intermediate grade MCTs[14], however Bostock does not include tumor depth in the histologic

classification of these tumors[12]. Variation in the inclusion of tumor depth in the histopathologic classification of canine MCTs has led to a marked degree of inter-observer variation when evaluating low to intermediate grade MCTs [20-22]. However, despite the controversy that surrounds the use of tumor depth in the histologic grading of canine MCTs, only one study has commented on the independent prognostic significance of tumor depth in canine cutaneous MCTs, and only as a minor side note in a larger study[23].

Despite the statistical significance of histologic grading, the utility and relevance of the current histologic grading system has been called into question on multiple occasions due to the predominance and questionable biology of intermediate grade tumors, which account for as many as 72% of the tumors in some studies[17,24], and the marked degree of inter-observer variation[20-22]. Specifically, the issue of inter-observer variability has been clearly demonstrated in 2 sets of studies that have evaluated variation among pathologists in the histologic grading of canine MCTs. In the first set of studies, 60 canine cutaneous MCTs were evaluated by 10 pathologists at a single institution. When each pathologist was allowed to grade according to his/her own set of histologic criteria,

there was only 50.3% agreement among the pathologists, and when a standardized set of histologic grading criteria was used, specifically the Patnaik histologic grading system, the total agreement only increased to 62.1%[20,21]. Similarly, in a second study in which 95 canine cutaneous MCTs were histologically graded by 31 pathologists from 16 different institutions, there was only a 63.1% concordance for grade I MCTs, 63% concordance for grade II MCTs, and 74% concordance for grade III MCTs[22]. Together these studies clearly demonstrate the limitations of the current histologic grading system and the need for novel and improved prognostic indicators for canine MCTs.

Clinical Progostic Markers

Over the last 30 years, several clinical, histologic, and molecular markers have been evaluated for the prognostication of canine MCTs. These markers include, but are not limited to, tumor location[25-27], stage[6,26,27], growth rate[12], duration[12], proliferation markers[28-32], DNA ploidy[33], intratumor vascular density[23], plasma histamine concentration[34], and morphometry[35]. However, the prognostic value and utility of many of these markers have been variable, and to date no marker has been established as a gold standard for prognosticating canine MCTs.

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Certain tumor locations, specifically the inguinal and perineal region, have been associated with a worse prognosis, although these associations have been largely based on the clinical impression of oncologists and pathologists[36-40], with relatively few statistical studies supporting this association[12,25-27,41]. In a recent retrospective study in which 68 cases of inguinal and perineal MCTs were evaluated for prognostic factors, the authors concluded that MCTs in the inguinal and perineal regions may have similar tumor-free intervals and survival times as compared to MCTs in other locations when treated with appropriate chemotherapy and radiation therapy[25]. It must be noted, however, that the authors of this study based these conclusions on a comparison of their survival data with that of the current literature and no control population consisting of MCTs in other anatomic locations were included in this study. Interestingly, the results of another recent study suggest that MCTs of the muzzle may be more locally aggressive, have an increased rate of local metastases, and may be associated with an increased incidence of MCT-related mortality[42]. This study, however, also lacked a control population and conclusions must be extrapolated from the current body of literature, thereby placing limitations on the conclusions that can be made.

Although many clinicians and pathologists still contend that inguinal, perineal, and muzzle-associated MCTs are associated with more aggressive mast cell disease, a true case-control study evaluating a large population of dogs undergoing a standardize treatment regimen is still needed to support or refute this hypothesis.

Clinical staging has also been used to prognosticate MCTs. However, the significance of multiple synchronous tumors, which are considered to be stage III disease according to the World Health Organization's staging system for canine MCTs, has been highly debated. Previously, no differences in survival were found between dogs with stage I and stage III MCTs when treated with prednisone and vinblastine[36]. However, in a second study, stage III MCTs were associated with an increased rate of metastases, but not an increased rate of local recurrence or a decrease in survival time, compared to stage I MCTs when treated with radiation therapy alone[37]. Additionally, in a recent study evaluating an eclectically treated group of multi-synchronous MCTs, Mullins et al. suggested that multi-synchronous MCTs do not offer a worse prognosis compared to solitary tumors[6], however a control population of solitary tumors was not included in this study. One potential cause for the discrepancies between these studies may be the fact

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that each of these studies evaluated populations of dogs treated with distinct therapeutic protocols. Animals with multi-synchronous mast cell tumors are likely to respond better to systemic chemotherapy than to local therapy alone, such as surgery alone or radiation therapy since their disease is more widespread. Therefore, these studies should be interpreted in light of the therapeutic protocol used in each study, and future studies should standardize the sampling populations used with regards to the treatment given to each individual patient in order to produce interpretable results.

Additional clinical parameters that have been used to prognosticate MCTs include tumor duration[12], rate of tumor growth[12], plasma histamine levels[34], and detection of mast cells in buffy coat smears[43,44]. Previously, Bostock has shown that both the duration that a tumor has been present and the rate of tumor growth per week are significantly associated with survival. Specifically, Bostock showed that dogs with tumors that grow less than 1 cm^3/ week and dogs with tumors that have been present longer than 28 weeks have significantly increased survival as compared to dogs with tumors that grow more than 1 cm^3/week and those that have been present for less than 28 weeks, respectively[12]. Recently, Ishiguro et al. investigated the

relationship between MCT progression and plasma histamine concentrations (PHC). Although initial PHC levels could not predict survival, the results of this study did show that seven of the seven dogs that died due to MCT-related disease developed a marked hyperhistaminemia, with a median value of 14ng/mL compared to 4 dogs that survived whose PHC levels remained less than 1ng/mL. These results suggest that PHC can be used to evaluate disease progression, but not for initial prognostication[34].

One prognostic measure for canine MCTs that has fallen by the wayside is the evaluation of buffy coat smears[43,44]. At one time, buffy coat smears were commonly used to detect mastocythemia in MCT patients in order to evaluate patients for systemic mast cell disease. However, it has been subsequently shown that mastocythemia may be associated with several dermatologic and inflammatory diseases, and that the mastocythemia associated with other diseases may in fact be greater than when it occurs secondary to MCTs, thereby extinguishing the promise of this prognostic tool[44,45,46].

Tumor-free Margins

Due to the high degree of inter-observer variability in histopathologic grading, several additional pathologic-based diagnostic markers have been evaluated in order to improve the prognostication of the current histologic grading system. Since mast cells tumors consist of round cells that invade and distribute themselves throughout a given tissue, evaluation of tumor margins is a critical part of the histologic evaluation of a canine MCT, as marginal mast cells are thought to be responsible for local tumor recurrence. In order to evaluate the significance of tumor-free vs. nontumor-free margins, Michels et al. compared the rate of local and distant relapse, and survival in 20 dogs with tumor-free margins, and in 11 dogs with nontumor-free margins[x]. This study found that dogs with nontumor-free margins had three-times greater incidence of local relapse (2/11 vs. 1/20, respectively), although this was not statistically significant, and a significantly increased rate of relapse at 12 and 24, but not 6, months post-surgery. Additionally, 2/11 of the dogs with nontumor-free margins died due to MCT-related diseases, compared to 0/20 dogs with tumor-free margins. Based on the raw data, it appears that complete surgical excision is likely to result in a reduced incidence of

relapsing disease, and a decreased incidence of MCT-related mortality. These conclusions coincide with the presumed biologic principles that leaving behind a single neoplastic cell could result in re-growth of the tumor. The major limitation of this study, however, is that the extremely small sample size, which is likely to be responsible for the lack of statistical significance. In this study a relatively low number of cases (2/11) with nontumor-free margins had local relapse. These results seem to be in striking contrast to the hypothesis that a single neoplastic cell left behind can reconstitute the tumor, and therefore place into question the significance of these marginal cells. Since mast cell tumors may produce chemokines that can attract additional normal mast cells to the tumor site[47-50], a major question continues to arise as to whether marginal mast cells represent neoplastic cells or recruited normal mast cells. It is critical to define the molecular phenotype of these marginal cells in order to define their biologic significance, and at this time few conclusions can be made based on the low number of cases and the relatively low rate of tumor recurrence and MCT-related mortality in this study.

Proliferation Markers

As uncontrolled cellular proliferation is considered a hallmark of cancer[51], proliferation markers, such as Ki67 indices, PCNA indices, and AgNOR counts, have been used to prognosticate several human[52-59] and canine[28-32,60-66] neoplastic diseases including canine cutaneous MCTs. Ki67 is a nuclear protein that is expressed during all phases of the cell cycle, but is absent in non-cycling cells[67,68]. The function of Ki67 during the cell cycle is unknown, although it appears to be necessary for cell cycle progression[68,69]. Since Ki67 is expressed in all phases of the cell cycle, the relative proportion of cells expressing this protein has been used as a measure of the proliferation index, or the relative proportion of neoplastic cells involved in cellular proliferation in a given tumor[64,67,68,70,71]. PCNA, or proliferating cell nuclear antigen, is an auxiliary subunit of DNA polymerase-delta, the major eukaryotic replicative DNA polymerase[72,73]. PCNA is also involved in other nuclear processes, most notably DNA repair[74]. Due to its role in DNA replication, PCNA is primarily expressed during the S-phase, or DNA synthesis phase of the cell cycle[75-77]. As such, PCNA has been used to determine the S-phase index, or the relative proportion of cells in the S-phase of the cell cycle[61,70]. Although PCNA expression peaks during the S-

phase, PCNA has a 20-hour half life[78] and therefore may be expressed at other phases of the cell cycle. Despite PCNA's long half-life, PCNA is still considered by many to be an S-phase specific marker[61,70]. AgNOR (agyrophilic nucleolar organizer region) histochemical staining is a silver-based staining technique that stains areas of ribosomal RNA transcription in the nucleus, called AgNORs, due to 2 RNA transcription-associated proteins' affinities for silver[79]. The number of AgNORs per nucleus is correlated with the rate of cell proliferation and tumor growth[80-85], which is thought to be due to the need for increased protein synthesis during cell cycle progression[79].

Ki67, AgNOR, and PCNA counts have all been shown to be independent prognostic factors for canine MCTs, although the utility of these markers has varied between studies[28-31,66]. In a study evaluating PCNA and Ki67 immunostaining in 120 MCTs treated with surgery alone, Abadie et al. found that the mean number of Ki67 positive cells/1,000 cells was significantly associated with survival. In this study, using cut-offs of 55 and 135 Ki67 positive cells/1,000 cells, MCTs were classified into 3 distinct classes that were significantly different from each other in terms of survival, and grade II MCTs with greater than 93 Ki67 positive cells/ 1,000 were found to have significantly

reduced survival as compared to grade II MCTs with less than 93 positive cells/1,000cells[30]. Additionally, in this study Abadie et al. found that the PCNA-positive cells were significantly greater in dogs that died due to MCT-related disease as compared to those that survived, but there was significant overlap between these groups thereby eliminating the prognostic utility of this technique[30].

In an earlier study, PCNA immunohistochemical staining was evaluated in a series 120 canine MCTs. Dogs with MCTs with greater than 261 PCNA-positive cells/5 high powered fields had significantly reduced survival as compared those with less than 261 PCNA-positive cells/5 high powered fields[66]. AgNORs were also evaluated in this study, and it was found that dogs with an AgNOR count greater than 2.25/cell, based on the evaluation of 100 cells, had significantly decreased survival durations as compared to those with less than 2.25[66]. In the original study evaluating AgNOR histochemical staining in canine MCTs, Bostock found a significant difference in survival between cases with >4 AgNORs/cell and those with less than 4 AgNORs/cell based on counting 100 cells. Additionally, in this study no dogs with an AgNOR count of less than 1.7 AgNORs/cell resulted in MCT-related mortality[68]. Recently all three markers were evaluated in a single study,

allowing for a comparison of these markers. In this study, AgNORs and Ki67 were both associated with disease progression, but Ki67 was determined to be the most useful as it could distinguish potentially benign and malignant histologic grade II tumors using distinct cut-off values[39]. Although these studies demonstrate the prognostic significance of these proliferation markers, the methods and results of these studies are highly varied, thereby confounding the interpretation and application of each individual study. Additionally, at this time, only 1 study has evaluated all three of these markers in a single cohort of animals[39], and no studies have evaluated the utility of using these markers as a panel rather than as individual indicators of malignancy. Since cellular proliferation is a result of both the number of cycling cells in a given tumor and the rate of cell cycle progression, it is necessary to evaluate both the proliferation index (Ki67) and the rate of cellular proliferation (AgNORs) in order to gain a full understanding of a tumor's cellular proliferation[45,58,70]. By looking at all three markers in a single cohort of animals the combined significance of these markers and a comparison of the prognostic significance of these markers can be made. Therefore, a single study evaluating all three of these markers in a cohort of dogs

treated with a standardized therapeutic protocol is needed in order to evaluate the true relevance of these markers, both alone and in combination.

Image Analysis

In addition to routine light microscopic techniques, image analysis software has been used to develop additional markers for the classification and prognostication of canine MCTs[23,35]. In a study evaluating the nuclear morphology of 24 canine MCTs using image analysis software, Strefezzi et al. found a significant difference in nuclear area, mean diameter, and nuclear perimeter between grade I and grade III and grade II and grade III MCTs. However, no differences were found between grade I and grade II tumors, which, as mentioned previously, are the most difficult to differentiate[35]. The utility of these findings is limited since no survival analysis was performed, and a relatively low number of cases were analyzed. Therefore, a larger study with complete survival analysis is needed in order to determine the biological significance of these variations in morphology. Image analysis software has also been used to determine the prognostic significance of intratumor microvessel density in canine MCTs[23]. Subsequent to immunohistochemically labeling endothelial cells with factor VIII-related antigen (von Willebrand's factor),

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Preziosi et al. used image analysis software to determine the number of microvessels/mm^2 in a series of MCTs in order to determine the association between intratumor vessel density and survival. Based on the cut-off value of 14.1 vessels/mm^2, dogs with MCTs with a high microvessel density were found to have a significantly decreased survival time and cancer-free interval, as compared to dogs with tumors with low microvessel density. In this study, intratumor micro-vessel density was found to be an independent prognostic indicator based on multivariate regression analysis. Although both of these studies are relatively small, they do demonstrate the potential power and utility of image analysis in cancer prognostication. There are, however, significant limitations to these techniques, such as the time constraints, cost, and the current availability of image analysis software at veterinary diagnostic laboratories. In the future, image analysis-based techniques may be routinely used in the prognostication of neoplastic diseases, but these current limitations prevent the routine use of image analysis in a veterinary diagnostic setting.

Despite the plethora of potential markers that have been evaluated for the prognostication of canine MCTs, no single marker has been clearly shown to be consistently

predictive of MCT behavior. In light of this, it is probably unrealistic to assume that a single marker will ever be able to accomplish such a daunting task. Instead, a prognostic panel for canine MCTs may be established that combines clinical, pathological, and molecular markers for the accurate prognostication and therapeutic determination of canine MCTs.

Molecular Pathology of Canine MCTs

p53

Despite the attention canine cutaneous MCTs have received in terms of prognostication, especially in terms of histopathologic evaluation, much less attention has been given to the molecular biology or molecular pathogenesis of these tumors. Using immunohistochemistry, expression of the p53 tumor suppressor gene in canine MCTs has been described in 2 independent studies[86,87]. The focus of each of these studies was to evaluate the prognostic significance of p53 expression in canine MCTs, and each study concluded that despite p53 expression in a large number of canine cutaneous MCTs, detection or the relative proportion of p53-positive cells was not useful in terms of prognostication. The major limitation of these studies, however, is that there were no attempts characterize the biologic significance of p53 expression in these tumors.

For example, null mutations in p53 may provide a chemo-resistant or radiation resistant phenotype by allowing the neoplastic cells to ignore DNA damage and to continue through the cell cycle. Additionally, as p53 mutations may be responsible for chemo-resistance and resistance to radiation therapy, these studies could have also been strengthened by assessing the prognostic significance of p53 expression and p53 mutations in a population of animals given a standardized therapeutic protocol, in order to determine the role these play on chemo-resistance in canine MCTs.

p21 and p27

Expression of the cyclin-dependent kinase inhibitors p21 and p27 has also been evaluated in canine MCTs in a single study[88]. In this study, 47 MCTs were evaluated for p21 and p27 expression using immunohistochemistry in order to test the hypothesis that p21 and p27 expression is lost in canine MCTs with advanced disease, as characterized by having a higher histologic grade. Although there was a tendency for higher histologic grade tumors to have a loss of p27 expression, there was also an increased relative proportion of grade II and grade III MCTs that had moderate to marked expression of p27. Additionally, this study found an increased p21 expression in higher histologic

grade MCTs, which is contrary to the authors' hypothesis that there should be loss of expression of these genes in canine MCTs. Overall, the significance of these results is very hard to determine, as no clear associations between histologic grade and p21 and p27 expression were found. Therefore, these results provide no evidence of a potential role of p21 and p27 in the progression of canine MCTs.

DNA ploidy

In order to determine the role that DNA ploidy and chromosomal aberrations play in canine MCTs, Ayl et al. conducted a retrospective study comparing DNA ploidy in 40 MCTs, using flow cytometry with propidium iodide staining, with patient survival, histopathologic grade, tumor-free survival, and clinical stage[33]. 72.5% of the MCTs evaluated in this study were diploid, suggesting a low incidence of chromosomal aberrations in canine MCTs. Although there was an overall low incidence of aneuploidy in the MCTs evaluated, the investigators did find that a relatively higher proportion of grade III MCTs were aneuploid, although this was not statistically significant. In this study, aneuploid tumors also tended to be associated with decreased survival times, although this was not statistically significant, and were found to be significantly associated with an increased clinical stage

when stage 1 tumors were compared to all other clinical stages combined. These results suggest that changes in DNA ploidy are rare events in canine MCTs, but they may be associated with more aggressive forms of mast cell disease when they do occur. Currently, the nature of aneuploidy in canine MCTs in unknown, but knowledge of the associated genetic changes may shed light on additional genes that may be responsible for the progression of canine MCTs.

ABC Transporters

Malignant canine MCTs commonly have a poor response rate to chemotherapeutics[5,7], suggesting that these tumors may express ATP-binding cassette transporters that are potentially responsible for drug efflux from the neoplastic cell and therefore the development a multi-drug resistance phenotype[89]. In a recent study, Miyoshi et al. evaluated a series of 44 canine MCTs for the expression of two of these transporters, P-glycoprotein and multidrug-resistance-associated protein[90]. In this study the authors found expression of at least one of these two proteins in 26% of the MCTs evaluated, and the expression of these proteins suggested an inverse correlation with histologic grade. At first glance these results are optimistic, suggesting that the majority of canine MCTs, especially those of higher histologic grade should be sensitive to standard

chemotherapeutics. However, the ABC transporter family is an extremely large protein family of which several members have been associated with multi-drug resistance[89]. Therefore, although P-glycoprotein and multi-drug-resistance-associated protein are not likely to play a significant role in the efficacy of chemotherapeutic treatment of canine MCTs, it is possible that other ABC transporters do play a role in the chemo-resistance of canine MCTs.

The Role of the c-KIT Proto-oncogene in Canine MCTs

c-KIT

Of the genes that have been evaluated for a potential role in canine MCTs, the role of the *c-KIT* proto-oncogene has been most clearly defined[16,91-96]. The *c-KIT* proto-oncogene encodes the type III receptor tyrosine kinase, KIT[97]. The KIT protein consists of an extracellular ligand-binding domain consisting of 5-immunoglobulin-like loops, a transmembrane domain, a negative-regulatory juxtamembrane domain, and a split cytoplasmic kinase domain[97-99]. The receptor tyrosine kinase KIT is expressed in multiple cell types including hematopoietic progenitor cells, melanocytes, mast cells, interstitial cells of Cajal, and germ cells, where KIT has been shown to be important for cell survival, differentiation, and proliferation[100-105].

Additionally, KIT has been shown to be important for mast cell chemotaxis, fibronectin adhesion, and degranulation[106-111]. The ligand for KIT is stem cell factor (SCF, also known as mast cell growth factor, KIT ligand, and steel factor)[106,112-115], which is also expressed by multiple cell types including fibroblasts, stromal cells and endothelial cells[116]. Mice with either *c-KIT* or SCF null mutations are characterized by hypopigmentation, sterility, anemia, and mast cell depletion, further demonstrating *c-KIT* and SCF's essential role in the survival, differentiation, and proliferation of hematopoietic cells, germ cells, melanocytes, and mast cells[113-115,117].

c-KIT in Human Cancer

In recent years, the *c-KIT* proto-oncogene has been implicated in several distinct neoplastic diseases, including gastrointestinal stromal tumors (GISTs)[118-121], mastocytosis[122,123], germ cell tumors[124-126], small cell lung cancer[127-129], prostate cancer[124,130], and acute myeloblastic leukemia[117,131] in humans, and mast cell tumors[91,92,94-96] and gastrointestinal stromal tumors[124,132] in canines. Potential activating mutations have been identified in several different exons of the *c-KIT* proto-oncogene in a variety of cancers. In human gastrointestinal stromal tumors patients, germline mutations have been identified in exon 8

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of the extracellular domain[133], exon 11 of the juxtamembrane domain[118,119], and exons 13 and 17 of *c-KIT*'s kinase domain[119]; however, the majority of sporadic *c-KIT* mutations in GISTs have been identified in exon 11, and to a lesser extent in exon 9 of the extracellular ligand-binding domain[119]. In human mastocytosis patients, point mutations occur most commonly in exon 17 at codon 816, usually resulting in the replacement of valine for aspartate. However, additional substitutions at codon 816 have been characterized in human mastocytosis patients, resulting in valine, tyrosine, and phenylalanine substitutions[134]. Additionally, a point mutation resulting in a lysine substitution for glutamate at codon 839 has been characterized in some pediatric mast cell patients[134], and a 3bp deletion at codon 419 in exon 8 has been identified in a family with a history of mastocytosis and GISTs[133]. In addition to those found in GISTs and mastocytosis patients *c-KIT* mutations have also been identified in exons 11 and 17 in human germ cell tumors[125,126].

Although the true biological significance of each of these mutations has not been thoroughly characterized, it has been previously shown that mutations in exons 11 and 17 of the *c-KIT* proto-oncogene result in constitutively activated KIT products in the absence of ligand[118,126,135].

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Additionally, different *c-KIT* mutations have been associated with distinct clinical forms of human mast cell disease[133,134,136], and with distinct tumor locations, phenotypes, and more aggressive disease in GIST patients[130,131,137], further suggesting a key role for *c-KIT* in the progression of these diseases. Interestingly, the presence and location of *c-KIT* mutations has also been associated with response to receptor tyrosine kinase inhibitors, such as imatinib mesylate (Gleevec). Specifically, it has been shown that tumors with *c-KIT* mutations in exon 11 are significantly more likely to respond to *c-KIT* inhibitors, such as Gleevec, as compared to tumors with exon 17 mutations or tumors that express KIT but lack *c-KIT* mutations altogether[131,137,138].

With the discovery of Gleevec, there has been an increased interest in identifying tumors that contain *c-KIT* mutations, or aberrantly express KIT. In light of this, aberrant KIT expression has been characterized in a number of human neoplastic diseases, including uterine leiomyosarcomas[139], small cell carcinoma of the urinary bladder[140], follicular thyroid carcinomas[101], endometrial carcinomas[101], acute myeloblastic leukemia[141], prostatic carcinomas[130], and small cell lung cancer[127-129]. The significance of aberrantly expressed KIT has been

characterized for some cancers, but for many, the role of KIT still needs to be elucidated. In some tumors, such as small cell lung cancer, concurrent SCF production by the neoplastic cells creates an autocrine/paracrine signaling loop which results in the constitutive activation of KIT[127,128].

A truncated isoform of KIT (TR-KIT) has been identified in other tumors, such as prostatic carcinomas and colon cancer[140,141,142]. In humans, TR-KIT originates in intron 15 of the *c-KIT* proto-oncogene and consists of a hydrophilic amino-terminus, and the phosphotransferase domain and carboxy-terminus of KIT[142]. Due to its lack of a ligand binding domain or an ATP binding site, TR-KIT is unable to either bind to its ligand or autophosphorylate. However, instead of dimerizing and signaling through traditional KIT pathways, TR-KIT acts as a scaffolding protein, which binds and activates the Src-like kinase Fyn. Upon activation, Fyn is able to bind and phosphorylate the STAR family RNA binding protein Sam68, which then acts as a scaffolding protein, facilitating the interactions between Fyn and Phospholipase Cγ1 (PLCγ1). This leads to the activation of PLCγ1[143]. In prostate cancer patients, TR-KIT has been found in neoplastic cells, but is absent from the surrounding normal tissue. Additionally, TR-KIT is more

commonly found in advanced forms of prostate cancer, thereby implicating TR-KIT in the progression of this disease[14].

c-KIT in canine cancer

Similar to human cancers, *c-KIT* expression has been described in multiple canine neoplastic diseases using immunohistochemistry, including mammary gland adenoma and carcinoma, malignant melanoma, seminoma, interstitial cell tumor, granulosa cell tumor, ovarian papillary adenocarcinoma, gastrointestinal stromal tumors, and mast cell tumors[91,94-96,124,132,144]. In the majority of these cancers, KIT expression has been described as being weak, with variable expression in the few cases that were evaluated[144]. Of the canine neoplastic diseases that have been shown to express KIT, KIT expression has been most consistently described in MCTs and GISTs, which tend to have strong diffuse KIT expression throughout the majority of these tumors[91,94,124,132].

In 2003, two laboratories independently described KIT expression in canine gastrointestinal stromal tumors. The relative frequency of KIT expression in GISTs varied between these two studies from 52% immuno-positivity (11/21 GISTs)[132] to 100% immuno-positivity (5/5 GISTs)[124], but both studies described strong, diffuse immuno-reactivity in the

majority of the tumors that expressed KIT. Aside from the identification of KIT expression in canine GISTs, Frost et al. also identified potential activating mutations in exon 11 of *c-KIT* in two of the four tumors evaluated. These mutations consisted of a six base pair deletion, deleting Try556 and Lys557, and a three base pair duplication of Gln555 in one case, and a T to C transition, substituting Pro575 for leu575 in a second case[132]. Although no large scale studies have evaluated the prevalence of *c-KIT* mutations in canine GISTs, the consistent expression of *c-KIT* in the majority of canine GISTs tumors, the identification of potential activating mutations, and the known role *c-KIT* plays in human GISTs suggests a potential role of *c-KIT* in the progression of canine GISTs. However, in the future, further studies are needed to characterize the prevalence and the biologic and clinical significance of both KIT expression and *c-KIT* mutations in canine GISTs.

Mutations in the *c-KIT* proto-oncogene and aberrant KIT expression, characterized by increased cytoplasmic protein localization, have been described in canine MCTs, and are thought to play a key role in the progression of this disease[91,92,94-96]. *c-KIT* mutations, consisting of internal tandem duplications and deletions, have been identified in exon 11 in canine MCTs. Internal tandem duplications in

canine MCTs consist of 44-69bp insertions that are primarily located at the 3′ end of exon 11. In some cases these mutations extend into intron 11, however the biologic significance of this extension and the effects it has on RNA splicing has not been elucidated at this time. Deletions, which range from 3-30bp in size, occur at a much lower frequency than ITD mutations in canine MCTs and tend to occur at the 5′ end of exon 11. All except for 1 of the ITDs and deletions that have been sequenced have been in-frame mutations. Additionally, of the mutations that have been characterized thus far, all of these have been shown to produce a constitutively activated KIT protein in the absence of ligand[91,95,145].

The reported frequency of *c-KIT* mutations in canine MCTs has varied between studies most likely reflecting variations in sample populations and methodologies used to detect *c-KIT* mutations. In a study evaluating the prevalence of *c-KIT* mutations in 88 canine MCTs submitted for histopathologic evaluation, primarily from private practitioners, 4.54% (4/88) and 9.1% (8/88) of the MCTs evaluated had deletions and ITDs, respectively[96]. In contrast to this, however, a second study reported a 33% prevalence of ITD *c-KIT* mutations in a series 157 MCTs that were treated at a referral veterinary teaching hospital[16].

The discrepancy between these studies is likely a result of differences in study populations, as the population of MCTs treated at a referral practice used in the latter study are more likely to represent more aggressive forms of mast cell disease and therefore may be more likely to have *c-KIT* mutations when compared to the entire spectrum of canine MCTs, as seen in the former study[16,96].

Additional discrepancies have also been reported in terms of the incidence of deletions in canine MCTs. As mentioned previously, in an evaluation of 88 canine MCTs Zemke et al. found deletions in 4.54% of the MCTs evaluated[96]. However, in a subsequent study Jones et al. failed to find any deletions in exon 11 of the *c-KIT* proto-oncogene in 25 MCTs[146]. Although this discrepancy may be a result of a small sample size, the forward primer used by Jones et al. for PCR amplification and sequencing was complementary to the sequence where Zemke et al. had previously reported deletions. Therefore, due to the design of this forward primer, these primers would not amplify the mutant allele in the majority of deletions and only the normal allele would amplify in heterozygotes, thereby inhibiting the detection of most, if not all deletions that were similar to those previously described[96,146].

In light of these discrepancies, it is likely that *c-KIT* mutations occur in 15-20% of all canine MCTs, but may occur in as many as 30-50% of high grade or malignant MCTs[16,96]. Despite the relatively low incidence of exon 11 *c-KIT* mutations, only two studies, in which only 6 and 11 MCTs were included, respectively, have screened additional *c-KIT* domains for potential activating mutations[91,95]. Therefore, future studies are needed to evaluate canine MCTs for additional *c-KIT* mutations, such as mutations in exon 17 of the kinase domain as seen in human mastocytosis patients[122,123,135] that may also play a role in the progression in canine MCTs.

Early studies suggest that *c-KIT* mutations play a significant role in the progression of canine mast cell disease. Previous studies by our laboratory have shown that deletions and ITD *c-KIT* mutations are significantly associated with higher histologic grade MCTs[96], and work from another group has shown that MCTs with ITD *c-KIT* mutations are twice as likely to locally recur and twice as likely to metastasize as compared to MCTs that lack ITD *c-KIT* mutations, although these differences were not statistically significant[16]. Together these data suggest that mutations in the *c-KIT* proto-oncogene may play a significant role in the progression of canine MCTs, and

therefore may serve as a potential target for treating MCTs. Additionally, the presence of these mutations may be used for prognostication and identification of MCTs that may respond to targeted therapies, such as receptor tyrosine kinase inhibitors.

MCT Treatment

Currently, canine cutaneous MCTs are primarily treated with surgical excision alone or in combination with radiation and/or chemotherapy depending on the clinical stage and histologic grade of the tumor. In the face of clean surgical margins, solitary low- and intermediate grade MCTs that do not involve the local lymph nodes are commonly treated with surgery alone, followed by careful observation of the surgical site for tumor recurrence[5,7]. Three centimeter margins lateral and deep to the MCT have classically been considered to be ideal for complete tumor resection, but a recent study suggest that 2-cm lateral margins and one fascial plan deep may be adequate for complete excision of histologic grade I and grade II MCTs[147]. However, no grade III MCTs were included in this study and since histologic grade is usually not determined by the time of surgical excision, the results of this study may not be applicable in a real world setting. If adequate surgical margins are not obtained from an intermediate or

high-grade MCT, re-operation and/or local radiation therapy is commonly used to prevent tumor recurrence. If regional lymph node or systemic metastases are present, or if the tumor is not amenable to resection, chemotherapeutics may also be added to the therapeutic regimen. Several single and multi-agent chemotherapeutic protocols have been used to treat canine MCTs. These include prednisone, vincristine or lomustine alone; prednisone and vinblastine in combination; prednisone, cyclophosphamide, and vinblastine in combination; or cyclophosphamide, vincristine, prednisone, and hydroxyurea in combination. Despite the wide array of chemotherapeutic protocols available, the overall response rate for canine MCTs extremely variable[5,7]. Coinciding with the discovery of *c-KIT* mutations in canine MCTs, recent focus has been directed towards the use of receptor tyrosine kinase inhibitors in the treatment of canine MCTs, especially those MCTs with activating *c-KIT* mutations, which are likely to have a better clinical response to these novel drugs. In a recent phase I clinical trial, the receptor tyrosine kinase inhibitor SU11654 was shown to cause objective tumor shrinkage in 11/22 canine MCTs and a total response rate of 55% with minimal and tolerable toxicities[148]. Interestingly, an increased response rate

was seen in canine MCTs with ITD *c-KIT* mutations (9/11) as compared to those that lacked *c-KIT* mutations (2/11). Furthermore, although not statistically significant, dogs with ITD *c-KIT* mutations tended to have a longer survival duration than those that lacked ITD *c-KIT* mutations (36.9 weeks vs. 15.4 weeks, respectively)[148]. This utility of SU11654 has been further validated, as it has been shown to modulate KIT phosphorylation and down-stream signaling pathways, such as ERK 1/2 in canine MCTs based on the evaluation of serial pre- and post-treatment biopsies[145]. Although SU11654 is not currently available, these data demonstrate the potential for targeting *c-KIT* in canine cutaneous MCTs in the future, which may provide the greatest hope for definitively treating aggressive canine MCTs.

Purpose

At the time of the inception of this dissertation project in late 2001, histologic grading and the overall clinical picture were the only criteria routinely used to prognosticate canine MCTs. At that time, the criteria used to evaluate a given patient's clinical picture was largely based on the clinical experiences and interpretations of oncologists and pathologists, with few studies available to support or refute these clinical assessments.

Additionally, as mentioned previously, the discrepancy between the histologic grading of pathologists and the question of the role tumor depth plays in the histologic grading of canine MCTs further confounded the prognostication of canine MCTs. Although studies had been published evaluating the use of proliferation markers for prognosticating MCTs, the discrepancies between studies, the use of image analysis software, and the labor intensive methods used to evaluate these markers prohibited their use in a routine diagnostic setting. Similarly, aside from the identification of KIT expression in canine MCTs, the identification of activating juxtamembrane domain mutations in the *c-KIT* proto-oncogene, and the associations between these mutations and histologic grade, minimal conclusive data were available with regards to the role of *c-KIT* in canine MCTs. The primary goals of the work described in the subsequent chapters of this dissertation were to 1. Identify and characterize prognostic markers for canine cutaneous MCTs; 2. Further define the role of the *c-KIT* proto-oncogene in canine cutaneous MCTs, and to better understand the molecular biology of canine MCTs. The following 7 chapters describe 7 studies that were carried out in order to address these two primary goals. In these studies we evaluated the prognostic significance of the

patient's signalment and clinical presentation, tumor depth, patterns of KIT and tryptase protein expression, internal tandem duplication *c-KIT* mutations, and the proliferation markers Ki-67, PCNA, and AgNOR in a series of dogs with canine cutaneous MCTs that were treated with surgical excision alone. In chapter 6 we describe a study evaluating the prognostic significance of ITD *c-KIT* mutations, KIT and tryptase protein expression patterns, histologic grade, and the proliferation markers Ki-67, PCNA, and AgNOR in a series of canine MCTs that were treated with a standardized chemotherapeutic protocol of vinblastine and prednisone. In addition to identifying and evaluating prognostic markers for canine cutaneous MCTs, a major goal of this dissertation was to better understand the role of the *c-KIT* proto-oncogene in canine MCTs. In light of this goal, in this dissertation project we have attempted to define the association between ITD *c-KIT* mutations and changes in KIT protein localization and the progression of canine cutaneous MCTs, and to begin to understand the biologic significance of these changes. As mentioned previously, *c-KIT* mutations primarily occur in the kinase domain of human mastocytosis patients. In order to determine the significance of kinase domain *c-KIT* mutations in canine MCTs, exons 16-20 of the *c-KIT* proto-

oncogene of 32 canine MCTs were sequenced in order to screen for potential activating mutations that may play a role in the progression of canine cutaneous MCTs. Together the studies described in this dissertation define the use and significance of the multiple prognostic markers that will further aid in defining the biologic behavior of a given MCT, and further define the role of the *c-KIT* proto-oncogene in canine cutaneous MCTs.

Kiupel M, Webster JD, Miller RA, Kaneene JB (2005). Impact of tumor depth, tumor location, and multiple synchronous masses on the prognosis of canine cutaneous mast cell tumors. Journal of Veterinary Medicine, Series A. 52:280-286.

CHAPTER 1

Impact of tumor depth, tumor location, and multiple synchronous masses on the prognosis of canine cutaneous mast cell tumors

Introduction

Cutaneous mast cell tumors (MCT) are one of the most common tumors in dogs, accounting for 7-21% of all cutaneous tumors[1-4]. Mast cell tumors have an extremely variable biologic behavior. Clinically, they can range from a benign mass that can be cured by surgical excision alone, to potentially fatal metastatic disease[12,17,39].

In the past, numerous clinical parameters, including tumor location, tumor volume, tumor stage, tumor duration, tumor free margins and age of affected dogs, have been evaluated for their predictive power with regards to the biological behavior of MCTs[8,12,27,41]. Results of these studies were highly variable, and no single parameter has been shown to clearly predict the biological behavior of canine cutaneous MCTs. Additionally, many of the parameters that are commonly used to evaluate MCTs have been based largely on the experience of oncologists and pathologists at various institutions, with only few studies statistically evaluating the significance of these parameters.

Currently, prognostic and therapeutic determinations of MCTs are based primarily on their histologic grade, using the criteria described by Patnaik et al.[14]. Tumor depth is an important component of this histologic grading system and can determine the dividing line between classifications as benign grade I tumors and guarded grade II tumors. Interestingly, another proposed grading system for canine cutaneous MCTs does not rely upon tumor depth[12]. Despite tumor depth being given much weight in the histologic evaluation of canine cutaneous MCTs, there are no published studies that have looked at the prognostic significance of tumor depth as a separate criterion.

Tumor location is a clinical parameter that has been suggested in a number of reports as an important prognostic factor for canine cutaneous MCTs[36-40]. Perineal/inguinal MCTs and MCTs at mucocutaneous junctions are commonly regarded as having a guarded prognosis. This assumption is commonly based on the clinical experiences of the authors, and is rarely based on statistical evaluations[36-40]. However, a few studies have analyzed tumor location as a prognostic factor for canine MCTs following various treatment protocols[13,26,27,41]. Of these studies, only one study found a poor prognosis for dogs with MCTs located on the trunk when compared to MCTs located on the extremities[27].

Clinical staging has also been suggested as a means to evaluate the prognosis associated with cutaneous MCTs[7,12]. The World Health Organization staging system places multiple cutaneous MCTs at a higher clinical stage than single cutaneous nodules, and therefore confers a worse prognosis. However, a few studies evaluating chemotherapy and radiation therapy have shown contradicting results[26,27]. One study found no difference in disease-free and overall survival times between dogs with stage I and stage III tumors when treated with chemotherapy[26]. Another study found only an increased rate of metastasis, but no higher risk of local recurrence or decrease in survival time, for dogs with stage III tumors treated with radiation as opposed to stage I tumors[27].

This study was designed as a retrospective study to evaluate the significance of tumor depth, tumor location and the presence of multiple synchronous tumors at the time of diagnosis for the prognostic evaluation of canine cutaneous MCTs following surgical removal.

Materials and Methods

One hundred cutaneous MCTs from 100 dogs that had been submitted to the Diagnostic Center for Population and Animal Health (DCPAH) at Michigan State University (MSU) between 1998 and 2001 were included in this study. These

MCTs had been submitted from 9 veterinary hospitals throughout Michigan. Inclusion criteria for this study required: surgical MCT removal as the only treatment modality; complete follow-up data and clinical history; tissues available for histologic evaluation.

Complete history and follow-up information were obtained for each case including: age at diagnosis; sex; breed; weight; diagnostics performed; adjunct medications given at the time of surgery; known tumor duration before removal; tumor location; number of tumor masses at the time of diagnosis; additional tumor development; survival time (time from diagnosis of MCT to time of death); and cause of death, if applicable. Additional tumor development was divided into two groups: MCTs recurring locally (at the surgical site) and MCTs occurring at distant sites (outside the original surgical margins).

All MCTs had been fixed in 10% neutral buffered formalin, and had been embedded in paraffin at the time of submission. Five micrometer sections were cut and routinely stained with hematoxylin and eosin for histologic evaluation and tumor grading. In addition, the tumor depth was evaluated for 98 of the 100 cases. Neoplastic mast cells completely effaced the entire tissue sections of 2

tumors, therefore these cases were not evaluated for tumor depth.

For the purpose of this study, 4 different categories of tumor depth were defined (Fig. 1A-D): 1. Superficial dermis only (neoplastic mast cells did not extend below the adnexal structures); 2. Superficial and deep dermis (neoplastic cells extended from just below the epidermis, to the deep dermis below the adnexa and into the subcutaneous adipose tissue); 3. Deep dermis alone (neoplastic cells were isolated in the deep dermis and did not extend into the superficial dermis and peri-adnexal region); 4. Deep invasion (neoplastic cells were invading into the underlying musculature).

Data pertaining to tumor location were available from the referring veterinarians' records on 98 of the 100 MCTs. For the purpose of this study 4 different tumor locations were defined (Fig. 2): 1. Head and neck; 2. Trunk; 3. Extremities; 4. Inguinal/ perineal region.

Statistics- *Descriptive Statistics*: Mantel-Haensel chi-square statistics were generated to assess associations between categorical risk factors (prognostic factors) and study outcomes (occurrence and recurrence of MCTs, and mortality due to MCT or due to any cause). Before developing multivariable survival models to assess

associations between risk factors and times to MCT outcome, each risk factor was evaluated singly for its association with MCT outcome. Univariable proportional hazards models were developed for each risk factor for each outcome, and the level of association was assessed through the risk factor's p-value in the model. Risk factors with p less than or equal to 0.30 were considered for inclusion in the multivariable model.

Multivariable Survival Analysis: Proportional hazards regression models were developed for survival analysis of different outcomes associated with MCTs. These outcomes included days to local recurrence of MCTs at the site of the primary tumor resection, days to detection of MCTs at additional distant sites (outside the original surgical margins), and days to death associated directly with MCTs or with complications arising from MCTs.

Results

The age of the 100 dogs in this study ranged from 1 to 14 years with a mean age of 7.2 years. Fifty-one of the dogs in this study were spayed females, 36 castrated males, 7 intact females and 6 intact males (Table 1). Based on univariable and multivariable survival statistics, older dogs and male dogs with cutaneous MCTs had significantly shorter survival times (Tables 6, 7 and 8). Also older dogs

with cutaneous MCTs were at higher risk to develop additional MCTs at distant sites (Table 7). Univariable analysis determined that sterilized dogs with cutaneous MCTs had shorter survival times (Table 6). However, these findings were not confirmed by multivariable analysis. A total of 23 breeds were represented including Labrador retrievers (n=23), mixed breed dogs (n=22), boxers (n=11), golden retrievers (n=10), Boston terriers (n=4), Cocker spaniels (n=3) and Bassett hounds (n=3). Additional breeds had 1-2 representatives in this study (Table 1). Based on univariable and multivariable survival statistics, Boxers with cutaneous MCTs were at higher risk to develop additional MCTs at distant sites (Tables 6 and 7). Body weight was of no prognostic significance.

Information regarding tumor depth was available for 98 dogs with MCTs (Table 2). Six of the 98 dogs had MCTs with neoplastic cells restricted to the superficial dermis, 54 dogs had MCTs with cells extending from the superficial dermis into the deep dermis, 10 dogs had MCTs with neoplastic cells located only in the deep subcutaneous tissues, and 28 dogs had MCTs with cells invading the underlying musculature. Twenty-nine of the 98 (29.6%) dogs with MCTs that had been evaluated for tumor depth developed additional MCTs after the primary tumor had been surgically

removed. In 5 dogs (17.2%), MCTs developed at the site of the primary, surgically removed MCT, 18 dogs (62.1%) developed MCTs at distant sites (outside the original surgical margins) and 6 dogs developed MCTs at the site of the primary surgical site as well as at distant sites (outside the original surgical margins). A total of 27 of the 98 dogs evaluated for tumor depth died during the follow-up period (27.6%), of which 16 died of mast cell-related disease (61.5%). Using univariable and multivariable analyses, no prognostic significance was found for tumor depth (Tables 5 and 6).

Information regarding tumor location was available for 98 dogs with MCTs (Table 3). A total of 95 dogs had single or multiple synchronous MCTs located within only one of the previously described body locations. Three dogs had multiple synchronous MCTs located within two different body regions, therefore a total of 101 body locations was used to evaluated the prognostic significance of tumor location. Of these 101 tumor locations, 43 were identified on the trunk, 36 on the extremities, 11 on the head or neck and 11 in the inguinal region. Of the 98 dogs with MCTs evaluated for tumor location, 29 (29.6%) dogs developed additional MCTs after the primary tumor had been surgically removed. In 5 dogs (17.2%) MCTs developed at the site of the

primary, surgically removed MCT, 18 dogs (62.1%) developed MCTs at distant sites (outside the original surgical margins) and in 6 dogs developed MCTs at the site of the primary surgical site as well as at distant sites (outside the original surgical margins). Twenty-six (26.5%) of these 98 dogs died during the follow-up period, of which 16 (61.5%) died of mast cell-related disease. Univariable analysis found an increased risk to develop MCTs at distant sites for dogs with MCTs located on the head and neck (Tables 5 and 6). However, multivariable analysis did not confirm this finding and there was no prognostic significance for tumor location in dogs with cutaneous MCTs (Tables 7 and 8).

Ten dogs had multiple synchronous masses present at the time of diagnosis (Table 4). Three dogs had multiple masses present on the extremities, 3 dogs had multiple masses present on the trunk, 1 dog had 3 masses on the extremities and 1 inguinal mass, 1 dog had 1 mass on the extremities and 3 masses on the trunk, 1 dog had 2 masses on the extremities and 2 masses on the trunk, and 1 dog had multiple masses in the peri-anal region. Using univariable and multivariable analysis, dogs with multiple synchronous MCTs present at the time of diagnosis had a significant decrease in survival time.

Discussion

This study determined the significance of tumor depth, tumor location and the presence of multiple synchronous tumors at the time of diagnosis for the prognostic evaluation of canine cutaneous MCTs. Multiple synchronous MCTs at the time of diagnosis are associated with a decreased survival time of dogs treated with surgical excision alone. Based on multivariable analysis, no significant associations were found between tumor depth or tumor location and additional MCT development or survival time.

Only cutaneous MCTs from dogs treated with surgical excision alone were included in this study. MCTs occurring in mucous membranes or MCTs from dogs treated with additional therapies, including radiation and chemotherapy were excluded. It is important to recognize that the prognostic significance of different clinical and biological variables may vary depending on the applied treatment. Each variable should be evaluated in the context of a single treatment modality. Therefore, the results of this study can only be used to predict the prognosis for dogs in which surgical excision is used as a single treatment modality.

It is unclear whether multiple synchronous MCTs represent independent spontaneous neoplastic events, are part of a systemic disease process, or even represent metastasic disease. The MCTs that developed in this study at the site of the surgically removed primary mass most likely represent recurrent MCTs, whereas additional MCTs developing at distant sites (outside the original surgical margins) may either have developed independently from the primary MCT or represent metastases. There are currently no techniques available that would have allowed us to further elucidate this question.

The most significant finding of this study is the association of multiple synchronous MCTs at the time of diagnosis with decreased survival time. Previous studies did not find a similar association in dogs with cutaneous MCTs that had been treated with radiation or chemotherapy[26,27]. In one study, no difference was found in survival time between dogs with single MCTs and those with multiple cutaneous MCTs when treated with prednisone and vinblastine[26]. These data indicate that in the face of multiple synchronous cutaneous MCTs, chemotherapy is a more appropriate and efficacious treatment protocol. Based on these findings further therapeutic considerations beyond surgical excision, such as chemotherapy, should be

considered in the face of multiple MCTs at the time of diagnosis in order to obtain the best results for the patient.

Tumor depth is a commonly used component in the histologic grading of canine MCTs[14], but the significance of tumor depth as a sole prognostic indicator has not been previously evaluated. This study did not find tumor depth to be a useful parameter for predicting survival time of dogs with cutaneous MCTs following surgical removal only. Due to the retrospective design of this study we were not able to accurately evaluate tumor margins for several cases. Although we were confident with our evaluation of tumor depth in the vast majority of cases, a prospective study using inked margins is necessary to validate our results regarding the prognostic significance of tumor depth for canine cutaneous MCTs.

Most comments in the veterinary literature regarding the prognostic significance of the tumor location for canine cutaneous MCTs are based on clinical observations rather than statistically confirmed survival data. MCTs arising in the perineal/inguinal region are commonly regarded as having a worse prognosis compared to MCTs arising on the trunk[36-40]. This study investigated the prognostic significance of tumor location for dogs with

surgically removed MCTs. Even though univariable analysis found dogs with MCTs located on the head and neck to have an increased risk for developing MCTs at distant sites, multivariable analysis did not confirm this finding. More importantly, there was no significant association between any of the selected tumor locations and additional MCT development or survival time confirming previous investigations[12]. Similar results have been noted for MCTs treated with chemotherapy[26].

Based on this study, boxers and older dogs are inclined to develop multiple MCTs and male dogs with MCTs have a decreased survival time as compared to females. Several previous studies have found that boxers have a predilection for the development of cutaneous MCTs[2,8,12] and, although not well described in the literature, boxers are commonly thought to be predisposed to the development of multiple MCTs in a single patient. Our study confirms the results of previous studies and supports the hypothesis that boxers are predisposed to multi-centric mast cell disease. The predisposition for multiple MCTs in older dogs may be explained by the fact that older dogs have accumulated more mutations in their genomic DNA and are therefore more likely to develop cancer in general. It would be logical that these dogs have an increased

likelihood of having multiple transformed mast cells and therefore multiple MCTs at the time of diagnosis. In this study male dogs were also found to have decreased survival duration as compared to females. Similarly, a previous study found that female dogs treated with a multi-agent chemotherapeutic protocol had a more favorable prognosis as compared to males[149]. Estrogen and progesterone receptors have been found in canine MCTs, and may explain this difference in survival between males and females, however, the exact role of hormone receptors in canine MCTs has not been elucidated[149,150]. Future studies are necessary to verify the sex-specific differences in survival for dogs with cutaneous MCTs and to determine the role of hormones and hormone receptors in the development of canine cutaneous MCTs.

This study defines the prognostic significance of tumor depth, location and multiple synchronous tumors for surgically removed canine cutaneous MCTs. It should be emphasized that no single clinical parameter can be used to accurately define the prognosis for every given patient. Only by evaluating multiple prognostically significant parameters concurrently can the biological behavior of canine cutaneous MCTs be more accurately predicted. Additionally, new technologies based on the molecular

biology of MCTs are needed to elucidate the relationship of multiple cutaneous MCTs occurring in a single dog as well as to identify MCTs with metastatic potential.

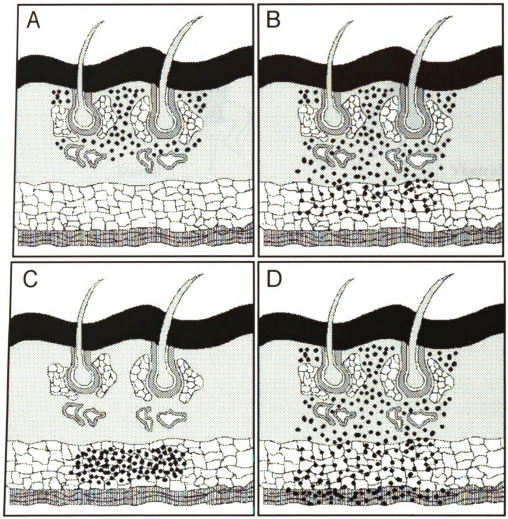

Figure 1 (A-D): Tumor depths identified for prognostic evaluation. A. superficial dermis. Neoplastic mast cells lie immediately below the epidermis and do not extend below the adnexal structures. B. Superficial and deep dermis. Neoplastic mast cells extend from the superficial dermis, immediately below the epidermis and extend into the deep dermis and subcutaneous adipose tissue. C. Deep dermis. Neoplastic mast cells are isolated in the deep dermis and do not extend into the peri-adnexal region. D. Muscularature invasion. Neoplastic mast cells extend and invade into the underlying muscularature.

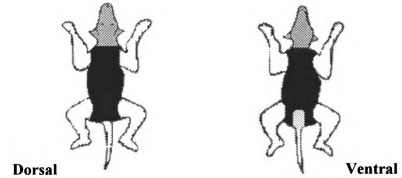

Figure 2: Tumor location was defined for prognostic purposes as: 1. Head and neck (dark gray); 2. Trunk (black); 3. Extremities (white); 4. Inguinal/ perineal region (light gray).

Table 1: Mast cell tumor outcomes by signalment

		Additional MCT Development						Death due to MCT		
		Local			Distant					
	Risk Factor	X^2 p	Odds Ratio	95% C.I.	X^2 p	Odds Ratio	95% C.I.	X^2 p	Odds Ratio	95% C.I.
Age	< 3 yrs	.9949	-	-	.9906	-	-	.9926	-	-
	3 – 5 yrs	.1673	.23	.03 – 1.85	.2447	.56	.21 – 1.50	.0520	.13	.02 – 1.02
	6 – 9 yrs	.3771	1.81	.49 – 6.75	.9023	1.05	.46 – 2.40	.2260	1.84	.69 – 4.95
	> 9 yrs	.3883	1.85	.46 – 7.54	.1004	2.06	.87 – 4.90	.1566	2.09	.75 – 5.78
Wt. (lbs)	< 10 lbs	.9954	-	-	.9913	-	-	.9935	-	-
	10 – 30 lbs	.0710	3.60	.90 – 14.45	.9895	-	-	.8064	1.21	.27 – 5.41
	30 – 50 lbs	.5105	.50	.06 – 4.01	.4230	1.47	.57 – 3.81	.5034	1.49	.47 – 4.75
	50 – 70 lbs	.2869	.32	.04 – 2.60	.4119	.63	.21 – 1.84	.1418	.22	.03 – 1.67
	> 70 lbs	.7477	1.24	.33 – 4.62	.1620	1.84	.78 – 4.35	.4221	1.54	.54 – 4.38
Sex	Male	.2815	2.07	.55 – 7.74	.6561	1.21	.53 – 2.75	.0206	2.23	1.13 – 4.37
	Sterile	.9644	1.05	.13 – 8.41	.2165	.51	.17 – 1.49	.0138	.26	.09 – .76
Breed	Mixed	.4530	1.70	.43 – 6.81	.2268	.47	.14 – 1.59	.8661	1.10	.36 – 3.42
	Labrador	.3315	.36	.04 – 2.87	.4667	1.41	.58 – 3.43	.9340	1.05	.34 – 3.26
	Golden Retriever	.7199	1.46	.18 – 11.77	.9384	1.06	.25 – 4.52	.5211	1.63	.37 – 7.16
	Boxer	.9941	.99	.12 – 7.96	.2768	1.82	.62 – 5.36	.8497	1.15	.26 – 5.09
	Other	.9518	1.04	.26 – 4.19	.7446	.86	.36 – 2.10	.4716	.66	.21 – 2.05

Table 2: Distribution of additional MCT development and mortality among dogs with MCTs of different tumor depths.

Depth	Additional MCT Development*		Mortality	
	Local	Distant	MCT Deaths	Total Deaths
Superficial Dermis (n=6)	1 (16.7%)	0 (0.0%)	1 (16.7%)	2 (33.3%)
Superficial/ Deep Dermis (n=54)	7 (13.0%)	15 (27.8%)	8 (14.8%)	16 (29.6%)
Deep Dermis (n=10)	0 (0.0%)	2 (20.0%)	0 (0.0%)	1 (1.0%)
Muscle Invasion (n=28)	3 (10.7%)	7 (25.0%)	7 (25.0%)	7 (25.0%)

* Six dogs had local and distant MCT development (total number of dogs in column is 29)

Table 3: Distribution of additional MCT development and mortality among dogs with MCTs in different body locations.

Location*	Additional MCT Development†		Mortality£	
	Local	Distant	MCT Deaths	Total Deaths
Head/ Neck (n=11)	3 (27.3%)	6 (54.5%)	3 (27.3%)	4 (36.4%)
Extremeties (n=36)	4 (11.1%)	8 (22.2%)	3 (83%)	10 (27.8%)
Trunk (n=43)	5 (11.6%)	11 (25.6%)	7 (16.3%)	12 (27.9%)
Inguinal/ Perineal (n=11)	0 (0.0%)	0 (0.0%)	3 (27.3%)	4 (36.4%)

* Three dogs had multiple synchronous MCTs at 2 distinct locations (total number of locations in column is 101)
† Six dogs had local and distant MCT development and 2 of the dogs with synchronous MCTs had also additional MCT development accounting for 2 additional locations (total number of dogs in column is 29)
£ Three dogs had multiple synchronous MCTs at 2 distinct locations and died during follow-up accounting for the 3 additional events in the table (total number of dogs in column is 27)

Table 4: Distribution of additional MCT development and mortality among dogs with multiple synchronous mast cell tumors.

	Additional MCT Development		Mortality	
	Local	Distant	MCT Deaths	Total Deaths
Multiple Synchronous Tumors (n=10)	3 (30%)	2 (20%)	2 (20%)	5 (50%)

Table 5: Univariate analysis of risk factors for MCT outcomes by body location and depth.

Outcome	Factor	Level	X^2 p	Odds Ratio	95% C.I.
Local MCT Develop.	Locat.	Head-neck	.2956	2.32	.48 - 11.19
		Extremities	.8650	.89	.22 - 3.55
		Trunk	.5227	1.54	.41 - 5.72
		Inguinal	.9946	-	-
	Depth	Dermis/ reticularis only	.5630	1.85	.23 - 14.83
		Dermis to subcutaneous tissues	.8161	1.07	.29 - 4.01
		Subcutaneous tissues only	.9933	-	-
		Skeletal muscle layers	.7103	1.30	.33 - 5.21
Distant MCT Develop.	Locat.	Head-neck	.0332	2.94	1.09 - 7.95
		Extremities	.6264	.80	.33 - 1.95
		Trunk	.8996	1.05	.46 - 2.40
		Inguinal	.4949	.50	.07 - 3.70
	Depth	Dermis/reticularis only	.9918	-	-
		Dermis to subcutaneous tissues	.4855	1.35	.58 - 3.12
		Subcutaneous tissues only	.6075	.68	.16 - 2.92
		Skeletal muscle layers	.7574	1.16	.48 - 2.83
Death due to MCT	Locat.	Head-neck	.2958	1.95	.56 - 6.86

Table 6: Unviariate analysis of risk factors for MCT outcomes (significant @ $p < 0.3$)

Outcome	Risk Factor	Chi-square p	Odds Ratio	95% C.I.
Local MCT Development				
	Age	.1282	1.98	.82 - 4.76
	Golden Retriever	.1286	1.46	.18 - 11.77
	Male	.2815	2.07	.55 - 7.74
	Location: head-neck	.2956	2.32	.48 - 11.19
Distant MCT Development	Animal age	.0503	1.70	1.00 - 2.91
	Mixed Breed	.2268	.47	.14 - 1.59
	Boxer	.2768	1.82	.62 - 5.36
	Sterilized	.2165	.51	.17 - 1.49
	Location: head-neck	.0332	2.94	1.09 - 7.95
Death due to MCT	Age	.0200	2.28	1.13 - 4.37
	Male	.0086	4.56	1.47 - 14.16
	Sterilized	.0138	.26	.09 - .76
	Depth: Skeletal muscle layer	.1711	1.96	.74 - 5.37
	Location: Head-neck	.2958	1.95	.56 - 6.86
	Location: Extremities	.1678	.41	.12 - 1.45
	Location: Inguinal	.1312	2.64	.75 - 9.34

Table 7: Reduced* multivariate proportional hazard regression model for survival analysis for days to distant MCT development after the original diagnosis.
* Initial model contained age, sex, sterilization, breed, multiple lesions, tumor location, and tumor depth

Risk Factor	Wald X2	p	Hazard Ratio	95% C.I.
Location: head-neck[a]	3.53	0.0601	3.33	0.95-11.69
Age	5.98	0.0145	2.16	1.17-4.01
Boxer	3.89	0.0487	2.92	1.01-8.49

Model Likelihood Ratio X^2 = 18.98, 5 d.f., p = 0.0019
a - Retained in model to control for confounding

Table 8: Reduced* multivariate proportional hazards regression model for survival analysis for death.
* - Initial model contained age, sex, sterilization, breed, multiple synchronous tumors, tumor location, and tumor depth

Risk Factor	Wald X2	p	Hazard Ratio	95% C.I.
Age	12.31	.0005	3.34	1.70 - 6.55
Male	5.42	.0199	2.85	1.18 - 6.89
Multiple synchronous tumors	6.51	.0107	4.60	1.42 - 14.83
Location: head-neck[a]	3.50	.0613	3.93	.94 - 16.43

Model Likelihood Ratio X2 = 39.94, 7 d.f., p < .0001
a - Retained in model to control for confounding

CHAPTER 2

Webster JD, Kiupel M, Kaneene JB, Miller RA, Yuzbasiyan-Gurkan V (2004). Use of KIT and tryptase expression patterns as prognostic tools for canine cutaneous mast cell tumors. Veterinary Pathology. 41:371-377.

CHAPTER 2

USE OF KIT AND TRYPTASE EXPRESSION PATTERNS AS PROGNOSTIC TOOLS FOR CANINE CUTANEOUS MAST CELL TUMORS

Introduction

Canine cutaneous mast cell tumors (MCT) are one of the most common neoplasms in dogs, accounting for 7-21% of all cutaneous tumors in dogs[1-4]. The biological behavior of canine MCTs is extremely variable. Clinically, MCTs can range from a single benign mass that may be cured with complete surgical excision, to fatal metastatic disease[12,14,96]. In one study of 114 dogs, 38% of the dogs died or were destroyed as a result of their MCT within 2½ years following diagnosis[12].

Currently, prognostic and therapeutic determinations of MCTs are based primarily on their histologic grade. Several histologic grading systems have been developed in an attempt to correlate the cellular morphology of the neoplasm with the overall survival of the dog[12,14]. The most commonly used grading system defines grade I MCTs as being the most differentiated, while grade III MCTs are the least differentiated. Grade II MCTs are of intermediate differentiation[14]. These grading systems have

shown a significant difference in survival time between well-differentiated tumors and poorly differentiated tumors[13,14], but there is still much debate over their relevance, especially when dealing with intermediate grade MCTs[8,17,24] that account for more than 40% of all MCTs[14]. The goal of this study was to evaluate the prognostic significance of KIT and tryptase immunohistochemical staining patterns in canine cutaneous mast cell tumors.

The KIT protein is a tyrosine kinase receptor that is a product of the *c-KIT* proto-oncogene[97], which is expressed in numerous tissues including glioblastoma cells, term placenta, brain, erythroid precursors, melanocytes, basophils and mast cells[97,100,107]. The ligand for the KIT receptor, stem cell factor (SCF), also called mast cell growth factor, has multiple effects on mast cells including proliferation, maturation, migration, degranulation, suppression of apoptosis and adhesion to fibronectin[106-111].

Mutations in the juxtamembrane coding region of the *c-KIT* proto-oncogene have been identified in several canine cutaneous MCTs[16,91,93,95,96]. Tandem duplication mutations in this region have been shown to constitutively activate the KIT tyrosine kinase despite the absence of the SCF

ligand[91,95]. It has been proposed that mutations in the juxtamembrane domain may play a critical role in the neoplastic transformation of mast cells tumors in dogs[91,95,96]. In one study, mutations in the juxtamembrane domain were more prevalent in histologic grade II and III MCTs compared to grade I MCTs[96]. In another study, MCTs with tandem duplication mutations were twice as likely to recur and twice as likely to metastasize as those without the mutation, although the association between recurrence and metastasis, and the presence of the mutation was not statistically significant[16].

Expression of the KIT receptor in MCTs, and the detection of the KIT receptor using immunohistochemistry, has been well established[92,94]. Different patterns of KIT expression have been described in normal mast cells and in neoplastic mast cells. Normal mast cells and some neoplastic mast cells express KIT mainly on the cell membrane, whereas in many neoplastic mast cells KIT accumulates in the cytoplasm, primarily adjacent to the nucleus[94]. A correlation between the expression of the KIT receptor and the histologic grade of MCTs has been made, with well-differentiated tumors weakly expressing KIT and poorly differentiated tumors having a high expression of KIT[94]. We hypothesized that the different patterns of KIT

receptor expression in canine cutaneous MCTs correlate with their biologic behavior. We also hypothesized that more benign tumor cells have weak KIT expression limited to the membrane only, and malignant tumor cells have stronger cytoplasmic expression of the KIT receptor.

Tryptase is one of the most common neutral proteases found in mast cells[151,152]. Identification of tryptase using immunohistochemistry has shown excellent specificity and sensitivity to mast cells[153,154] Poorly differentiated MCTs tend to have fewer granules, and a generally poorer staining pattern when stained with hematoxylin and eosin, toluidine blue, alcian blue and giemsa, when compared to well differentiated tumors[155]. It has been assumed that this decrease in the staining pattern is due to the lack of production or storage of cellular components that react with the stain[155]. We hypothesized that well differentiated MCTs have increased cytoplasmic expression of tryptase than poorly differentiated MCTs, and that such a difference in expression could be used to predict the biologic behavior of canine cutaneous MCTs. To test these hypotheses a retro-prospective study was conducted.

Materials and Methods

Source of MCTs

One hundred cases of canine cutaneous MCTs were selected from over 1,000 cases of MCTs submitted to the Diagnostic Center for Population and Animal Health at Michigan State University between 1998 and 2001. All cases were submitted as routine biopsy specimens from a total of 8 different veterinary hospitals. Tissues had been fixed in 10% neutral buffered formalin for an average of 24-30 hrs and were routinely dehydrated in graded alcohol and paraffin embedded. Case selection criteria included: 1. original diagnosis (by an MSU pathologist) of a cutaneous MCT, 2. surgical excision as the only treatment, 3. availability of sufficient amounts of tissue (formalin-fixed, paraffin embedded) for additional testing, and 4. complete history and follow-up data. The original diagnosis of canine cutaneous MCT was independently confirmed and histologically graded based on the Patnaik histologic grading system by 2 board certified pathologists. A complete history and follow-up information for each case was obtained from the referring veterinarians including age, sex, breed, weight, number of masses, location of mass, time before excision, medication at the time of surgery, diagnostic tests that were performed, recurrence,

tumor margins, metastasis, survival time and cause of death. For the purpose of the study, recurrence was defined as clinical reappearance of a mass at the initial tumor site. A distant recurrence was defined as the development of an additional mass at a site distant from the original mass observed. Ninety-six of the tumors were stained with both anti-tryptase antibodies and anti-KIT antibodies. Two of the tumors were stained with anti-tryptase antibodies only and 2 of the tumors were stained with anti-*c-KIT* antibodies only.

Immunohistochemistry

All MCTs had been fixed in 10% neutral buffered formalin. Sections were cut at a thickness of 5 μm, deparaffinized in xylene, rehydrated in graded ethanol and rinsed in distilled water. Endogenous peroxidases were neutralized with 3% hydrogen peroxide for 5 minutes followed by rinsing for 5 minutes in distilled water. Antigen retrieval was achieved by incubating slides in antigen retrieval solution in a steamer (Black & Decker, Towson, MD) for 20 min. Non-specific immunoglobulin binding was blocked by incubation of slides for 10 min with a protein-blocking agent (Dako, Carpinteria, CA) prior to application of the primary antibody. Using an autostainer, slides were incubated for 30 minutes with a mouse anti-human mast cell tryptase

antibody (Dako, Carpenteria, CA) at a dilution of 1:100, and a rabbit anti-human *c-KIT* antibody (Dako, Carpenteria, CA) at a dilution of 1:100, respectively. A streptavidin-immunoperoxidase staining procedure (Dako, Carpinteria, CA) was used for immunolabeling. The immunoreaction was "visualized" with 3,3'-diaminobenzidine substrate (Dako, Carpinteria, CA). Sections were counterstained with Mayer's haematoxylin. Positive immunohistochemical controls included known MCTs. Negative immunohistochemical controls (Figure 3D) were known MCTs treated identically as routine sections, with 20 minute antigen retrieval and 10 minute protein blocking, except the 30 minute incubation with primary antibodies was replaced with a 30 minute incubation with buffer.

Grading of c-KIT staining pattern

Non-neoplastic mast cells exhibit faint staining of their membrane for KIT and have no cytoplasmic staining. MCTs were divided into 3 groups based on their KIT immunostaining pattern (Figures 3A-D). MCTs with KIT staining pattern I consisted of neoplastic mast cells that stained primarily along the cytoplasmic membrane with only minimal cytoplasmic staining (Figure 3A). The staining intensity for this pattern varied from neoplastic mast cells with extremely faint membrane-associated staining and

no cytoplasmic staining, to cells with intense membrane-associated staining and small amounts of cytoplasmic staining. KIT staining pattern II was characterized by neoplastic mast cells with either an intense, focally clustered cytoplasmic KIT staining or strong positive stippling throughout cytoplasm (Figure 3B). Membrane-associated staining was not as prominent of a feature of these cells. KIT staining pattern III was defined by neoplastic mast cells with diffuse cytoplasmic staining, obscuring all other cytoplasmic features (Figure 3C). Each MCT was assigned one of these 3 staining patterns based on the highest staining pattern (staining pattern I vs. II vs III) present in at least 10% (estimated based on 100 neoplastic cells in a high power field) of the neoplastic cell population or being present in large clusters of neoplastic cells within the tumor. Cells on the margins of the tissue sections were not considered for classification to avoid possible artifactual staining.

Grading of tryptase staining pattern

MCTs were classified into 3 groups based on their different tryptase staining patterns. Tryptase staining pattern I was characterized by neoplastic mast cells that were diffusely positive for tryptase throughout the cytoplasm, obscuring all other cytoplasmic features (figure

3E). MCTs with tryptase staining pattern II consisted of neoplastic mast cells with moderate amounts of stippling throughout the cytoplasm. In MCTs with tryptase staining pattern III, neoplastic mast cells had weak cytoplasmic stippling (Figure 3F). Due to the presence of occasional degranulated cells within MCTs, each MCT was assigned to 1 of these 3 tryptase staining patterns based on the predominate staining found in the majority of neoplastic cells throughout the section, instead of being classified based on the highest staining pattern present. Cells on the margins of the sections were not considered for classification to avoid possible artifactual staining.

Statistics

Univariable Analyses: Before developing multivariable models, each risk factor (prognostic factor) was evaluated for its association with MCT outcomes. Univariable proportional hazards models were developed for each risk factor for each outcome, and the level of association was assessed through the risk factor's p-value in the model. Risk factors with p less than or equal to 0.20 were considered for inclusion in the multivariable model.

Multivariable Survival Analysis Models: Proportional hazards regression models were developed for survival analysis of different outcomes associated with MCTs. These

outcomes included days to local recurrence of MCT, days to distant recurrence, and days to death associated directly to MCT or to complications arising from MCT. Additionally, models were developed for days to death by any cause (including MCT), for comparison with days to death associated with MCT.

Results

A total of 100 cases of canine cutaneous MCTs were included in this study. There was no age or sex predilection in this study. The sex distribution of the cases consisted of 7 intact and 49 spayed female dogs, as well as 5 intact and 38 castrated male dogs and 1 male dog with an unknown alteration status. A total of 22 dog breeds were represented in this study, including 24 Labrador retrievers, 12 boxers, 9 golden retrievers, 5 Boston terriers, 3 basset hounds, 3 cocker spaniels and 22 mixed breed dogs. Fifteen additional breeds were represented by 1 or 2 dogs each. The distribution of the cases according to the Patnaik histologic grading system consisted of 17 grade I MCTs, 72 grade II and 11 grade III MCTs.

The majority of MCTs in this study had either KIT staining pattern I (42.9%) or II (43.9%) (Table 9). A total of 28 of 98 (28.6%) dogs died during the follow-up period. Of these, 17 (60.7%) dogs died due to complications

associated with their MCTs. Thirty-four (34.7%) of 98 MCTs from 98 dogs that had been stained for KIT had recurrent MCTs.

Fifteen of 98 (15.3%) MCTs were classified as having tryptase staining pattern I, 59 (60.2%) MCTs as having tryptase staining pattern II and 24 (24.5%) MCTs as having tryptase staining pattern III (Table 10). Thirty-five (35.7%) of 98 MCTs from 98 dogs that had been stained for tryptase had recurrent MCTs. Eleven (31.4%) of these dogs had local recurrence at the site of the previous tumor, whereas 24 (68.6%) of these dogs had recurrence at a distant site. A total of 29 of 98 (29.6%) dogs died during the follow-up period. Of these, 18 (62.1%) dogs died due to complications associated with their MCTs. Using uni- and multi-variate survival statistics, the KIT staining patterns II and III were both significantly associated with a higher risk of local recurrence of MCTs (Figure 4) and a shorter overall survival time due to mast cell disease (Figure 5). Of the 3 classes of tryptase staining patterns, none were found to be significantly associated with disease-free survival, overall survival of the animal or local recurrence (Table 11).

Discussion

Our current results offer a new variable upon which a novel classification of canine cutaneous mast cell tumors can be made. In this study, we have shown that peri-membrane KIT staining of neoplastic mast cells is not associated with recurrence or a decrease in survival time, but cytoplasmic stippling with decreased peri-membrane staining and diffuse cytoplasmic KIT staining are associated with both an increased rate of recurrence and a decreased survival time. With these results we propose a new system for the prognostic grading of canine cutaneous mast cell tumors based on KIT immunostaining patterns. This system can be used as a valuable tool for the routine prognostic evaluation of canine cutaneous mast cell tumors, and can be used to help clarify the ambiguity of the current histologic grading system. Additionally, our results show that tryptase staining patterns have no prognostic value in the evaluation of canine MCTs.

The KIT receptor is a transmembrane protein[97], and as such, immunoreactivity of this protein is localized to the cytoplasmic membrane of non-neoplastic mast cells. In dogs, non-neoplastic mast cells have been shown to express KIT exclusively along the cell membrane, while MCTs

histologically graded as II or III according to Patnaik et al.[14] predominately expressed KIT in their cytoplasm[94]. Our results confirm that a more aggressive biological behavior of canine cutaneous MCTs is associated with the increase of cytoplasmic staining for KIT. In this study, membrane-associated staining for KIT was not associated with local or distant recurrence of MCTs or decreased survival time. This coincides with the idea that the KIT receptor is present along the cytoplasmic membrane of well-differentiated mast cells, and therefore neoplastic mast cells with predominately have membrane-associated KIT expression have a lower degree of malignant transformation and exhibit benign biological behavior. In contrast, this study demonstrated that canine cutaneous MCTs with an increased expression of KIT in the cytoplasm of neoplastic mast cells had an increased risk of local recurrence, and a decreased survival time, both overall and due to complications of the mast cell disease.

The more aggressive biologic behavior of MCTs with an increased KIT expression may be explained by the functional roles that KIT and its ligand, stem cell factor, (SCF) play in mast cell development. KIT and SCF have been shown to mediate numerous roles in mast cell development, including proliferation, maturation, inhibition of apoptosis,

adhesion and migration[97,106,108-111]. The exact mechanism by which increased KIT expression causes the malignant transformation of MCTs is unknown. As has been suggested by others[94], we hypothesize that the cytoplasmic isoform of KIT may be activated by soluble SCF or it may contain constitutively activating mutations, which then lead to the inhibition of apoptosis, and mast cell adhesion, migration and proliferation. Further studies are needed to define the exact molecular role that this increase in KIT expression plays in the neoplastic transformation of canine MCTs.

Several studies of canine MCTs have described mutations in the *c-KIT* proto-oncogene[16,91,93,95,96]. These mutations produce a constitutively activated product[91,95], and have also been suggested to play a role in the malignant transformation of MCTs[96]. Our results suggest that on over-expression of the *c-KIT* gene may be a key factor in the malignant transformation of MCTs. It is currently unknown whether mutations in the *c-KIT* proto-oncogene and the increased expression of the *c-KIT* gene are co-dependent or independent events. Additionally, if these are independent events it is unknown whether there is a synergistic relationship between the 2 events, resulting in a more aggressive biological behavior.

Cellular differentiation has been commonly used as an indicator of the biologic behavior of neoplasms. In previous studies it has been demonstrated that poorly differentiated MCTs tend to have fewer cytoplasmic granules than well-differentiated tumors[155]. Since tryptase is one of the most common neutral proteases present in mast cell granules, we hypothesized that MCTs with decreased staining with anti-tryptase antibodies are poorly differentiated and therefore have a more malignant biological behavior and a worse prognosis. However, this study did not demonstrate a significant association between the tryptase staining pattern and the biologic behavior of canine cutaneous MCTs following surgical removal.

The biological behavior of canine cutaneous MCTs is highly variable, and our current ability to give an accurate prognosis for these tumors is hampered by the large number of intermediate grade MCTs according to the histological classification. Our studies demonstrate that the evaluation of the immunohistochemical staining pattern of KIT can improve the prognostication of canine cutaneous MCTs. Expression of KIT was prognostically significant for surgically removed canine cutaneous MCTs. Therefore, we propose a new prognostic classification of canine cutaneous MCTs based on their KIT staining pattern. For canine

cutaneous MCTs that have been treated with surgical removal only, an increased cytoplasmic KIT staining is a strong indicator for local recurrence and shorter survival, therefore supporting a more aggressive therapy of such neoplasms, such as radiation. Further studies are necessary to validate this classification for other treatment protocols and to elucidate the role of an increased KIT expression in the tumorigenesis of canine cutaneous MCTs.

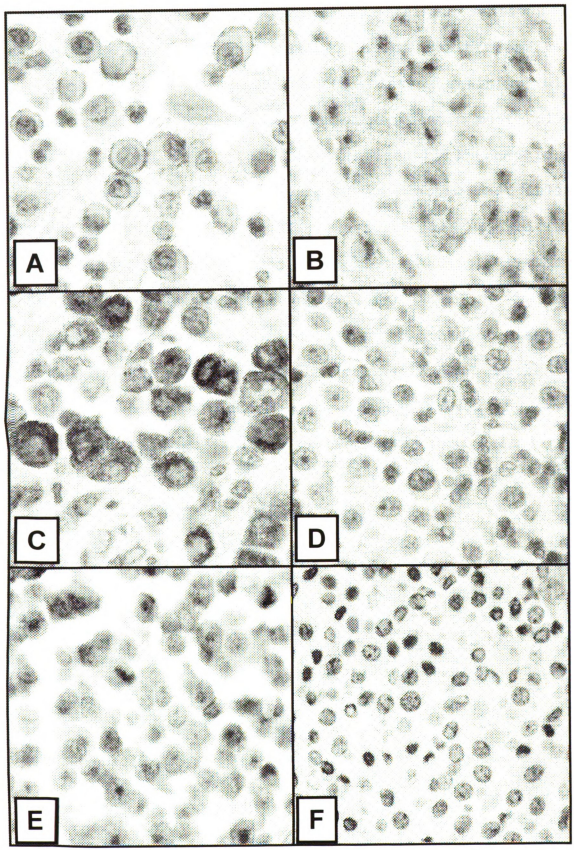
A
B
C
D
E
F

Figure 3(A-F): Immunohistochemically stained sections of canine cutaneous mast cell tumors. A: KIT staining pattern I, characterized by membrane associated staining with little to no cytoplasmic staining of neoplastic mast cells. B: KIT staining pattern II, characterized by intense focal or stippled cytoplasmic staining of neoplastic mast cells. C: KIT staining pattern III, characterized by diffuse cytoplasmic staining of neoplastic mast cells. D: Negative control of cutaneous mast cell tumor with no visible staining. E: Tryptase staining pattern I, characterized by diffuse cytoplasmic staining of neoplastic mast cells. F: Tryptase staining pattern III, characterized by weak cytoplasmic stippled staining of neoplastic mast cells.

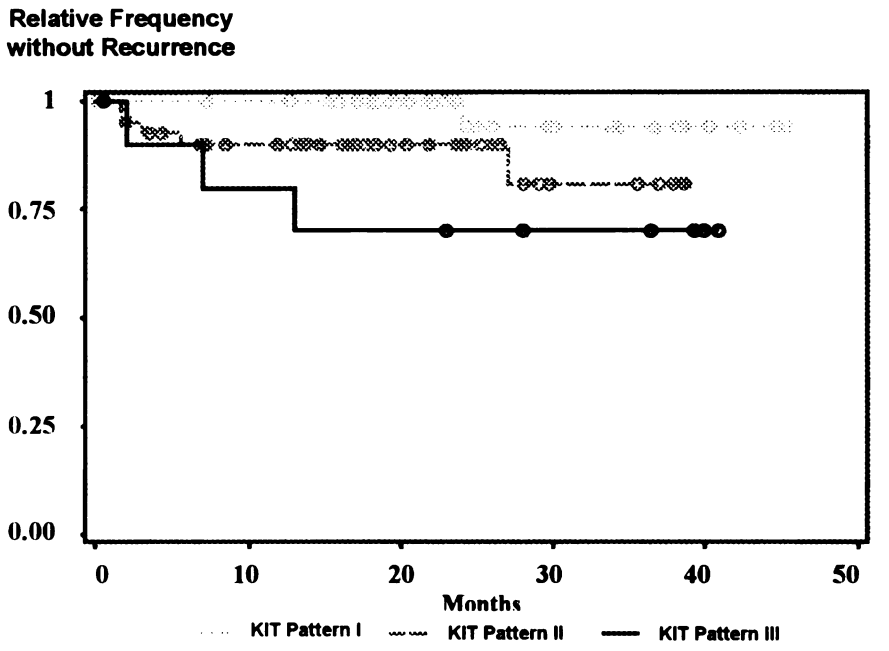

Figure 4: Local recurrence survival curve for dogs with cutaneous mast cell tumors with different KIT staining patterns.

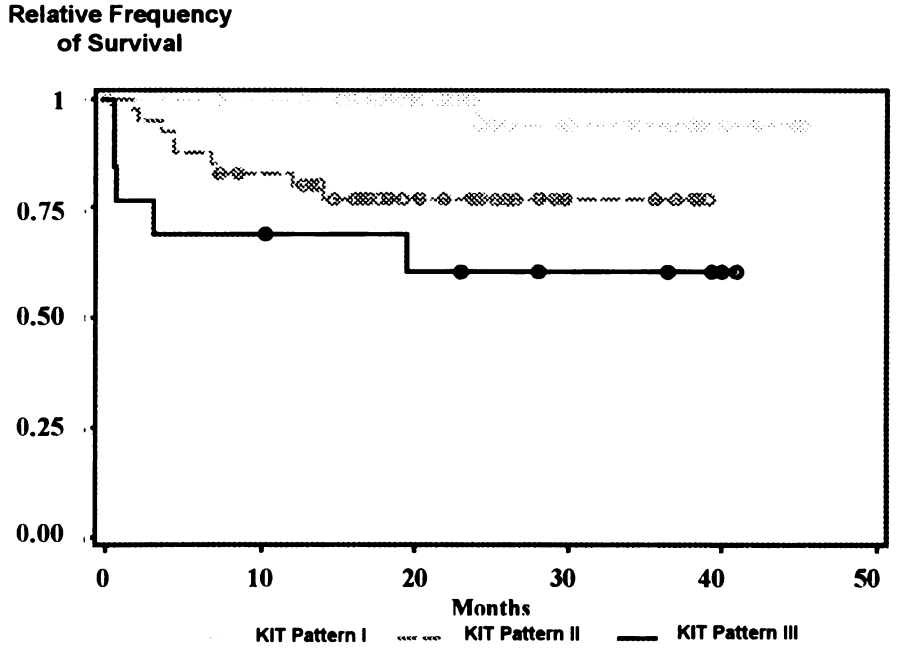

Figure 5: Overall survival curve for dogs with cutaneous mast cell tumors with different KIT staining patterns.

Table 9: Distribution of recurrent disease and deaths among KIT staining patterns of canine cutaneous MCTs. Percents are listed as the number of recurrences or deaths within each staining pattern.

KIT Staining Pattern	Recurrent Disease		Mortality	
	Local	Distant	Mast Cell Disease	Total
Staining Pattern I (n=42)	1 (2.4%)	6 (14.3%)	1 (2.4%)	6 (14.3%)
Staining Pattern II (n=43)	6 (14.0%)	13 (31.0%)	11 (25.6%)	17 (39.5%)
Staining Pattern III (n=13)	3 (23.1%)	5 (38.5%)	5 (38.5%)	5 (38.5%)
Total	10	24	17	28

Table 10: Multivariate analysis results of KIT staining patterns II and III in canine cutaneous MCTs predictive ability for recurrence, death due to MCT and death due to any cause.

KIT Staining Pattern	Local recurrence		Death due to MCT		Death due to any cause	
	p-value	Hazard ratio	p-value	Hazard ratio	p-value	Hazard ratio
Staining Pattern II	0.0079	35.82	0.0056	20.10	0.004	7.52
Staining Pattern III	0.0131	51.14	0.0004	76.54	0.0192	6.03

Table 11: Distribution of recurrent diseases and deaths among tryptase staining patterns of canine cutaneous MCTs. Percents are listed as the number of recurrences or deaths within each staining pattern.

Tryptase Staining Patterns	Recurrent Disease		Mortality	
	Local	Distant	Mast Cell Disease	Total
Staining Pattern I (n=15)	2 (13.3%)	7 (46.7%)	3 (20.0%)	6 (13.7%)
Staining Pattern II (n=59)	7 (11.9%)	12 (20.3%)	8 (13.6%)	14 (23.7%)
Staining Pattern III (n=24)	2 (8.3%)	5 (20.8%)	7 (29.2%)	9 (37.5%)
Total	11	24	18	29

CHAPTER 3

Webster JD, Yuzbasiyan-Gurkan V, Kaneene JB, Miller RA, Resau JH, Kiupel M (2006). The role of *c-KIT* in tumorigenesis: evaluation in canine cutaneous mast cell tumors. Neoplasia 8:104-111

CHAPTER 3

THE ROLE OF *c-KIT* IN TUMORIGENESIS: EVALUATION IN CANINE CUTANEOUS MAST CELL TUMORS

Introduction

The *c-KIT* proto-oncogene encodes the receptor tyrosine kinase KIT, which consists of an extracellular ligand binding domain composed of 5 immunoglobulin-like loops, a transmembrane domain, a negative regulatory juxtamembrane domain, and a split cytoplasmic kinase domain[97-99]. The ligand for KIT is stem cell factor, which is also known as steel factor, KIT ligand, or mast cell growth factor[106,113-115]. The receptor tyrosine kinase KIT is expressed by multiple cell types including hematopoietic progenitor cells, germ cells, interstitial cells of Cajal, melanocytes, and mast cells, where it has been associated with cell survival, proliferation, and differentiation[100-105,196]. In addition to these functions, in mast cells, KIT has been shown to be important for fibronectin adhesion, chemotaxis, and degranulation[106-111].

Recently, *c-KIT* has been implicated in the pathogenesis of multiple human neoplastic diseases. *c-KIT* mutations, which lead to a constitutively activated KIT product in the absence of ligand, have been identified in the juxtamembrane domain of gastrointestinal stromal tumors

(GISTs) in humans[118], and in the kinase domain at codon 816 of human mastocytosis patients[122,123,135]. Additionally, aberrant KIT expression is increasingly being identified in multiple neoplasms including small cell lung cancer, prostate cancer, and acute myeloblastic leukemia[127-131,157]. The significance of this aberrant expression has been determined for some of these cancers, such as small cell lung cancer where autocrine and paracrine signaling loops have been identified[127,129], and in prostate cancer, where truncated isoforms of KIT that signal through phospholipase C-γ1 have been characterized[130,143]. However, for several other cancers, the significance of this aberrant expression has not been elucidated.

Activating *c-KIT* mutations[13,91,95,96] and aberrant KIT expression has also been described in canine cutaneous mast cell tumors (MCTs)[92,94,144,158,159], therefore implicating *c-KIT* in their pathogenesis. Unlike mastocytosis in humans, which is a rather rare condition and usually has a positive prognosis[9-11], canine cutaneous MCTs are one of the most common neoplastic diseases in dogs, accounting for 7-21% of all cutaneous neoplasms[1-4], and have an extremely variable biologic behavior ranging from a benign mass to a fatal metastatic disease[12-14]. Canine cutaneous MCTs commonly present as a solitary neoplastic mass in the skin and/or

subcutaneous tissue of older dogs, with mean age of onset of approximately 9 years of age. There is no reported sex predilection[5,7]. All breeds of dogs are affected by MCTs, but several breeds such as the boxer, Boston terrier, bulldog, Weimaraner, and Labrador retriever have been suggested to have an increased incidence of the disease[2,8]. Prognostic and therapeutic determinations for canine cutaneous MCTs are commonly based on histologic grading. Several histologic grading systems have been developed for the evaluation of canine cutaneous MCTs[12,14]. The most commonly used system is that proposed by Patnaik et al.[14] and defines grade I MCTs as being well-differentiated tumors with a good prognosis, grade III MCTs as being poorly-differentiated tumors with a poor prognosis, and grade II MCTs as being of intermediated differentiation with an intermediate prognosis.

c-KIT mutations have been identified in the juxtamembrane domain, primarily in exon 11, of canine MCTs and consist of internal tandem duplications (ITDs) and deletions[13,16,91,93,95,96,145,146]. Internal tandem duplication *c-KIT* mutations were identified in 9% of canine MCTs in one study that looked at the mutation status of 88 randomly selected MCTs[96], but these mutations may occur in as many as 30-50% of all intermediate to high grade MCTs[16]. All except

for one of the previously described ITDs are in-frame duplications that range from approximately 39-69 bp in size[13,16,91,95,96,145,146] and all of the mutations that have been characterized thus far produce a constitutively activated form of KIT in the absence of ligand[91,95,145]. Previous work by our laboratory has shown that *c-KIT* mutations are significantly associated with histologically higher grade canine MCTs[96]. Recently, our laboratory has also shown that increased cytoplasmic localization of KIT in canine MCTs is significantly associated with a decreased survival duration and disease-free interval as compared to MCTs with peri-membrane KIT localization[159].

The goal of this study was to define the prognostic significance of *c-KIT* mutations, and the associations between *c-KIT* mutations, KIT localization, and KIT expression levels in canine MCTs. Mutations in the *c-KIT*'s juxtamembrane domain were identified in 15% of the MCTs examined, using laser capture microdissection and PCR amplification. This is the first study to show that *c-KIT* mutations in canine MCTs are significantly associated with decreased disease-free and overall survival, and that a significant relationship between KIT protein localization and the presence of *c-KIT* mutations exists in canine MCTs. These data clearly implicate an important role of *c-KIT* in

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the progression of canine cutaneous MCTs. Considering the high prevalence of MCTs in dogs and the central role *c-KIT* appears to play in the tumorigenesis of many canine MCTs, canine cutaneous MCTs provide an excellent spontaneous *in vivo* model for studying the molecular biology of *c-KIT* in human and animal neoplastic diseases. Furthermore, canine cutaneous MCTs are an excellent model for the treatment of cancers that are driven by *c-KIT* and can be used in clinical trials for testing chemotherapeutics aimed at targeting the *c-KIT* proto-oncogene.

Materials and Methods

Case selection, tissue samples, and survival data

Sixty canine cutaneous MCTs from 60 different dogs submitted to Michigan State University's Diagnostic Center for Population and Animal Health between 1998 and 2001 were included in this study. Cases were included in this study solely based on the meeting of all inclusion criteria. Inclusion criteria for this study were as follows: 1. all cases were previously diagnosed as canine cutaneous MCT. The diagnosis of canine cutaneous mast cell tumor and the histologic grade of each tumor was confirmed by a veterinary pathologist. 2. all cases were treated with surgical excision as the only primary treatment modality; i.e., no chemotherapy or radiation therapy was used. 3.

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complete follow-up data from the referring veterinarian was available. 4. adequate formalin-fixed paraffin embedded tissue for DNA extraction and immunohistochemistry was available. Complete follow-up data for each case included age, sex, breed, weight, number of masses, location of mass, time before excision, medication at the time of surgery, diagnostic tests that were performed, recurrence, tumor margins, metastasis, survival time and cause of death. Histologic grading of canine MCTs was performed in conjunction with a multi-institutional review of the current histologic grading system for canine cutaneous MCTs, in which 31 pathologists participated in the histologic grading of 95 canine MCTs[22]. Histologic grades represent a consensus of those results.

Laser capture microdissection and DNA extraction

Laser capture microdissection (LCM) was used to isolate neoplastic mast cells for DNA extraction and subsequent PCR amplification of *c-KIT* exon 11 and intron 11 in order to identify ITD *c-KIT* mutations. Five to 7 µm sections of each formalin-fixed, paraffin-embedded MCT was dehydrated and stained with hematoxylin for laser capture microdissection. Two-thousand to four-thousand neoplastic mast cells were extracted from each tumor sample using the Pixcell laser capture microdissection system with Macro LCM

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caps (Arcturus, Mountain View, CA) (Figure 6). Extracted cells adhered to the Macro LCM caps were incubated overnight in 50 µl of DNA extraction buffer (10 mM Tris pH 8.0, 1 mM EDTA, 1% Tween) and 1.5 µl of 15 mg/ml Proteinase K (Roche, Indianapolis, IN) at 37°C. Samples were centrifuged at 1,306 x g for 5 minutes, and Proteinase K was inactivated by heating at 95°C for 8 minutes.

PCR amplification of c-KIT exon 11 and intron 11

Polymerase chain reaction (PCR) amplification was performed using a previously described primer pair that flanks exon 11 and the 5′ end of intron 11[146], which includes the previously described ITD region of the *c-KIT* proto-oncogene in canine MCTs[13,16,91,95,96,145,146]. Polymerase chain reactions were prepared in a 25 µl total reaction volume, with 5 µl LCM extracted DNA, 5 pmol of each primer, 0.5 units of Taq polymerase (Invitrogen, Carlsbad, CA), and final concentrations of 80 µM dNTPs, 2 mM $MgCl_2$, 20 mM Tris-HCl, and 50 µl KCl. Cycling conditions were as follows: 94°C for 4 minutes; 35-45 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; 72°C for 5 minutes. Amplified products and ITD mutations were visualized by agarose gel electrophoresis on a 2% agarose gel after ethidium bromide staining (Figure 7).

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DNA sequencing

Mutant *c-KIT* alleles were identified by agarose gel electrophoresis and DNA fragments were excised for DNA purification. DNA was purified using the Qiaex II gel purification kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. DNA fragments were subcloned into Topo vectors using the Topo cloning kit (Invitrogen, Carlsbad, CA) and subsequently chemically transformed into competent *E. coli* cells according to the manufacturer's protocol. *c-KIT* clones were sequenced either using an automated sequencing technique using fluorescently labeled dideoxy-nucleotides, with capillary electrophoresis and detection using an ABI sequence analyzer (Foster City, CA) at Michigan State University's Genomics Technology Support Facility, or by manually sequencing with the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing kit (USB Corporation, Cleveland, OH) and ^{33}P-labelled dideoxy-nucleotide triphosphates according to the manufacturer's protocol, followed by 48-72 hr exposure to Biomax MR Scientific Imaging Film (Kodak, Rochester, NY).

Immunohistochemistry

Tissue sections of canine cutaneous MCTs were used for immunohistochemical evaluation of KIT protein localization as previously described[159]. In brief, 5 µm sections of

formalin-fixed paraffin-embedded tissue were deparaffinized in xylene, rehydrated in graded ethanol and rinsed in distilled water. Endogenous peroxidase was neutralized with 3% hydrogen peroxide for 5 minutes. Antigen retrieval was achieved by incubating slides in a citric buffer antigen retrieval solution (Dako, Carpinteria, CA) in a steamer (Black & Decker, Towson, MD) for 20 min, and non-specific immunoglobulin binding was blocked by incubation of slides for 10 min with a protein-blocking agent (Dako, Carpinteria, CA). Using an autostainer, slides were incubated for 30 minutes with a rabbit anti-human *c-KIT* antibody (Dako, Carpinteria, CA) at a dilution of 1:100. A streptavidin-immuno peroxidase staining procedure (Dako, Carpinteria, CA) was used for immunolabeling. The immunoreaction was visualized with 3,3'-diaminobenzidine substrate (Dako, Carpinteria, CA). Sections were counterstained with Mayer's hematoxylin. Positive and negative immunohistochemical controls were included in each run. Known canine MCTs were used as positive controls. Negative controls were canine MCTs that were treated identically as routine sections, except the 30 minute incubation with primary antibodies was replaced with a 30 minute incubation with buffer. KIT staining patterns and protein localization for each MCT was characterized as

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being peri-membrane (KIT staining pattern I), focal or stippled cytoplasmic (KIT staining pattern II), or diffuse cytoplasmic protein localization (KIT staining pattern III) as previously described[159] (Figure 8). The evaluation of KIT protein localization was performed by a single investigator (JDW) in order to eliminate inter-observer variability.

Tissue microarray and immunofluorescence

One millimeter cores that were microscopically selected to be representative of each tumor were taken from paraffin-embedded MCT tissue blocks and were placed in a common recipient paraffin block. Mast cell tumors included in the tissue array were chosen based on the availability of tissue for transferring to the recipient block. This resulted in 42 MCT samples from 42 cases being represented on the tissue microarray. The recipient block was subsequently heated at 37°C for approximately 1-hour in order to create a cohesive block. Five micrometer sections were cut and deparaffinized in xylene, and subsequently dehydrated in graded alcohol with a final rinse in distilled water. Twenty minute steam retrieval in a citric buffer solution (Dako, Carpinteria, CA) was used for antigen retrieval. Non-specific antibody binding was performed with 5% donkey serum with blocking buffer.

Slides were incubated with primary rabbit anti-human *c-KIT* (Dako, Carpinteria, CA) antibodies at a dilution of 1:100 overnight in a humidity chamber at 4°C. Sections were then incubated with Cy-3 labeled secondary antibodies and nuclei were counter stained with 4',6-Diamidino-2-phenylindole (DAPI). Mean immunofluorescence was quantified for each tumor sample using a Perkin Elmer Scan Array (Perkin Elmer, Wellesley, MA).

Statistics

Univariable Analyses: Before developing multivariable models, each risk factor was evaluated for its association with MCT outcomes. Univariable proportional hazards model were developed for each risk factor for each outcome, and the level of association was assessed through the risk factor's p-value in the model. Risk factors with p less than or equal to 0.20 were considered for inclusion in the multivariable model, which included the two variables, *c-KIT* mutation status and KIT staining patterns.

Multivariable Logistic Regression Models: Logistic regression models were developed for the occurrence of outcomes associated with MCTs, including recurrence of local MCTs, occurrence of distant MCTs, and death associated with MCTs. In addition to risk factors of interest, animal signalment (age, sex, weight) were

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included in the multivariable model to account for their effects on model outcome. Results were reported as odds ratios: an odds ratio less than one means the likelihood of the occurrence of an event is reduced, while an odds ratio greater than one indicates the likelihood of an event is increased. Odds ratios equal to 1 indicate that the risk factor neither increases nor decreases the likelihood of the outcome.

Multivariable Survival Analysis Models: This study used the Cox proportional hazards models (SAS PROC PHREG) (SAS Version 9.13, SAS Institute, Inc., Cary, N.C.) for survival analysis, using survival times (time-to-event) as the model outcome, and produces point estimates of the hazard ratio (risk ratio) for risk factors in the model. Proportional hazards regression models were developed for survival analysis of different outcomes associated with MCTs. These outcomes were days to recurrence of local MCTs, days to occurrence of distant MCTs, and days to death resulting from MCT. In addition to risk factors of interest, animal signalment (age, sex, weight) were included in the multivariable model to account for their effects on model outcome. The effects of risk factors on days to events were reported as hazard ratios. Comparable to odds ratios, hazard ratios less than one indicate that

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the risk factor increases time to outcome, while hazard ratios greater than one indicate that the risk factor decreases time to outcome.

Associations between *c-KIT* mutation status and KIT staining patterns were tested using Mantel-Hanzel chi square analysis. Associations between *c-KIT* mutation status and mean immunofluorescence and KIT staining patterns and mean immunofluorescence were tested using Wilcoxon Rank-sum tests.

Results

Study population

Sixty canine cutaneous MCTs from 60 dogs that met the inclusion criteria were included in this study. The age of these dogs ranged from 2-14 years, with a mean age of 7.84 years. Thirty-six dogs were females and 24 dogs were males. A total of 19 different breeds were represented by the study population. There were 13 mixed-breed dogs, 12 Labrador retrievers, 10 boxers, 6 golden retrievers, 3 pugs, 2 bassett hounds, 2 springer spaniels and 12 other breeds were represented by single dogs. According to the Patnaik histologic grading system for canine MCTs[14] 8 MCTs were grade I, 45 MCTs were grade II, and 7 MCTs were grade III.

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c-KIT mutations in canine mast cell tumors

DNA fragments representing exon 11 of the *c-KIT* proto-oncogene were amplified and visualized for each tumor. *c-KIT* mutations were identified in 9 of 60 MCTs (15%). Mutations in cases 1-8 were similar to previously described ITD *c-KIT* mutations[15,16,91,95,96,145,146]. All of these ITD mutations were in-frame mutations that ranged from 45-60 bp in size. In cases 5, 6, 7, and 8, duplications extended 1, 2, 3, and 4 nucleotides into intron 11, respectively. The mutation in case 9 was located entirely in intron 11. This mutation was tentatively identified as a duplication based on its banding pattern on agarose gel electrophoresis, but when sequenced it was found to consist of a 24 nucleotide poly-T insertion followed by a 15 nucleotide duplication of the sequence preceding the poly-T insertion. Additionally, a G to A transition was found in the duplicated sequence that preceded the poly-T insert. Four of the MCTs in which mutations were identified were histologic grade II and 5 were grade III (Table 12).

According to multivariable analysis, patients with MCTs containing ITD *c-KIT* mutations had significantly decreased survival times (p= 0.0068, hazard ration (HR) = 6.23 (1.66-23.4)) and an increased incidence of mortality due to MCT-related disease (p=0.0011, odds ratio (OR) = 15

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(2.95-76.31)) (Figure 9). Additionally, patients with MCTs containing ITD *c-KIT* mutations were also significantly associated with an increased incidence of recurrence at the original tumor site (p= 0.0255, OR= 5.4 (1.23-23.75)) and at sites outside of the original tumor margins (p= 0.0016, OR= 6.13 (1.99-18.92)), and with a decreased disease-free interval both at the site of the original tumor (p=0.0157, HR= 5.78 (1.40-23.99)) and at sites outside of the tumor margin (p=0.0012, HR=6.14 (2.06-18.37)).

KIT protein localization and c-KIT mutations

KIT protein localization was examined in each MCT using immunohistochemical staining with anti-KIT antibodies. Twenty-five of the 60 MCTs examined had KIT staining pattern I, which is characterized by peri-membrane KIT protein localization, as seen in non-neoplastic (inflammatory) mast cells. Twenty-four of the 60 MCTs in this study had KIT staining pattern II, which is characterized by stippled to focal cytoplasmic KIT localization, often with a decrease in peri-membrane protein localization; and the remaining 11 MCTs had KIT staining pattern III, which is characterized by diffuse cytoplasmic KIT localization. Seven of the 9 MCTs (77.8%) with ITD *c-KIT* mutations also had aberrant KIT protein localization (KIT staining patterns II or III). Two of the

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MCTs with ITD *c-KIT* mutations had KIT staining pattern I, 3 cases had KIT staining pattern II, and 4 cases had KIT staining pattern III. A significant trend was identified between the presence of ITD *c-KIT* mutations and increased cytoplasmic localization of KIT (p= 0.046) (Figure 10) as evidenced by higher KIT staining patterns.

KIT protein expression

The tissue microarray representing 42 of the 60 samples was used to quantify KIT immunofluorescence. Relationships between immunofluorescence, and *c-KIT* mutations and KIT protein localization were investigated. No significant relationships were identified (data not shown).

Discussion

The goal of this study was to look at the *c-KIT* proto-oncogene and its product KIT at both the gene and the protein levels in order to better define the role this gene plays in the pathogenesis of canine cutaneous MCTs. This is the first study to demonstrate a significant association between *c-KIT* ITD mutations and an increased rate of recurrent disease and mortality in dogs with canine cutaneous MCTs. Additionally; this is the first study to identify a significant relationship between the presence of ITD *c-KIT* mutations and the aberrant localization of KIT in

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canine MCTs. These data document the importance of the *c-KIT* proto-oncogene in the tumorigenesis of canine cutaneous MCTs and clearly identifies the *c-KIT* proto-oncogene as a potential target for the treatment of canine MCTs.

The *c-KIT* proto-oncogene was first implicated in the progression of canine cutaneous MCTs when activating mutations were identified in the juxtamembrane domain of *c-KIT*[91,96]. Following the identification of *c-KIT* mutations in canine MCTs, work by our lab has shown that the presence of *c-KIT* mutations is significantly associated with higher histologic grade MCTs[96]. The results of this paper further demonstrate the association of *c-KIT* mutations with higher histologic grade MCTs in dogs. All of the MCTs with *c-KIT* mutations identified in this study were of histologic grade 2 and 3, while no grade 1 MCTs were found to have *c-KIT* mutations. In this paper we have further defined the significance of ITD *c-KIT* mutations in canine MCTs, by showing that *c-KIT* ITD mutations are significantly associated with an increased incidence of MCT-related death, and an increased occurrence of MCTs at the original or distant cutaneous or extracutaneous locations.

The prognostic value and biologic significance of molecular markers can be confounded by variation in the treatment protocols used in a given study population. In

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order to overcome this source of bias, only cases that were treated with surgical excision alone (i.e. no chemotherapy or radiation therapy) were included in this study. This is the only study that has looked at the significance of *c-KIT* mutations in a population of dogs treated with a single therapeutic protocol.

In this study, ITD *c-KIT* mutations were found in 15% of the MCTs that were examined. The incidence of ITD *c-KIT* mutations varied from 9% to 33% in the two previous studies, which consisted of randomly selected and referral high grade tumors, respectively[16,96]. The predominance of intermediate and high grade tumors in the later study[16] is likely to account for the high incidence of *c-KIT* mutations in their study population. In the current study, cases were randomly selected, and represented the entire spectrum of canine cutaneous MCTs[8,12,14]. Based on the results of this study and previous studies, the true incidence of ITD *c-KIT* is likely to be between 9-15% in all MCTs. However these mutations may occur in as many as 50% of high grade canine MCTs[16,96].

Previously our laboratory has shown that increased cytoplasmic KIT protein localization in neoplastic mast cells is associated with both a decreased disease-free and overall survival of dogs with cutaneous MCTs[159]. In this

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study, we identified a significant association between the presence of ITD *c-KIT* mutations and changes in KIT localization in canine cutaneous MCTs. Seven of 9 MCTs with *c-KIT* mutations had aberrant KIT protein localization. Although the significance of this relationship is not currently clear, this may suggests that ITD *c-KIT* mutations may be responsible for aberrant KIT localization in a subset of canine MCTs.

Two cases with *c-KIT* mutations did not have aberrant KIT localization, and remain as outliers to this hypothesis. However, the mutation in one of these MCTs was located within intron 11 only, and therefore could be spliced out during mRNA processing and may not be biologically significant (case 9). It is also important to note that the dog with the intronic *c-KIT* mutation (case 9) was still alive with no report of local or distant recurrence at 20 months post-surgery. Furthermore, significant statistical relationships between ITD *c-KIT* mutations and both the incidence of ($p=0.0052$) and time until MCT-related deaths ($p=0.0267$) are preserved when this mutation is not considered as a biologically significant mutation.

A potential explanation for the absence of cytoplasmic KIT localization in the other MCT that had an ITD *c-KIT*

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mutation may be that this tumor only recently acquired the mutation, and the changes in KIT localization may not have occurred yet at the time of surgical excision. However, ITD *c-KIT* mutations and changes in KIT localization may represent separate events that occur independent of one another in the progression of canine cutaneous MCTs. This hypothesis is supported by the fact that 26 MCTs included in this study had aberrant KIT localization without the presence of ITD *c-KIT* mutations.

This data could also indicate that in addition to a direct causal relationship between the ITD mutations and aberrant KIT localization, other factors may be responsible for aberrant KIT localization in canine cutaneous MCTs without ITD *c-KIT* mutations. The primers that were used in this study do not allow for the detection of the previously reported deletions in canine MCTs, since the forward primer is located in the region of *c-KIT* that has been reported to be deleted in a small subset of canine MCTs[95,96]. Therefore, although rare, other *c-KIT* mutations such as deletions in the juxtamembrane domain may be responsible for the aberrant protein localization in those cases in which we did not identify ITD *c-KIT* mutations. In summary, the correlation between ITD *c-KIT* mutations and aberrant KIT localization leads to many interesting questions regarding

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the functional significance of this relationship and the overall functional significance of aberrantly localized KIT when ITD *c-KIT* mutations are not present. Current work in our laboratory is focused on the further characterization of aberrantly localized KIT and on functional studies to better elucidate the relationship between ITD *c-KIT* mutations and the aberrant localization of KIT.

No significant relationship was found in this study between the presence of ITD *c-KIT* mutations or the aberrant localization of KIT and the level of KIT protein expression as measured by mean immunofluorescence in a tissue microarray. These results suggest that constitutive activation of KIT due to ITD mutations or changes in signaling pathways through aberrant KIT localization may be more important in the pathogenesis of canine MCTs than over-expression of KIT and subsequent increases in receptor sensitivity to its ligand. In order to clarify these observations, these results need to be verified using additional techniques to quantify KIT protein levels in canine MCTs. Additionally, further studies need to be conducted in order to elucidate the functional significance of aberrantly localized KIT and the effects it has on signaling in neoplastic mast cells.

Spontaneous neoplastic diseases are commonly seen in dogs[2,3], and in many cases share similar morphologic, clinical, and molecular characteristics to human neoplastic diseases. Therefore, these tumors are an excellent *in vivo* model of spontaneous neoplasia that may be utilized to better understand the roles of various genes and proteins in the progression of neoplastic diseases, and to serve as model systems for testing the safety and efficacy of novel therapeutic agents[160,161]. Canine cutaneous MCTs are one of the most common neoplasms in dogs and, unlike human mastocytomas, often have an aggressive behavior that can result in death. Due to the high incidence of canine MCTs, and the central role that *c-KIT* plays in MCT tumorigenesis, canine MCTs can serve as an excellent *in vivo* model for studying its role in the progression of this and other human and animal neoplastic diseases. We propose canine MCTs as a spontaneous *in vivo* model for clinical trials aimed at determining the safety and efficacy of novel targeted chemotherapeutic agents involving *c-KIT* signaling pathways.

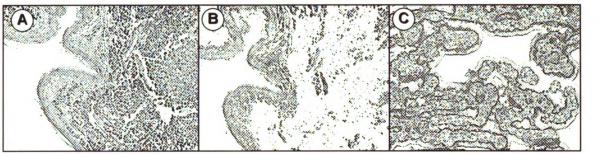

Figure 6: *Laser capture microdissection of neoplastic canine cutaneous mast cell tumors* (magnification: 10X). Laser capture microdissection (LCM) was performed using archival formalin-fixed paraffin-embedded tissue sections. DNA was extracted from captured cells and PCR amplification was performed in order to identify *c-KIT* mutations. A: Hematoxylin stained section of MCT prior to microdissection. B: Section of MCT following microdissection. C: Laser capture microdissected cells adhered to cap.

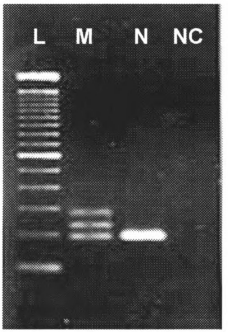

Figure 7: *2% agarose gel of PCR amplified c-KIT exon 11 and intron 11 from LCM extracted DNA from canine MCTs.* L: 100 bp ladder; M: heterozygous for normal allele (191bp) and mutant allele (250bp) with a upper band representing heterodimerization of normal and mutant alleles; N: 191 bp homozygous normal allele; NC: negative control (no template).

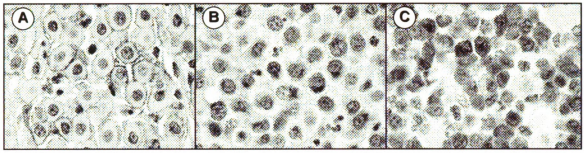

Figure 8: *Sections of canine cutaneous mast cell tumors (skin) stained with anti-KIT antibodies and counterstained with hematoxylin (magnification: 100X oil) representing three patterns of KIT localization identified in neoplastic canine mast cells.* A: KIT staining pattern I, consisting of peri-membrane protein localization with little to no cytoplasmic protein localization; B: KIT staining pattern II, consisting of focal to stippled cytoplasmic staining; C: KIT staining pattern III, consisting of diffuse cytoplasmic staining.

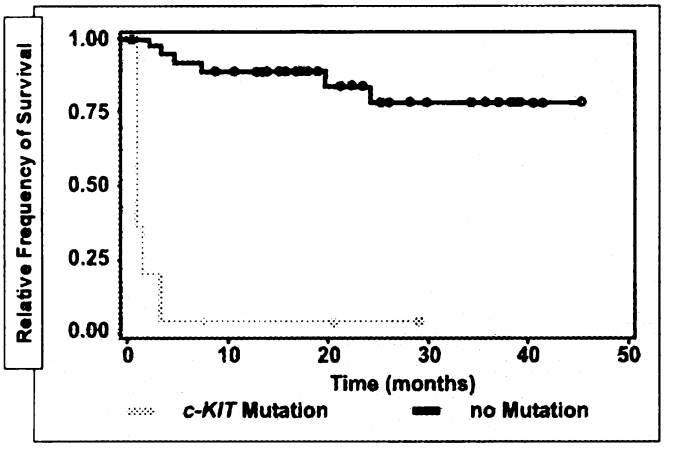

Figure 9: *Kaplan-Meier Survival Curve:* Relative frequency of survival vs. time in months for canine cutaneous mast cell tumor patients with and without identified *c-KIT* mutations. The presence of duplication mutation in the *c-KIT* proto-oncogene was significantly associated with a decreased survival duration (p= 0.0068, HR= 6.23 (1.66-23.40)).

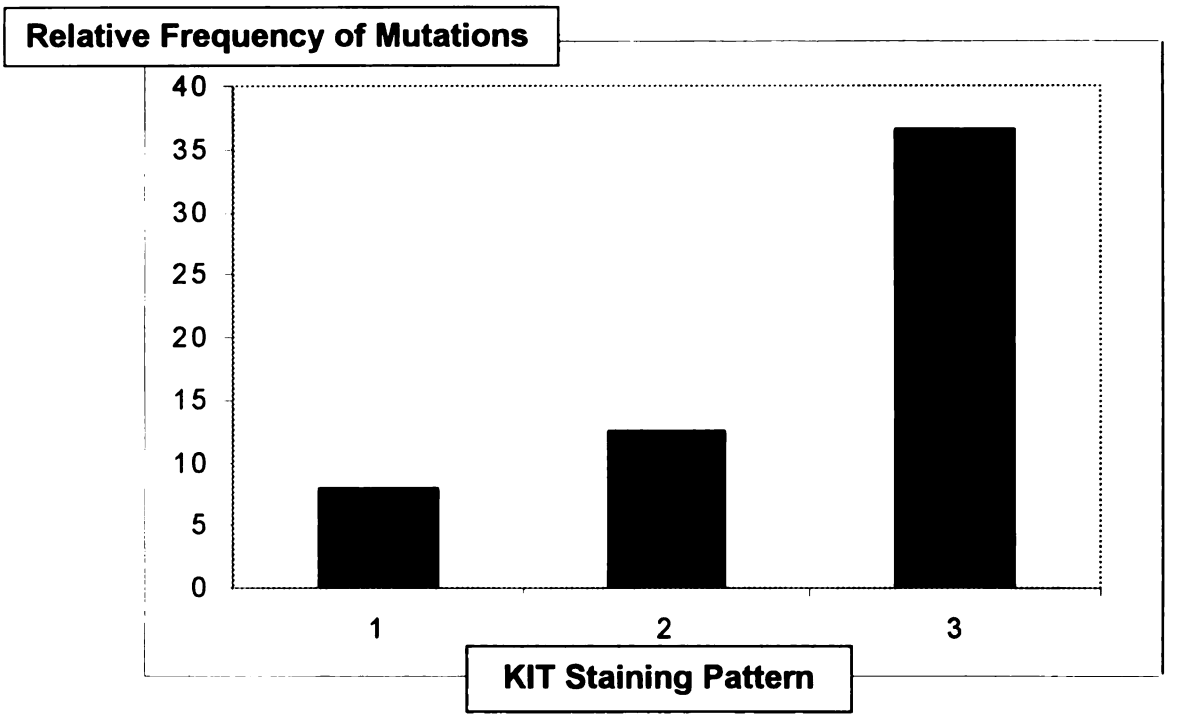

Figure 10: *Correlation between ITD c-KIT mutations and KIT protein localization in canine MCTs.* A significant association was found between the presence of *c-KIT* mutations and the cellular localization of KIT in canine MCTs (p= 0.046). 7/9 (77.8%) of MCTs with ITD *c-KIT* mutations had aberrant KIT localization in neoplastic MCTs.

Table 12: Mutation and case description for cases with ITD *c-KIT* mutations.

Case No.	Size (bp)	Location	Grade*	KIT Staining Pattern†	Local Recur. (months)	Distant Recur. (months)	MCT-related Death (months)
1	45	Exon 11	3	3	None	None	0.5
2	45	Exon 11	2	2	None	None	None at 29.1
3	45	Exon 11	3	3	None	0.5	0.5
4	45	Exon 11	2	2	0.5	0.5	0.5
5	60	Exon 11/ Intron 11	3	2	1	1	1
6	54	Exon 11/ Intron 11	3	3	2	2	3
7	60	Exon 11/ Intron 11	3	3	None	0.6	0.6
8	57	Exon 11/ Intron 11	2	1	None	None	None at 7.3‡
9	15	Intron 11**	2	1	None	None	None at 20.4

· Histologic grading was performed based on the Patnaik histologic grading system for canine cutaneous MCTs (45).

· KIT staining patterns were classified as described by Webster et al., 2004 (36).

‡ Dog no.8 died at 7.3 months due to causes unrelated to mast cell disease.

·· Mutation in dog no. 9 consisted of a 24bp poly-T insert with a 15 bp duplication, which was located entirely in intron 11. An additional A to G transition was also identified in the duplicated sequence preceding the poly-T insert.

CHAPTER 4

Webster JD, Kiupel M, Yuzbasiyan-Gurkan V (2006). Evaluation of the kinase domain of *c-KIT* in canine cutaneous mast cell tumors. BMC Cancer 6:85.

CHAPTER 4

EVALUATION OF THE KINASE DOMAIN OF *c-KIT* IN CANINE CUTANEOUS MAST CELL TUMORS

Introduction

The *c-KIT* proto-oncogene encodes the type III receptor tyrosine kinase KIT, which consists of an extracellular ligand binding domain, a transmembrane domain, a negative regulatory juxtamembrane domain and a split kinase domain[97-99] (Figure 11). In healthy humans as well as in dogs, *c-KIT* is expressed by multiple cell types including mast cells, germ cells, melanocytes, and hematopoietic precursor cells[100,101,103,105,106]. Notably, in mast cells KIT and its ligand stem cell factor (SCF, also known as mast cell growth factor)[106,114-115] have been shown to be involved in cell survival, proliferation, differentiation, chemotaxis, degranulation, and fibronectin adhesion[106-111]. In human patients, mutations in the *c-KIT* proto-oncogene have been implicated in the pathogenesis of multiple neoplastic diseases, including mastocytosis, germ cell tumors, and gastrointestinal stromal tumors (GISTs)[118,122,123,125,126,135]. The locations of these *c-KIT* mutations vary between the different neoplastic diseases. The vast majority of mutations characterized in human patients with mastocytosis

occur at codon 816 in exon 17, which encodes a portion of the kinase domain of KIT[123,134,136]. Mutations in germ cell tumors have been found in both the juxtamembrane domain and the kinase domain of *c-KIT*[125,126], while mutations in GISTs tend to occur in exon 11 of the juxtamembrane domain of *c-KIT*[118,120,121]. Despite their variation in location, both juxtamembrane domain and kinase domains *c-KIT* mutations result in a constitutively activated KIT protein that is phosphorylated in the absence of the ligand[118,123,125,126].

In recent years, *c-KIT* has been implicated in the pathogenesis of canine cutaneous mast cell tumors (MCTs), which are one of the most common neoplasms in dogs[1-5]. Internal tandem duplications (ITDs), deletions, and point mutations have been identified in the juxtamembrane domain of *c-KIT* in canine cutaneous MCTs[91,95,96]. The reported incidence of *c-KIT* mutations has varied between different studies. In two studies that screened randomly selected cases submitted, mutations were identified in 15% of canine MCTs[96,162]. However, another study reported *c-KIT* mutations in 50% of canine MCTs that were seen in a referral oncology practice[16]. This discrepancy in the incidence of *c-KIT* mutations in canine MCTs is most likely due to the variations in case selection between these studies with the higher percentage reflecting ascertainment bias at the

referral practice. Internal tandem duplication *c-KIT* mutations are the most common[96,146], and therefore the most extensively studied *c-KIT* mutation in canine MCTs. All of the ITD mutations that have been studied thus far have been found to produce a constitutively activated product, thereby implicating *c-KIT* in the progression of canine MCTs[91,95,145]. Previous work by our laboratory has shown that *c-KIT* mutations are significantly associated with higher histologic grade MCTs[96], and that MCT patients with ITDs have a significantly worse prognosis as compared to patients without ITD mutations, thereby further implicating *c-KIT* in the progression of canine MCTs[162].

In addition to *c-KIT* mutations, aberrant KIT expression and more specifically, the aberrant localization of KIT has been described in canine cutaneous MCTs[94,158,159]. Recently our laboratory has identified 3 patterns of KIT protein localization in canine MCTs: 1. peri-membrane KIT localization (KIT pattern 1); 2. cytoplasmic stippling to focal KIT localization (KIT pattern 2); 3. diffuse cytoplasmic KIT localization (KIT pattern 3), as shown in Figure 12. Recent studies have shown that canine MCTs that have a primarily cytoplasmic pattern of KIT protein localization (KIT patterns 2 and 3) have a significantly worse prognosis, in terms of both their disease-free

interval and survival duration, as compared to MCTs that have a primarily peri-membrane pattern of KIT localization[159]. Furthermore, we have found that ITD *c-KIT* mutations in canine MCTs are significantly associated with aberrant KIT localization in neoplastic mast cells. However, a substantial number of canine MCTs have aberrant KIT localization, but do not have ITD mutations[162]. This suggests that additional factors, aside from ITD *c-KIT* mutations may be responsible for aberrant KIT localization in neoplastic mast cells.

Despite the high incidence of *c-KIT* kinase domain mutations in human patients with mastocytosis, only a total of 18 canine MCTs and 3 canine MCT cell lines have been evaluated for kinase domain mutations in *c-KIT*[91,95]. For a more detailed evaluation of possible *c-KIT* kinase domain mutations in canine MCTs, we screened exons 16-20 of the phospho-transferase portion of the kinase domain of *c-KIT* for mutations that may contribute to the progression of these tumors. Additionally, in order to determine if mutations in exon 17 of *c-KIT*, where the majority of mutations occur in human mastocytosis patients, were responsible for aberrant KIT localization, exon 17 was screened in 18 MCTs with aberrant KIT localization that lack ITD *c-KIT* mutations. However, no mutations or

polymorphisms were identified in exons 16-20 of any of the canine MCTs that were examined.

Materials and Methods

Study Population

Samples from 33 canine cutaneous MCTs from 33 dogs were obtained from archival formalin-fixed paraffin-embedded tissues that had been submitted to the Diagnostic Center for Population and Animal Health at Michigan State University. Single paraffin blocks with neoplastic tissue were selected for each tumor and each tumor was histologically graded based on the Patnaik histologic grading system[34] for canine cutaneous MCTs.

DNA Isolation from formalin-fixed paraffin embedded tissues

Tissue samples for DNA isolation were selected within the tumor boundaries, as identified by histologic evaluation. Approximately 2 mm^3 tissue samples were obtained from each block for DNA extraction. DNA was isolated as previously described[96,163]. In brief, 400 µl of digestion buffer (50 mM Tris, pH 8.5, 1 mM EDTA, 0.5% Tween) was added to each sample. Samples were heated to 95°C for 10 minutes followed by heating in a microwave at full power twice for 30 seconds. Samples were thoroughly vortexed between each heating step. Five µl of 15 mg/ml proteinase K was added to each sample, and the samples were subsequently

incubated overnight at 42°C. Proteinase K was inactivated at 95°C for 10 minutes. Samples were then centrifuged and 200 µl of the reaction was aliquoted for use as template in PCR.

Amplification of c-KIT exons 16-20

PCR amplification was carried out using primer pairs that flanked exons 16, 17, 18, 19, and 20 in order to sequence each exon in its entirety and the 10-20 nucleotides that flank each exon (Table 13, Figure 13). Twenty-five microliter PCR reactions were prepared with 5 µl DNA at a 1:25 dilution, 5 pmol of each primer, 0.5 units of Taq polymerase (Invitrogen, Carlsbad, CA), and final concentrations of 80 µM dNTPs, 2 mM $MgCl_2$, 20 mM Tris-HCl, and 50 µl KCl. Cycling conditions for amplifying exons 16, 17, 18, and 20 were as follows: 94°C for 4 minutes; 40 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute; 72°C for 5 minutes. Cycling conditions were similar for exon 19, except a 54°C annealing temperature was used instead of a 60°C annealing temperature. Amplified products were visualized by agarose gel electrophoresis on a 2% agarose gel stained with ethidium bromide.

Sequencing of c-KIT exons 16-20

Amplified products were pooled in groups of 7-10 for DNA sequencing whenever possible, as previously described

[redacted]. This pool and sequence method has been shown to allow for the detection of minor alleles at frequencies as low as 5%. If clean sequences were not obtained from pooled samples, then samples were sequenced individually. DNA to be sequenced was separated on a 2% agarose gel, and DNA fragments were excised for DNA purification. DNA was purified using the Qiaex II gel purification kit (Qiagen, Valencia, CA) according to manufacturer's protocol. DNA sequencing was carried out using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham, Piscataway, NJ), following manufacturer's instruction. Sequence reactions were separated on a 6% denaturing polyacrylamide gel, which was dried and exposed to BioMax MR scientific imaging film (Kodak, Rochester, NY) for 72 hours for visualization.

DNA isolation from laser capture microdissected tumor samples

Eighteen MCTs with aberrant KIT localization, but no ITD *c-KIT* mutations were identified as part of a previous study[redacted] (Figure 12). Seven micrometer sections of each MCT were dehydrated and stained with hematoxylin for laser capture microdissection. Two-thousand to four-thousand neoplastic mast cells were extracted from each tumor sample using the Pixcell laser capture microdissection system with

Macro LCM caps (Arcturus, Mountain View, CA). LCM caps adhered to extracted cells were incubated inverted overnight in 50 µl of DNA extraction buffer (10 mM Tris pH 8.0, 1 mM EDTA, 1% Tween) and 1.5 µl of 15 mg/ml Proteinase K at 37°C. Samples were centrifuged at 1306 x g for 5 minutes, and Proteinase K was inactivated by heating at 95°C for 8 minutes. 5 µl of DNA was used for each 25 µl reaction. PCR reactions were prepared using primers flanking *c-KIT* exon 17 as described above.

Results

Thirty-three cutaneous MCTs from 33 dogs were included in this study. The age of these dogs ranged from 2.5-15 years with an average of 7.42 years. Twenty of the 33 dogs were female and 13 were male. The breed distribution of this study population included 10 boxers, 9 Labrador retrievers, 5 golden retrievers, 1 Boston terrier, 1 Bichon Frise and 7 mixed breed dogs. All MCTs were graded according to the Patnaik histologic grading system for canine cutaneous mast cell tumors[14]. Twelve of the 33 MCTs were histologic grade 1, 18 were grade 2, and 3 were grade 3. All 33 MCTs included in this study were previously screened for mutations in the juxtamembrane domain of *c-KIT*. No internal tandem duplications or deletions were

identified in the juxtamembrane domain of *c-KIT* in any of the MCTs included in this study (data not shown).

In order to identify potential activating mutations, *c-KIT* exons 16, 17, 18, 19, and 20, which encode the phospho-transferase region of the kinase domain of the KIT protein, were amplified using PCR amplification and subsequently sequenced. Exons 16 to 20 were identical to previously published cDNA sequences of the canine *c-KIT* gene [Genbank:AF448148] in all canine MCTs examined. No mutations or polymorphisms were identified in *c-KIT* exons 16 to 20 in any of the canine cutaneous MCTs screened. One single nucleotide polymorphism was identified at the 7th nucleotide of intron 18 consisting of a C to A transversion.

Previous work by our laboratory has identified a correlation between ITD *c-KIT* mutations and aberrant KIT localization in canine cutaneous MCTs. However, we have also found that a subset of canine cutaneous MCTs have aberrant KIT localization without having ITD *c-KIT* mutations[162]. In human mastocytosis patients, mutations of *c-KIT* are commonly seen in exon 17[123,134,136]. In order to test the hypothesis that mutations in *c-KIT* exon 17 are responsible for aberrant KIT localization in canine MCTs that lack ITD *c-KIT* mutations, exon 17 was amplified and

sequenced from 18 such cases. Exon 17 was identical to previously published *c-KIT* sequences [Genbank:AF448148] and no mutations or polymorphisms were identified in exon 17 in any of the canine MCTs evaluated.

Discussion

This study did not identify any mutations of the phospho-transferase domain of *c-KIT* in 33 canine cutaneous MCTs examined. These findings are also supported by reports from London et al. in which 11 MCTs were examined for kinase domain mutations[91]. Therefore, it is highly unlikely that the phospho-transferase domain of *c-KIT* plays a role in the progression of canine MCTs. Furthermore, mutations in exon 17 of the *c-KIT* proto-oncogene, where mutations are most commonly seen in human patients with mastocytosis[113,134,136], do not contribute to the aberrant localization of KIT in canine cutaneous MCTs.

The kinase domain of *c-KIT* is highly conserved between humans and canines. In a comparison of the human [Genbank: NM_000222] and canine [Genbank:AF448148] amino acid sequences corresponding to exons 16-20, there is only a single difference in which a glutamate in the human is replaced with an aspartate residue in exon 20 of the dog, giving greater than 99% identity between these species (figure 14). The high degree of conservation between the

human and the dog suggests that a change in a single amino acid of the kinase domain could alter the function of the KIT protein, potentially producing a constitutively activated protein, as is seen in human mastocytosis patients. However, unlike human mastocytosis patients, kinase domain *c-KIT* mutations do not appear to play a role in the progression of canine MCTs.

Internal tandem duplications and deletions in the juxtamembrane domain of *c-KIT* have been identified in approximately 15% of canine MCTs[162]. The internal tandem duplication mutations are the most frequent and best characterized *c-KIT* mutations in canine MCTs[96,146]. All of the characterized ITD mutations result in a constitutively activated KIT product, which is characterized by the constitutive phosphorylation of the receptor in the absence of ligand binding[91,95,145]. Considering the role of *c-KIT* in mast cell survival and proliferation[106,111] and the association between ITD *c-KIT* mutations and decreased disease-free interval and survival duration of dogs[162], *c-KIT* appears to play a key role in the progression of canine MCTs[162]. Therefore, *c-KIT* may also represent a potential therapeutic target for canine cutaneous MCTs[16,96,145,159,162,165].

Juxtamembrane domain *c-KIT* mutations have been found in only approximately 15% of all canine MCTs[96,162] and 50% of

high grade MCTs[16]. We hypothesized that phospho-transferase domain *c-KIT* mutations may play a role in the progression of canine cutaneous MCTs that lack ITD *c-KIT* mutations. The goal of this study was to investigate the presence of any activating mutations in the phospho-transferase domain of the *c-KIT* proto-oncogene. The results of this study suggest that *c-KIT* mutations of the phospho-transferase domain do not play a significant role in the progression of canine MCTs. Since some small molecule kinase inhibitors have shown a greater efficacy in tumors with mutations in the target receptor tyrosine kinase (e.g. KIT)[145,166,167], MCTs lacking *c-KIT* mutations may not be candidates for the treatment with these drugs. Therefore, other genetic or epigenetic changes that play a role in the progression of canine MCTs need to be identified in order to develop targeted treatment strategies in those MCTs that lack ITD *c-KIT* mutations. However, additional domains of the *c-KIT* proto-oncogene still need to be evaluated for the presence of activating mutations in canine MCTs. One area of particular interest is the extracellular ligand-binding domain where mutations have been identified in exon 8 of human familial mastocytosis and GIST patients[133] and exon 9 of human GIST patients[119,147,167].

Additional work from our laboratory has shown a correlation between ITD *c-KIT* mutations and the aberrant localization of KIT in canine MCTs[162]. These results suggest that ITD *c-KIT* mutations may lead to the aberrant cytoplasmic localization of KIT in at least a subset of canine MCTs. Despite this correlation between ITD mutations and aberrant KIT localization, we have identified some MCTs that have aberrant KIT localization, but do not have ITD *c-KIT* mutations[162]. We hypothesized that point mutations in exon 17 of the *c-KIT* proto-oncogene, similar to those seen in human patients with mastocytosis, are responsible for the aberrant KIT localization in MCTs without ITD *c-KIT* mutations. However, in this study we did not find any mutations in exon 17 of the *c-KIT* proto-oncogene in 18 canine cutaneous MCTs with aberrant KIT localization. These results suggest that other changes to *c-KIT*, such as mutations in other *c-KIT* domains, changes in transcriptional regulation or alternative splicing, or other cellular changes, such as changes in golgi processing or intracellular trafficking may be responsible for aberrant KIT localization and may therefore play a role in the progression of canine cutaneous MCTs.

In this study, no kinase domain *c-KIT* mutations were found in any canine cutaneous MCT examined and therefore these mutations do not appear to play a significant role in the progression of this disease. In accordance with these data, exon 17 *c-KIT* mutations do not appear to be responsible for aberrant KIT localization in canine MCTs with aberrant KIT localization that lack ITD *c-KIT* mutations. Due to the relatively low incidence of juxtamembrane domain *c-KIT* mutations, and the lack of kinase domain mutations in canine cutaneous MCTs, further studies are necessary to identify additional factors and genes that lead to the progression of canine cutaneous MCTs. Although targeting constitutively activated KIT protein in canine MCTs offers great promise for the treatment of canine MCTs[145,165], such a therapeutic approach may not be justified for all MCTs. A significant number of MCTs seem to have no alterations in any of the domains that have been assessed in the *c-KIT* proto-oncogene, and targeted inhibition of this gene will therefore have little or no effect on these tumors. Therefore, additional studies need to be performed in order to determine other factors that may be involved in the progression of canine MCTs without ITD *c-KIT* mutations in order to identify potential targets in those tumors.

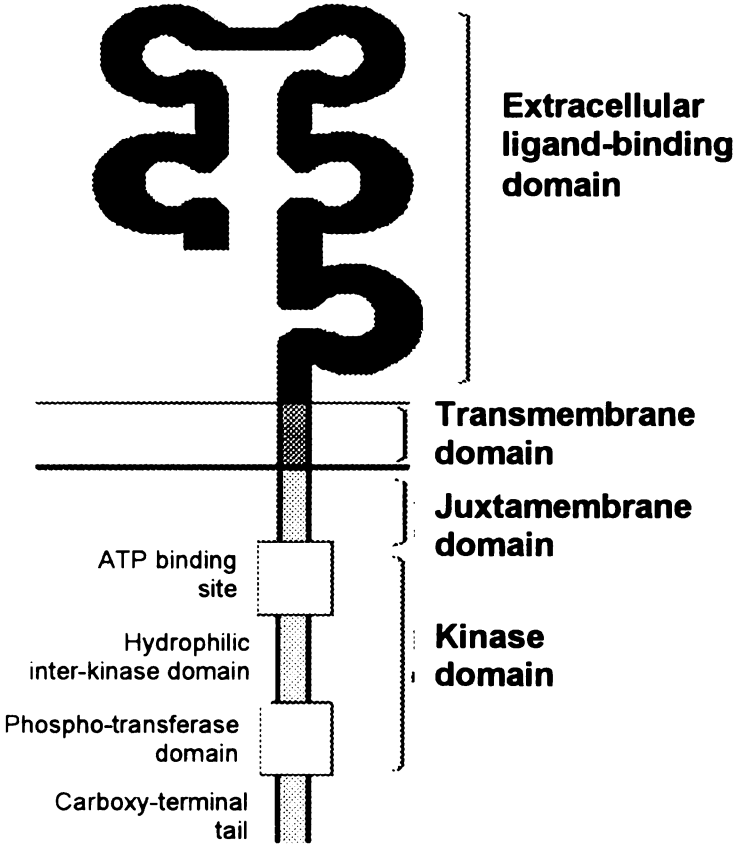

Figure 11: Schematic diagram of the receptor tyrosine kinase KIT.

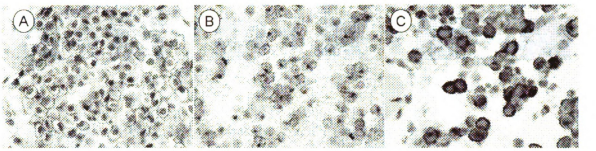

Figure 12: Immunohistochemical staining patterns of canine cutaneous MCTs staining with anti-KIT antibodies. A. KIT staining pattern 1: peri-membrane KIT protein localization with little to no cytoplasmic protein localization; B. KIT staining pattern 2: focal or stippled cytoplasmic KIT protein localization; C. KIT staining pattern 3: Diffuse cytoplasmic KIT localization. MCTs with KIT staining patterns 2 and 3 that lacked ITD *c-KIT* mutations were screened for mutations in *c-KIT* exon 17. B and C show MCTs with aberrant KIT localization that lack ITD *c-KIT* mutations.

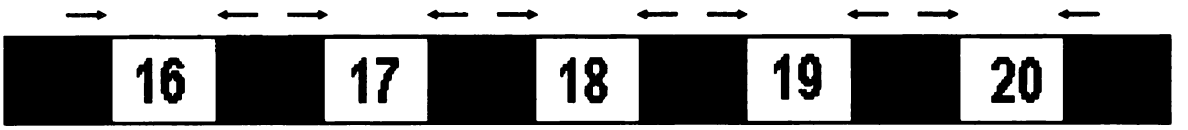

Figure 13: Schematic Diagram of Primer Design for Polymerase Chain Reaction. Forward and reverse primers (arrows) were designed in introns (black boxes) flanking exons 16, 17, 18, 19, and 20 (white boxes).

```
Canine  GSYTERDVTPAIMEDDELALDLEDLLSFSYQVAKGMAFLASKNCIHRDLAARNILLTHGRITKICDFGLA
Human   ............................................................

Canine  RDIKNDSNYVVKGNARLPVKWMAPESIFNCVYTFESDVWSYGIFLWELFSLGSSPYPGMPVDSKFYKMIK
Human   ............................................................

Canine  EGFRMLSPEHAPAEMYDIMKTCWDADPLKRPTFKQIVQLIEKQISDSTNH
Human   ..........................................E.....
```

Figure 14: Amino acid alignment of the kinase domain (exons 16-20) of canine and human KIT. Canine and human amino acid alignments demonstrate a 99.5% identity between the phospho-transferase portion of the kinase domain of these species. Codon 816, which is commonly mutated in human mastocytosis patients (D816V; shaded residue) is also conserved in canine KIT.

Table 13. Primers used for PCR amplification of *c-KIT* exons 16-20 and size of expected PCR products for each primer pair.

Exon	Primer Number	Direction	Sequence	Product Size (bp)
16	1202	Forward	CTT TGA GGC TTA ATT GCT AAG AA	256
	1203	Reverse	ACT ATG AAC TCT AAA ATG CGC CA	
17	1204	Forward	ATA GCA GCA TTC TCG TGT TG	261
	1205	Reverse	AAC TAA AAT CCT TCA CTG GAC TG	
18	1206	Forward	AAC ATT GCC GGA TCT GTT GT	189
	1207	Reverse	AGA TGC TCT CGC CCA ACC A	
19	1208	Forward	GGG TCC TGC TTG CTT ATT	188
	1209	Reverse	AGC ATG ATC TCA AGG GAA	
20	1210	Forward	AGG CTA AGG GCG TTG AGG	189
	1211	Reverse	GCA GGG AGG TTC TAC GGC T	

CHAPTER 5

CELLULAR PROLIFERATION IN CANINE CUTANEOUS MAST CELL TUMORS: ASSOCIATION WITH *c-KIT* AND PROGNOSTIC MARKERS

Introduction

Canine cutaneous mast cell tumors (MCTs) are one of the most common neoplasms in dogs[1-4], and have an extremely variable biologic behavior ranging from a single benign mass that can be cured with surgical excision to a potentially fatal, multi-systemic disease[5,13,14,26]. Due to their high prevalence and variable biologic behavior, accurate prognostication and a thorough understanding of the molecular biology of canine MCTs are critical for the successful treatment of this disease. Currently histologic grading is the primary prognostic and therapeutic determinant for canine cutaneous MCTs. The most commonly used histologic grading system defines grade I MCTs as well-differentiated tumors with a positive prognosis; grade II MCTs as intermediately differentiated tumors with a cautionary prognosis; and grade III MCTs as poorly differentiated tumors with a negative prognosis[14]. Although histologic grades have been shown to be significantly associated with prognosis[12,14], the ambiguity of intermediate grade tumors[17,24] and the marked degree of inter-observer

variation has led to questioning of the relevance of the current histologic grading system[20-22].

The propensity for uncontrolled cellular proliferation is a hallmark of cancer[51] and, as such, measures of cellular proliferation have been used extensively to prognosticate both human[52-59] and veterinary neoplastic diseases[18-31,60-66]. In veterinary medicine, the most commonly used methods to evaluate cellular proliferation in a tumor include immunohistochemical staining with anti-proliferating cell nuclear antigen (PCNA) antibodies, immunohistochemical staining with anti-Ki67 antibodies, and AgNOR (agyrophilic nucleolar organizing region) histochemical staining[63,64]. Proliferating cell nuclear antigen, or PCNA, is the auxillary subunit of DNA polymerase delta[72,73], and is also involved in several additional processes in the nucleus, most notably DNA repair[74]. Although PCNA has an extended half life of 20 hours[6], and is involved in multiple nuclear functions[74], maximal PCNA expression is commonly seen in the S-phase[71,75,77], or DNA synthesis phase, of the cell cycle and immunohistochemical staining with anti-PCNA antibodies is commonly used to characterize the relative proportion of the cells in, or recently in, S-phase[61,70]. Ki67 is a nuclear protein that is expressed in all phases of the cell

cycle, but is not expressed in non-cycling cells[67,68]. Although it has been shown that Ki67 is necessary for cell cycle progression, its exact function during the cell cycle has not been characterized[68,69]. Since Ki67 is expressed in all phases of the cell cycle but not in non-cycling cells, the relative number of Ki67 positive cells in a given tissues is used to determine the proliferation index, or the relative number of cells actively involved in the cell cycle[63,64,67,68,70,168,17,28,33,55]. Agyrophilic nucleolar organizer regions, or AgNORs, are nucleolar substructures that are involved in ribosomal RNA transcription[79]. AgNORs can be identified in histologic sections as discrete black nucleolar foci using a silver-based staining method, due to the silver affinity of associated proteins[169]. The quantity of AgNORs per nuclei has been shown to be correlated with the rate of cell proliferation or the cell doubling time in vitro[12,48,59] and the rate of tumor growth in vivo[7-,33,35].

The rate of tumor growth is influenced by both the rate of cell cycle progression and the proportion of actively dividing cells in a given tumor, or the proliferation index[58,70]. Immunohistochemical staining with anti-Ki67 and anti-PCNA antibodies identify cells at various phases of the cell cycle and therefore may be used

to determine the proportion, or relative number, of cells that are actively proliferating (i.e. the proliferation index); however they do not give any indication as to how fast the cells are progressing through the cell cycle. Conversely, the average number of AgNORs per nucleus is correlated with the rate of cell cycle progression, but does not indicate what phase of the cell cycle a given cell is in, or even differentiate between cycling and non-cycling cells. Since AgNOR, PCNA, and Ki67 staining provide mutually exclusive and complementary information, these measures may provide more useful prognostic and biologic information when used in concert as opposed to when they are used independently[58,70].

In previous studies, evaluation of PCNA and Ki67 immunostaining and AgNOR histochemical staining have been shown to be independent prognostic markers for canine MCTs[28-31,66]. Although these studies have demonstrated the prognostic significance of these proliferation markers, the methods and results of these studies are highly varied, thereby confounding the interpretation and application of each individual study. Additionally, at this time, only 1 recent study has evaluated all three of these markers in a single cohort of animals[29], and no studies have evaluated the prognostic value of these thee markers in combination.

Since cellular proliferation is a result of both the number of cycling cells in a given tumor and the rate of cell cycle progression it is necessary to evaluate both the proliferation index (Ki67) and the rate of cellular proliferation (AgNORs) in order to gain a full understanding of a tumor's cellular proliferation[58,70]. By looking at all three markers, Ki67, PCNA, and AgNORs in a single cohort of animals the combined effect of these markers and a comparison of the prognostic significance of these markers can be made. A primary goal of our laboratory is to develop a prognostic classification system for canine cutaneous MCTs using multiple cellular and molecular markers, including histologic grading [22], KIT immunostaining patterns[159], *c-KIT* mutation status[162], and cellular proliferation analysis. Due to the variation in the methodologies used to assess cellular proliferation and a similar variation in results[28-31,66], we wanted to re-evaluate the utility of PCNA and Ki67 immunohistochemical staining and AgNOR histochemical staining, either independently or in conjunction with one another, as prognostic markers for canine cutaneous mast cell tumors in a population of dogs that were treated with surgical excision alone.

An additional goal of our laboratory is to understand the molecular pathogenesis of canine MCTs. Specifically, our laboratory is interested in understanding the role that the c-*KIT* proto-oncogene plays in the development of canine MCTs. The c-*KIT* proto-oncogene encodes the receptor tyrosine kinase KIT[68], which has been shown to be important for normal mast cell survival, proliferation, differentiation, and migration[106-111]. Previously, our laboratory has shown that juxtamembrane domain c-*KIT* mutations are significantly associated with higher histologic grade MCTs[96], and internal tandem duplication (ITD) mutations in the juxtamembrane domain of c-*KIT* are significantly associated with both decreased disease-free and overall survival[162]. Additionally, our laboratory has shown that changes in KIT protein localization, namely a change from peri-membrane protein localization as seen in normal, non-neoplastic mast cells to cytoplasmic localization in canine MCTs is significantly associated with decreased disease-free and overall survival[159]. Due to the fact that KIT plays an important role in normal mast cell proliferation[106,110,111], we hypothesize that ITD c-*KIT* mutations and aberrant KIT protein localization are associated with an increased proliferation index and rate of cellular proliferation in vivo. Therefore, an

additional goal of this study was to evaluate cellular proliferation as measured by Ki67 and PCNA immunohistochemical staining and AgNOR histochemical staining in a series of canine cutaneous MCTs, both with and without ITD c-*KIT* mutations and with different patterns of KIT protein localization in order to test the hypothesis that ITD c-*KIT* mutations and changes in KIT protein localization are associated with increased cellular proliferation in canine cutaneous MCTs.

Materials and Methods

Case selection and Follow-up data

Fifty-six canine cutaneous mast cell tumors from 56 dogs were included in this study. All tumors included in this study were submitted to the Diagnostic Center for Population and Animal Health at Michigan State University between 1998 and 2001 for routine diagnostic histopathologic examination. All cases were included in this study based on the following inclusion criteria: 1. confirmed diagnosis of canine cutaneous mast cell tumor; 2. treatment with surgical excision alone (no radiation or chemotherapy at the time of the initial tumor treatment); 3. availability of follow-up data; 4. adequate formalin-fixed paraffin-embedded material available for all analyses. Follow-up data including age, sex, breed,

weight, number of masses, location of mass, time before excision, medication at the time of surgery, diagnostic tests that were performed, subsequent local and distant tumor occurrences, tumor margins, survival time, and cause of death were obtained from referring veterinarians. The diagnosis and histologic grade of each MCT was independently re-evaluated according to the Patnaik histologic grading system for canine cutaneous MCTs[14] prior to inclusion in this study.

c-KIT and PCNA immunohistochemical staining

For *c-KIT* and PCNA immunostaining 5-µm sections of formalin-fixed paraffin-embedded tissue were cut, deparaffinized in xylene, rehydrated in graded ethanol, and rinsed in distilled water. Endogenous peroxidases were blocked by incubating section in 3% hydrogen peroxide for 5 minutes and subsequently rinsed in distilled water. For *c-KIT* immunostaining antigen retrieval was performed by incubating tissue sections in a citrate buffer antigen retrieval solution (Dako, Carpenteria, CA) in a steamer for 20 minutes and cooled for 20 minutes. No antigen retrieval was used for PCNA immunostaining. Using a commercial autostainer (Dako, Carpenteria, CA) non-specific antibody binding was blocked by incubating sections with a protein blocking agent (Dako, Carpenteria, CA) for 10 minutes.

Sections were either incubated with mouse monoclonal anti-PCNA antibodies (PC10 clone; Dako Cytomation, Carpenteria, CA) (Figure 15) at a 1:100 dilution or rabbit polyclonal anti-c-*KIT* (Dako Cytomation, Carpenteria, CA) at a 1:100 dilution for 30 minutes. A strepavidin-biotin labeling system (Dako, Carpenteria, CA) was used for immunolabeling. The immuno-reaction was visualized with 3'3'-diaminobenzidine (Dako, Carpenteria, CA) and all slides were counterstained with Mayer's hematoxylin. Negative controls, consisting of canine cutaneous MCTs that were treated identically to the other tissue sections except buffer was used in place of primary antibody, were included in each run. Known canine cutaneous mast cell tumor sections were included in each run as a positive control for c-*KIT* immunohistochemical staining. The basal layer of the epidermis served as an internal positive control for PCNA immunohistochemical staining.

Ki67 *immunohistochemical staining*

Using the Benchmark immunohistochemical staining platform (Ventana, Tucson, AZ) 5 µm sections of formalin-fixed paraffin-embedded tissue were deparaffinized in xylene, rehydrated in graded ethanol, and rinsed in distilled water. Antigen retrieval was performed using the Ventana medium cell conditioner protocol (Ventana, Tucson, AZ).

Sections were subsequently incubated with mouse monoclonal anti-Ki67 primary antibodies (MIB1, Dako Cytomation, Carpenteria, CA) at a 1:50 dilution for 32 minutes. The immuno-reaction was detected using a commercial alkaline phosphatase-based enhanced strepavidin-biotin secondary antibody system (Ventana, Tucson, AZ), and slides were then counter stained with Mayer's hematoxylin. Negative controls, consisting of canine cutaneous MCTs that were treated identically to the other tissue sections except buffer was used in place of primary antibody, were included in each run. The basal layer of the epidermis served as an internal positive control for Ki67 immunohistochemical staining (Figure 15).

AgNOR histochemical staining

AgNOR histochemical staining was performed using a previously described modified one-step silver staining technique[169]. In brief, 5 µm sections of formalin-fixed paraffin-embedded tissue were cut, deparaffinized in xylene, rehydrated in graded ethanol, and rinsed in distilled water. Slides were incubated for 30 minutes at room temperature in the dark with freshly made AgNOR staining solution consisting of 0.02 g gelatin in 1mL of 1% formic acid and 1 g silver nitrate in 2 mL of distilled water. Following AgNOR staining, slides were rinsed with

distilled water, dehydrated with graded ethanol and xylene and coverslipped (Figure 15).

Evaluation of KIT immunostaining Patterns

KIT immunohistochemical staining was evaluated as previously described for canine cutaneous MCTs[159]. In brief, 3 patterns of KIT protein localization were identified: 1. KIT pattern I, which consisted of a predominately perimembrane pattern of KIT protein localization with minimal cytoplasmic KIT protein localization; 2. KIT pattern II, which consisted of focal to stippled cytoplasmic KIT protein localization; and 3. KIT pattern III, which consisted of diffuse KIT cytoplasmic KIT protein localization. Each MCT was classified as having one of these 3 immunostaining patterns based on the highest staining pattern present in at least 10% (estimated based on 100 neoplastic cells in a high power field) of the neoplastic cell population or being present in large clusters of neoplastic cells within the tumor. Cells on the margins of the tissue sections were not considered for classification due to possible artifactual staining.

Evaluation of PCNA and Ki67 *immunostaining*

In order to evaluate PCNA and Ki67 immunohistochemical staining, areas with the highest proportion of immuno-positive neoplastic mast cells were identified at 100X

magnification using an American Optical light microscope. Upon identification of highly proliferative areas the number of immuno-positive cells present in a 10 mm x 10 mm grid area was counted using a 1 cm^2 10 x 10 grid reticle at 400X magnification. The number of immuno-positive cells per grid area was evaluated over 5 high powered fields and subsequently averaged in order to obtain an average S-phase index in the case of PCNA immunostaining, and the proliferation index in the case of Ki67 immunostaining.

Evaluation of AgNOR histochemical staining

In order to determine the average AgNOR count/cell in each tumor, AgNORs were counted in 100 randomly selected neoplastic mast cells throughout the tumor at 1000x magnification. Individual AgNORs were resolved by focusing up and down while counting within individual nuclei. Average AgNOR counts/cell was then determined based on averaging the counts within these 100 random neoplastic cells.

Laser capture microdissection and analysis of c-KIT mutations

Laser capture microdissection (LCM) was used to isolate neoplastic mast cells for DNA extraction and subsequent PCR amplification of c-*KIT* exon 11 and intron 11 in order to identify ITD c-*KIT* mutations as previously

described[162]. Five to 7 µm sections of each formalin-fixed, paraffin-embedded MCTs were stained with hematoxylin and dehydrated in graded alcohol for laser capture microdissection. Two-thousand to four-thousand neoplastic mast cells were extracted from each tumor sample using the Pixcell laser capture microdissection system with Macro LCM caps (Arcturus, Mountain View, CA). Extracted cells adhered to the Macro LCM caps were incubated overnight in 50 µl of DNA extraction buffer (10 mM Tris pH 8.0, 1 mM EDTA, 1% Tween) and 1.5 µl of 15 mg/ml Proteinase K (Roche, Indianapolis, IN) at 37°C. Samples were centrifuged at 1,306 x g for 5 minutes, and Proteinase K was inactivated by heating at 95°C for 8 minutes.

PCR amplification of c-KIT exon 11 and intron 11

Polymerase chain reaction (PCR) amplification was performed using a previously described primer pair that flanks exon 11 and the 5′ end of intron 11[146], which includes the previously described ITD region of the c-*KIT* proto-oncogene in canine MCTs[16,91,95,96,145,146,162]. Polymerase chain reactions were prepared in a 25 µl total reaction volume, with 5 µl LCM extracted DNA, 5 pmol of each primer, 0.5 units of Taq polymerase (Invitrogen, Carlsbad, CA), and final concentrations of 80 µM dNTPs, 2 mM $MgCl_2$, 20 mM Tris-HCl, and 50 µl KCl. Cycling conditions were as follows:

94°C for 4 minutes; 35-45 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; 72°C for 5 minutes. Amplified products and ITD mutations were visualized by agarose gel electrophoresis on a 2% agarose gel after ethidium bromide staining.

Statistical Analysis

Univariable Analyses: Before developing multivariable models, each risk factor was evaluated for its association with MCT outcomes. Univariable proportional hazards model were developed for each risk factor for each outcome, and the level of association was assessed through the risk factor's p-value in the model. Risk factors with p less than or equal to 0.20 were considered for inclusion in the multivariable model.

Multivariable Logistic Regression Models: Logistic regression models were developed for the occurrence of outcomes associated with MCTs, including recurrence of local MCTs, recurrence of distant MCTs, and death associated with MCTs. In addition to risk factors of interest, animal signalment (age, sex, weight) were included in the multivariable model to account for their effects on model outcome. Results were reported as odds ratios: an odds ratio less than one means the likelihood of the occurrence of an event is reduced, while an odds ratio

greater than one indicates the likelihood of an event is increased. Odds ratios equal to 1 indicate that the risk factor neither increases nor decreases the likelihood of the outcome.

Multivariable Survival Analysis Models: This study used the Cox proportional hazards models (SAS PROC PHREG) (SAS Version 9.13, SAS Institute, Inc., Cary, N.C.) for survival analysis, using survival times (time-to-event) as the model outcome, and produces point estimates of the hazard ratio (risk ratio) for risk factors in the model. Proportional hazards regression models were developed for survival analysis of different outcomes associated with MCTs. These outcomes were days to recurrence of local MCTs, days to recurrence of distant MCTs, and days to death resulting from MCT. In addition to risk factors of interest, animal signalment (age, sex, weight) were included in the multivariable model to account for their effects on model outcome. The effects of risk factors on days to events was reported as hazard ratios. Comparable to odds ratios, hazard ratios less than one indicate that the risk factor increases time to outcome, while hazard ratios greater than one indicate that the risk factor decreases time to outcome.

Results

Eighteen dog breeds were represented in this study including 12 mixed breed dogs, 12 Labrador retrievers, 10 boxers, 6 golden retrievers, 2 pugs, 2 basset hounds, and 12 additional breeds that were represented by single dogs. The median age of the dogs included in this study was 7.93 years and ranged from 2 to 14 years of age. Thirty-three female and 23 male dogs were included in this study. Eight of the fifty-six MCTs included in the study were histologic grade 1, 41 MCTs were histologic grade 2 and 7 MCTs were histologic grade 3.

The average PCNA counts of the MCTs included in this study ranged from 6.00 to 243.20 positive cells per grid area with average count of 64.94 positive cells/grid area and a median of 44.50 positive cells. AgNOR counts ranged from 1.25 AgNORs/cell to 4.05 AgNORs/cell, with an average count of 2.29 AgNORs/cell and a median count of 2.21 AgNORs/cell. Ki67 counts ranged from 3 to 97 positive cells/grid area, with an average of 24.66 positive cells/ grid area and a median Ki67 count of 17.10 cells.

The prognostic significance of Ki67, PCNA, and AgNOR counts were first evaluated in a univariable statistical analysis in order to identify potentially significant variables for inclusion in a multivariable

statistical model controlling for age, gender, and breed (Tables 14 and 15). Additionally, since tumor growth is determined by both the number of proliferating cells within a given tumor and the rate of cellular proliferation[58,70], derived variables were created by multiplying the AgNOR counts by either the Ki67 counts (Ag67) or PCNA counts (AgPCNA) in order to determine the utility of evaluating the proportion of cycling cells, as measured by either the proliferation index (Ki67 count) or S-phase index (PCNA count), and the rate of cellular proliferation as measured by AgNORs in concert. According to multivariable analysis increased Ki67 counts were significantly associated with both an increased incidence and rate of subsequent tumor occurrence at the original surgical site ($p=0.0111$; $p=0.0017$, respectively), an increased rate of MCT occurrence at sites distant from the original tumor site ($p=0.0081$), and an increased incidence of MCT-related mortality ($p=0.0022$). Increased AgNOR counts were significantly associated with both an increased rate of subsequent tumor occurrence at the original surgical site and at sites distant from the original surgical site ($p=0.0435$; $p=0.0121$, respectively), and with an increased incidence and rate of MCT-related mortality ($p=0.0028$; $p=0.0017$, respectively). Additionally, increased Ag67

counts were significantly associated with an increased incidence and rate of MCT occurrence at the original surgical site(p=0.0230; p=0.0021, respectively), an increased rate of MCT occurrence at distant sites (p=0.0026), and the incidence and rate of MCT-related mortality (p=0.0053; p=0.0318, respectively), whereas AgPCNA counts were only associated with an increased incidence and rate of MCT-related mortality (p=0.048; p=0.0325, respectively). PCNA counts were not found to be of any prognostic significance following multi-variable analysis.

In order to utilize these proliferation markers distinct cut points for each marker that differentiate between tumors with a favorable prognosis and those with a poor prognosis are needed. Cut-off values that discriminated MCTs associated with patient mortality from those that were not associated with mortality were determined for each proliferation marker. These cut-off values were based on the mean value and 95% confidence interval of each marker for MCT associated with patient mortality and those that were not. Only the Ki67 and the Ag67 indices had distinct cut off points, of 23.08 and 53.95, respectively, that allowed for a clear differentiation between these two populations of tumors

with non-overlapping confidence intervals. According to multivariable analysis, MCTs with a Ki67 index greater than 23.08 cells/grid area or an Ag67 index greater than 53.95 were significantly associated with an increased incidence (p=0.0262; p=0.0130, respectively) and rate (p= 0.0074; p=0.0109, respectively) of MCT occurrence at the original surgical site (Figures 16 and 17, respectively) and with an increased incidence of MCT-related mortality (p=0.0016; p=0.0012, respectively) (Figure 18). Additionally, MCTs with a Ki67 index greater than 23.08 were also significantly associated with an increased rate of MCT occurrence at distant sites (p=0.0138), and an increased rate of MCT-related mortality (p=0.0171; Figure 19).

In order to determine the associations between ITD c-*KIT* mutations and aberrant KIT protein localization, and cellular proliferation in canine MCTs, each MCT included in this study was evaluated for ITD c-*KIT* mutations using PCR and aberrant KIT protein localization using immunohistochemistry. Internal tandem duplication mutations were identified in the juxtamembrane domain of the c-*KIT* proto-oncogene of 9 of the 56 MCTs included in this study. According to multivariable analysis, canine MCTs with ITD c-*KIT* mutations had a significantly greater proliferation index (p=0.0015), as measured by Ki67

immunostaining, and a significantly greater rate of cell cycle progression ($p=0.0025$), as measured by AgNOR histochemical staining, than MCTs that lack such mutations. Additionally, we found that increased cytoplasmic KIT protein localization, as compared to peri-membrane protein localization that is seen in normal, non-neoplastic mast cells, was also significantly associated with an increased proliferation index ($p=0.0027$), as measured by Ki67 immunostaining, and with an increased rate of cell cycle progression ($p=0.0038$), as measured by AgNOR staining (Figure 20).

Discussion

The results of this study demonstrate the significant role cellular proliferation plays in the progression of canine MCTs, as we have shown that both the rate of cellular proliferation, as measured by AgNORs, and the proportion of cycling cells, as measured by Ki67, are both significantly associated with the progression of canine MCTs. In light of these results, we recommend that AgNOR and Ki67 indices should be routinely evaluated in canine MCTs patients in conjunction with other prognostic markers, such as histologic grading, *c-KIT* mutations, and KIT staining patterns.

In order to utilize proliferation indices in a routine diagnostic setting distinct break points are needed to differentiate between tumors that are likely to have a favorable prognosis and those that are likely to have a poor prognosis. Such distinct break points, with non-overlapping, or minimally over-lapping 95% confidence intervals could only be defined for the Ki67 index, which had a break point of 23.08 immuno-positive cells/ grid area, and the Ag67 index, which had a break point of 53.95. Based on these breakpoints Ki67 was better in terms of identifying MCTs that were associated with a decreased survival duration, whereas Ag67 was a better marker for identifying MCTs with a decreased disease-free interval. Therefore, based on these results MCTs should be evaluated for both their Ki67 and Ag67 indices, and should be evaluated in light of these break-points, in order to best identify patients that are likely to have subsequent local and distant MCT occurrences, and in order to identify patients that are likely to succumb to their mast cell disease.

Although ITD c-*KIT* mutations and aberrant KIT protein localization have been well-characterized in canine cutaneous MCTs[16,91,95,96,145,146,162], little is known about downstream consequences of these mutations on the cellular

level. All of the ITD c-*KIT* mutations that have been characterized to date have been shown to produce a constitutively activated KIT protein in the absence of ligand[91,99,145] and have also been associated with downstream ERK phosphorylation[145]. Previous studies by our laboratory have shown that both ITD c-*KIT* mutations and aberrant KIT protein localization in canine MCTs are associated with a worse prognosis, as compared to MCTs that lack such mutations or have normal peri-membrane protein localization, respectively[159,162]. The results of this study suggest that a downstream consequence of c-*KIT* mutations and aberrant KIT protein localization in canine MCTs may be an increase in cellular proliferation, by both increasing the rate at which the cells enter the cell cycle and by increasing the rate at which the neoplastic cells progress through the cell cycle. Although these results are extremely intriguing and provide preliminary in vivo data that support the hypothesis that c-*KIT* mutations and aberrant KIT protein localization cause an increase in cellular proliferation in canine cutaneous MCTs, further in vitro studies are needed to support these in vivo results.

The results of this study demonstrate the utility of evaluating cellular proliferation, specifically Ki67 immunostaining and AgNOR histochemical staining, in the

routine prognostication of canine MCTs. Although the results of this study confirm the results of previous studies that have shown the prognostic significance of cellular proliferation in canine MCTs[28-31,66], cellular proliferation should not be evaluated as a single prognostic factor for canine MCTs, but should be evaluated in tandem with additional prognostic markers such as histologic grade, c-*KIT* mutations, and aberrant KIT protein localization. To date no single marker will definitively distinguish between benign and malignant MCTs. Therefore, each prognostic marker should be considered in light of additional makers in order to evaluate the full scope of the disease and in order to make the best therapeutic determinations for a given patient.

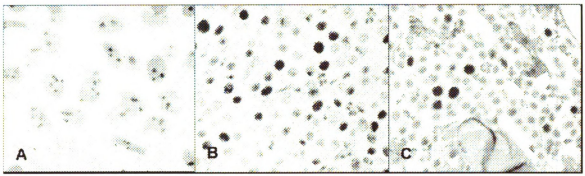

Figure 15: Histochemical and immunohistochemical staining for proliferation markers in canine cutaneous MCTs. A: AgNOR histochemical staining: AgNORs are identified as discrete, black nuclear foci (orginal magnification: 1,000X); B: PCNA immunohistochemical staining. Cells expressing PCNA are identified by dark brown nuclear staining (original magnification: 400X); C: Ki67 immunohistochemical staining. Cells expressing Ki67 are identified by magenta nuclear staining (original magnification: 400x).

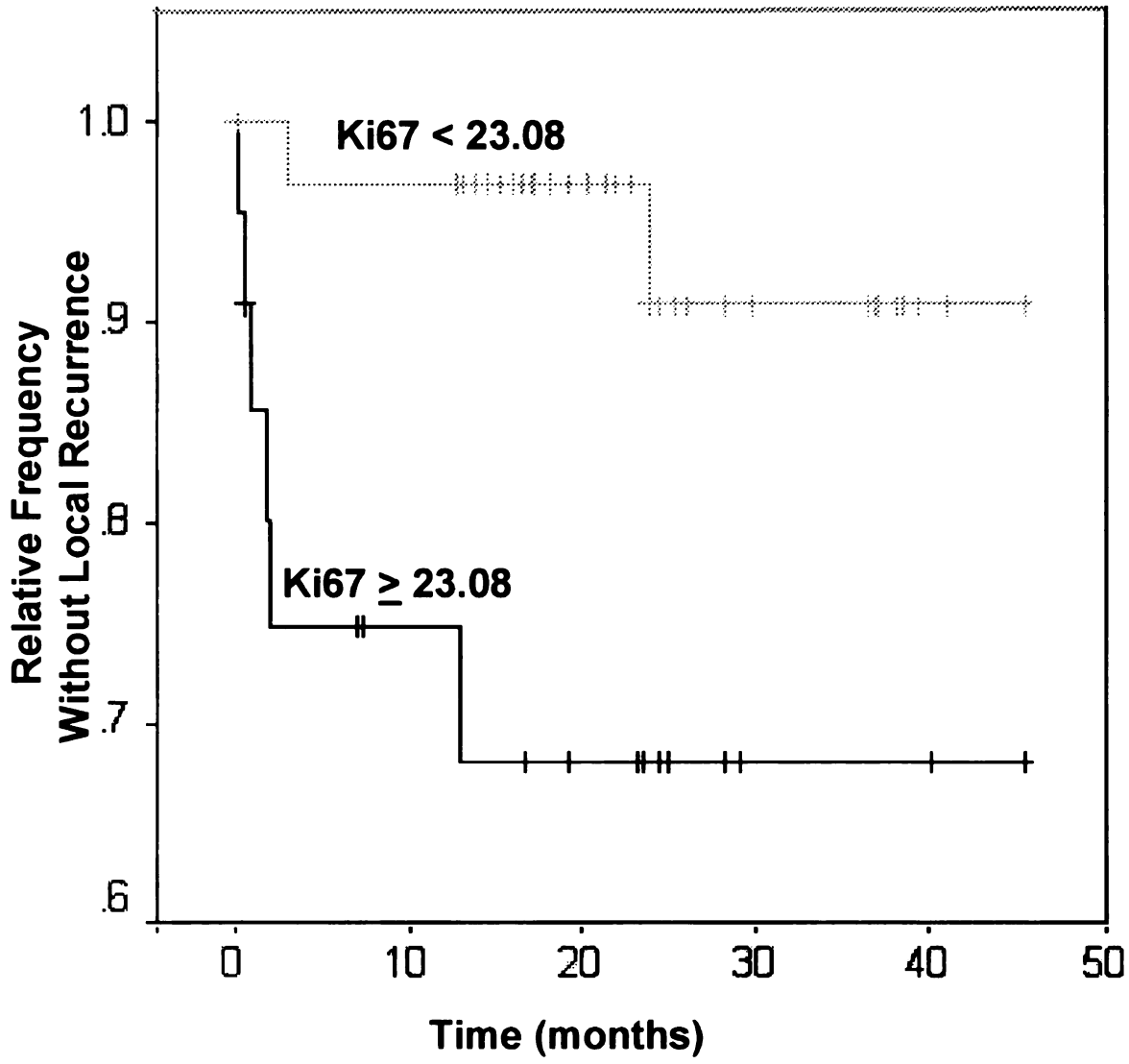

Figure 16: Kaplan-Meier survival curve evaluating the time until local recurrence of canine MCT patients classified based on Ki67 protein expression. Patients with greater than or equal to 23.08 Ki67 positive cells per grid area had a significantly shorter time to local recurrence compared to those with less than 23.08 Ki67 positive cells (p=0.0074).

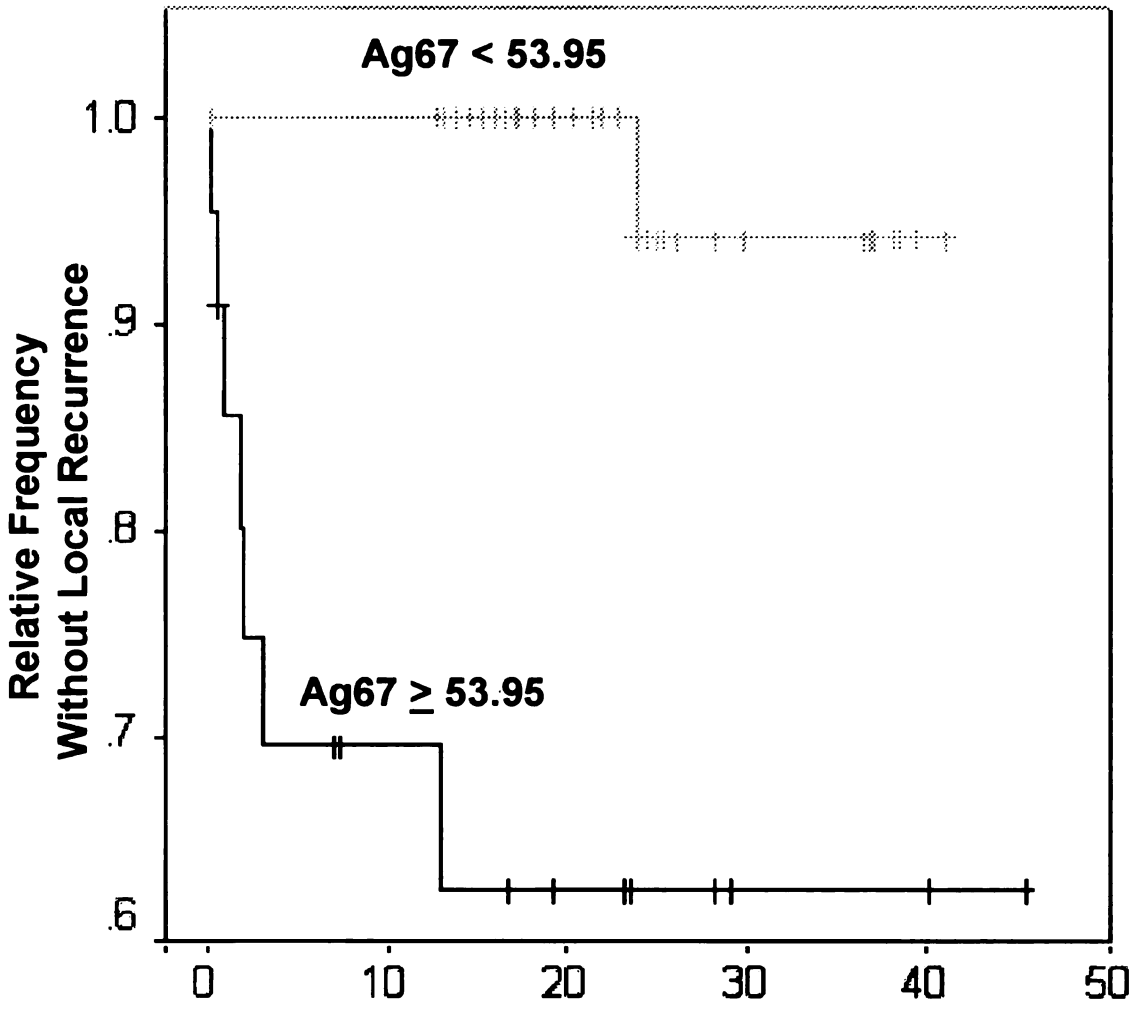

Figure 17: Kaplan-Meier survival curve evaluating the time until local recurrence of canine MCTs patients classified based on Ag67 values. Patients with Ag67 counts greater than or equal to 53.95 had a significantly shorter time to local recurrence compared to those with less than 53.95 (p=0.0109).

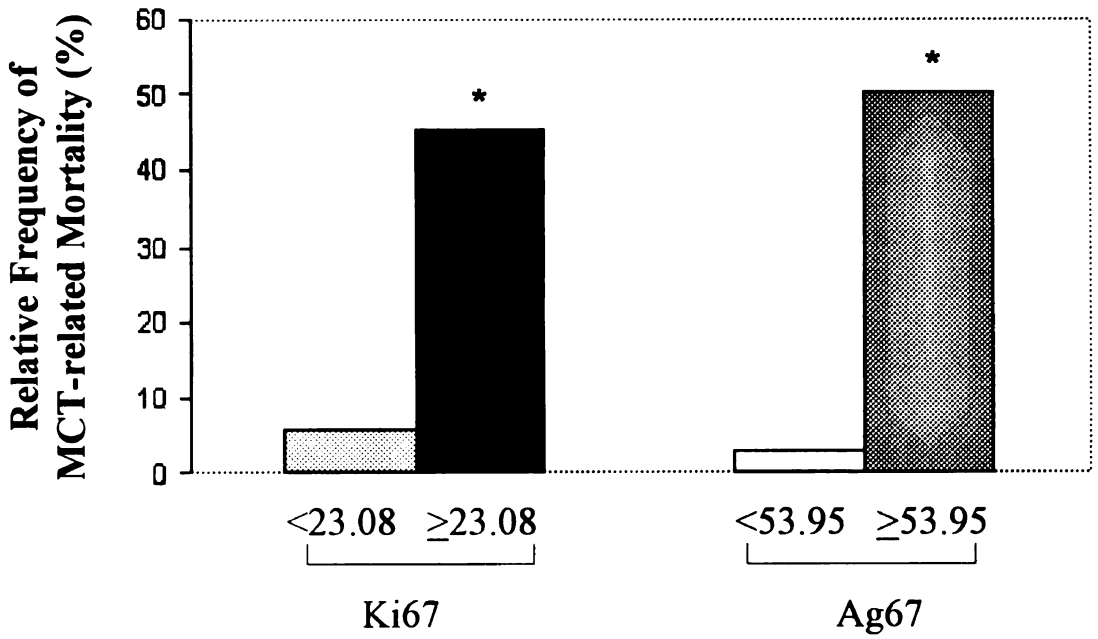

Figure 18: Relative frequency of MCT-related mortalities in canine cutaneous MCTs based on their Ki67 and Ag67 indices. Canine MCTs with greater than or equal to 23.08 Ki67 or 53.95 Ag67 were associated with a significantly increased incidence of MCT-related mortality compared to those that have less than 23.08 or 53.95, respectively ($p=0.0016$; 0.0012, respectively).

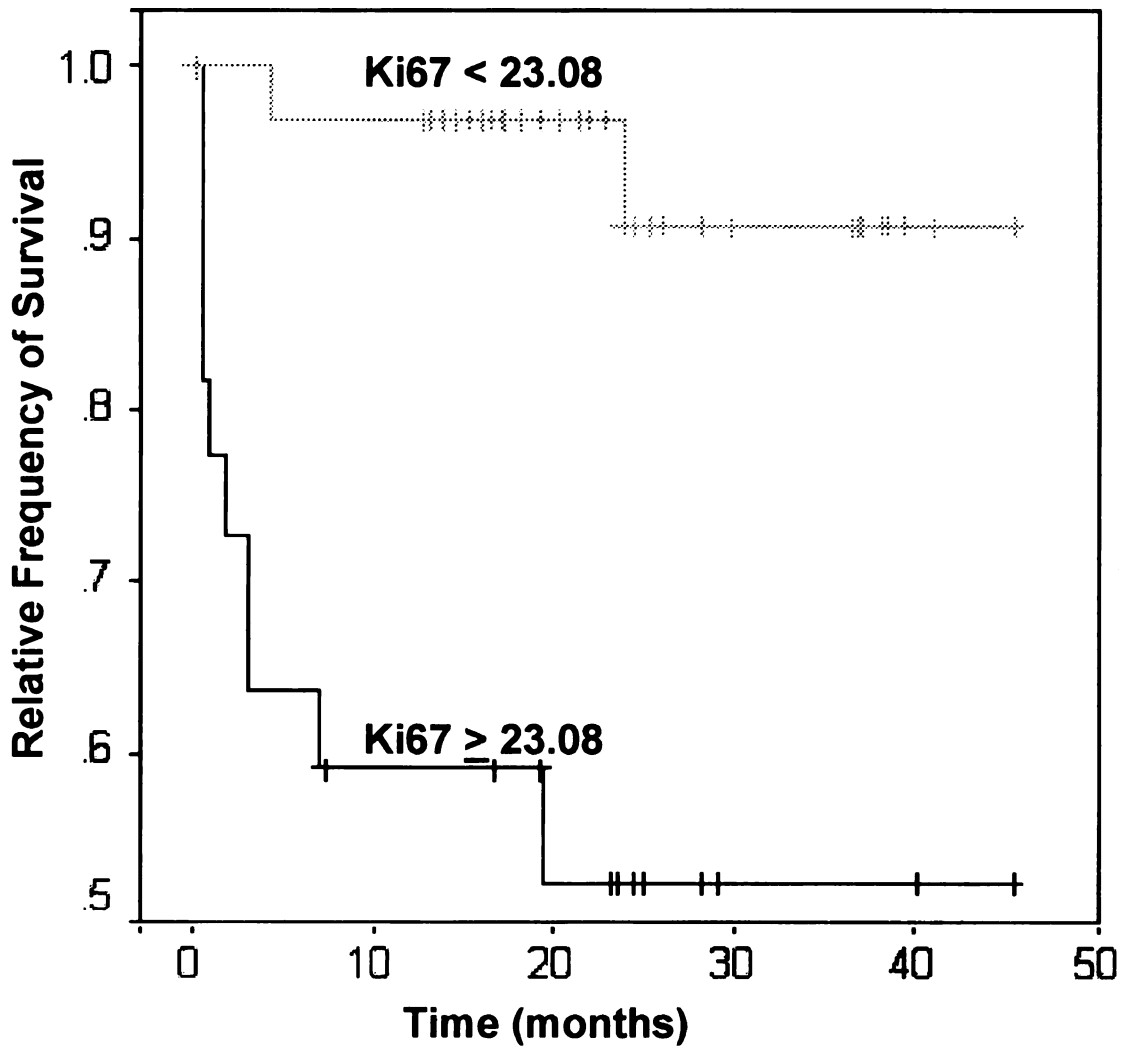

Figure 19: Kaplan-Meier survival curve evaluating the time until MCT-related mortality of canine MCT patients classified based on Ki67 protein expression. Patients with greater than or equal to 23.08 Ki67 positive cells per grid area had a significantly shorter survival duration compared to those with less than 23.08 Ki67 positive cells (p=0.0171).

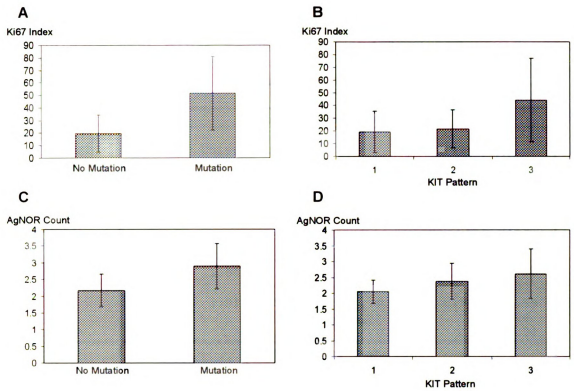

Figure 20: Association between cellular proliferation and the presence of *c-KIT* mutations or aberrant KIT protein localization. Canine cutaneous MCTs with ITD *c-KIT* mutations were associated with a significantly increased proliferation index (figure A; p=0.0015) and a significantly increased rate of cellular proliferation (figure C; p=0.0025). Similarly, canine cutaneous MCTs with aberrant cytoplasmic KIT protein localization were significantly associated with an increased proliferation index (figure B; 0.0049) and a significantly increased rate of cellular proliferation (figure D; p=0.0065).

Table 14: Univariate and multivariate logistic regression analysis between Ki67, AgNOR, PCNA, and Ag67 and incidence of subsequent tumor development and MCT-related mortality.

Proliferation marker	Local Tumor Occurrence					
	Univariate			Multivariate		
	p	Odds Ratio	95% C.I.	p	Odds Ratio	95% C.I.
PCNA	0.0814	1.01	0.99 - 1.02	0.153	0.99	0.98 - 1.00
AgNOR	0.4552	1.61	0.46 - 5.56	0.246	0.42	0.10 - 1.83
Ki-67	0.0124	1.04	1.01 - 1.08	0.0111	0.93	0.88 - 0.98
Ag67	0.074	1.01	0.99 - 1.02	0.023	0.98	0.97 - 0.99
AgPCNA	0.0914	1	0.99 - 1.01	0.143	1	0.99 - 1.01
Proliferation marker	Distant Tumor Occurrence					
	Univariate			Multivariate		
	p	Odds Ratio	95% C.I.	p	Odds Ratio	95% C.I.
PCNA	0.1878	1.01	0.99 - 1.02	0.3807	0.99	0.98 - 1.01
AgNOR	0.2345	1.86	0.67 - 5.16	0.1849	0.48	0.16 - 1.42
Ki-67	0.1258	1.02	0.99 - 1.05	0.1317	0.98	0.94 - 1.01
Ag67	0.2391	1.01	0.99 - 1.01	0.2019	0.99	0.98 - 1.00
AgPCNA	0.1611	1	0.99 - 1.01	0.3011	1	0.99 - 1.00
Proliferation marker	MCT-related Mortality					
	Univariate			Multivariate		
	p	Odds Ratio	95% C.I.	p	Odds Ratio	95% C.I.
PCNA	0.0848	1.01	0.99 - 1.02	0.1768	0.99	0.98 - 1.00
AgNOR	0.0046	7.09	1.83 - 27.49	0.0028	0.06	0.01 - 0.38
Ki-67	0.0018	1.07	1.02 - 1.11	0.0022	0.93	0.88 - 0.97
Ag67	0.0009	1.003	1.001- 1.009	0.0053	0.96	0.94 - 0.99
AgPCNA	0.0255	1.005	1.001 - 1.009	0.048	0.995	0.991 -1.00

Table 15: Univariate and multivariate proportional hazards analysis between Ki67, AgNOR, PCNA, and Ag67 and time until subsequent tumor development and MCT-related mortality.

Proliferation marker	Local Tumor Occurrence					
	Univariate			Multivariate		
	p	Hazard Ratio	95% C.I.	p	Hazard Ratio	95% C.I.
PCNA	0.047 4	1.01	1.00 - 1.02	0.0942	1.01	0.99 - 1.02
AgNOR	0.196 7	2.33	0.65 - 8.20	0.0435	4.48	1.05 - 19.17
Ki-67	0.001 3	1.04	1.02 - 1.07	0.0017	1.08	1.03 - 1.13
Ag67	0.003 4	1.01	1.004 - 1.02	0.0021	1.03	1.01 - 1.04
AgPCNA	0.037 1	1.004	1.000 - 1.007	0.0713	1.003	1.00 - 1.006
Proliferation marker	Distant Tumor Occurrence					
	Univariate			Multivariate		
	p	Hazard Ratio	95% C.I.	p	Hazard Ratio	95% C.I.
PCNA	0.105 7	1.006	0.99 - 1.01	0.1835	1.005	0.99 - 1.013
AgNOR	0.042 3	2.79	1.04 - 7.50	0.0121	3.9	1.35 - 11.31
Ki-67	0.014 0.007	1.03	1.01 - 1.05 1.00 -	0.0081	1.03	1.01 - 1.06 1.00 -
Ag67	4 0.045	1.01	1.02 1.00 -	0.0026	1.01	1.02 1.00 -
AgPCNA	7	1.003	1.01	0.0837	1.002	1.01
Proliferation marker	MCT-related Mortality					
	Univariate			Multivariate		
	p	Hazard Ratio	95% C.I.	p	Hazard Ratio	95% C.I.
PCNA	0.350 5	1.004	.99 - 1.01	0.11	1.01	0.99 - 1.03
AgNOR	0.001 3	9.46	2.41 - 37.13	0.0017	17.63	2.93 - 106.05
Ki-67	0.116 8	1.02	.99 - 1.04	0.1484	1.02	0.99 - 1.05
Ag67	0.009 3	1.01	1.002 - 1.02	0.0318	1.01	1.00 - 1.02
AgPCNA	0.100 5	1.002	1.00 - 1.01	0.0325	1.007	1.001 - 1.013

CHAPTER 6

EVALUATION OF PROGNOSTIC MARKERS ASSOCIATED WITH THE PROGRESSION OF CANINE CUTANEOUS MAST CELL TUMORS IN DOGS TREATED WITH THE COMBINED CHEMOTHERAPY

Introduction

Canine cutaneous mast cell tumors (MCTs) are one of the most common neoplastic diseases in dogs, accounting for 7-21% of all cutaneous neoplasms[1-4]. Canine MCTs have an extremely variable biologic behavior, ranging from a solitary benign mass that can be cured with surgery alone, to a systemic and potentially fatal metastatic disease[5,7,13,14,36]. Currently, surgical excision is considered to be the primary treatment modality for canine MCTs, and in the event of incomplete surgical excision or a non-resectable tumor, radiation therapy may be used as an adjunct therapy. Additionally, in the face of multi-centric, metastatic, or aggressive MCTs, chemotherapy is commonly employed. Although numerous chemotherapeutic protocols have been evaluated for the treatment of canine cutaneous mast cell tumors, the response rates of these protocols are extremely variable and no protocol has been shown to be clearly superior for treating canine MCTs[5,7].

In light of the variable and somewhat poor response rates of canine MCTs to chemotherapy and the potentially fatal side effects that can be associated with chemotherapy, it is critical to identify patients that will benefit most from such treatment. Therefore, it is critical to identify prognostic markers that are associated with the disease progression of canine MCTs treated with a given chemotherapeutic protocol. In order to properly evaluate the prognostic significance of a given marker, each marker needs to be evaluated in a population of animals treated with a single treatment protocol.

Previously, our laboratory has evaluated the value of multiple markers in the prognostication of canine MCTs following treatment with surgical excision alone. In these studies we have found that KIT staining patterns[159], *c-KIT* internal tandem duplication (ITD) mutations[162], and cellular proliferation as measured by Ki67 immuno-staining and AgNOR histochemical staining (Webster et al. unpublished results) are significantly associated with the progression of canine MCTs when treated with surgery alone, whereas tryptase immunohistochemical staining patterns[159] and PCNA indices were not found to be of prognostic significance (Webster et al., unpublished results). Although the results of these studies demonstrate the potential for the application of

these markers as diagnostic and prognostic tools for MCTs treated with surgery alone, they do not provide any information regarding their association with the outcome of patients treated with adjunct radiation or chemotherapy.

The combination chemotherapy of vinblastine and prednisone has been previously shown to be efficacious as an adjunct therapy in a subset of canine cutaneous MCTs[26]. However, no clear determinates have been identified to discriminate between MCTs that are likely to benefit from vinblastine and prednisone and those that are not. Therefore, the primary goal of this study is to evaluate the prognostic significance of histologic grade, *c-KIT* ITD mutation status, KIT staining patterns, tryptase staining patterns, and the proliferation markers Ki67, PCNA, and AgNORs for dogs treated with vinblastine and prednisone.

Currently, most veterinary studies evaluating novel therapeutic protocols are retrospective in nature and lack a true control, untreated population from which comparisons can be made[26,170]. Instead, patient survival is subjectively compared to historical survival data. In order to better determine the efficacy of vinblastine and prednisone in the treatment of canine MCTs, an additional goal of this study is to compare the survival of dogs treated with vinblastine

and prednisone to those treated with surgery alone, when stratified based on known prognostic markers.

Materials and Methods

Case and tissue selection

Twenty-eight canine cutaneous mast cell tumors from 28 dogs included in this study were identified as part of a larger retrospective study[16] with the goals of: 1. evaluating the combination of vinblastine and prednisone as an adjunct chemotherapy for canine MCTs following surgical excision; and 2. identifying clinical prognostic factors associated with this treatment. All cases were treated at the University of Wisconsin-Madison Veterinary Medical Teaching Hospital and were included in this study based on the following inclusion criteria: 1. absence of known severe concurrent disease; 2. complete staging; 3. no concurrent systemic anti-neoplastic therapy other then prednisone and vinblastine; 4. absence of measurable disease following surgery; 5. confirmed histologic diagnosis of canine cutaneous MCT; 6. adequate tissues available for *c-KIT* polymerase chain reaction and immunohistochemistry. Histologic grade of each tumor was confirmed according to the Patnaik histologic grading system for canine cutaneous MCTs by a single pathologist[14]. Complete clinical staging was performed in all patients

including a complete physical exam, a complete blood count and a serum biochemistry profile, abdominal ultrasound, and regional lymph node palpation with or without fine needle aspirate cytology. Lymph nodes were only considered positive for lymph node metastasis if they contained clusters or sheets of mast cells. Scattered individual mast cells were not sufficient in order to diagnose lymph node metastasis.

c-KIT, tryptase and PCNA immunohistochemical staining

For c-*KIT*, tryptase, and PCNA immuno-staining 5-µm sections of formalin-fixed paraffin-embedded tissue were cut, deparaffinized in xylene, rehydrated in graded ethanol, and rinsed in distilled water. Endogenous peroxidases were blocked by incubating section in 3% hydrogen peroxide for 5 minutes and subsequently rinsed in distilled water. For c-*KIT* and tryptase immuostaining antigen retrieval was performed by incubating tissue sections in a citrate buffer antigen retrieval solution (Dako, Carpenteria, CA) in a steamer for 20 minutes and cooled for 20 minutes. No antigen retrieval was used for PCNA immuno-staining. Using a commercial autostainer (Dako, Carpenteria, CA) Non-specific antibody binding was blocked by incubating sections with a protein blocking agent (Dako, Carpenteria, CA) for 10 minutes. Sections

were either incubated with mouse monoclonal anti-PCNA antibodies (PC10 clone; Dako Cytomation, Carpenteria, CA) at a 1:100 dilution, rabbit polyclonal anti-c-*KIT* antibodies (Dako Cytomation, Carpenteria, CA) at a 1:100 dilution, or mouse anti-human tryptase antibodies (Dako Cytomation, Carpenteria, CA) for 30 minutes. A strepavidin-biotin labeling system (Dako, Carpenteria, CA) was used for immunolabeling. The immuno-reaction was visualized with 3'3'-diaminobenzidine (Dako, Carpenteria, CA) and all slides were counterstained with Mayer's hematoxylin. Negative controls, consisting of canine cutaneous MCTs that were treated identical to the other tissue sections except buffer was used in place of primary antibody, were included in each run. Known canine cutaneous mast cell tumors sections were included in each run as a positive control for c-*KIT* immunohistochemical staining. The basal layer of the epidermis served as an internal positive control for PCNA immunohistochemical staining

Ki67 *immunohistochemical staining*

Using the Benchmark immunohistochemical staining platform (Ventana, Tucson, AZ) 5 µm sections of formalin-fixed paraffin-embedded tissue were deparaffinized in xylene, rehydrated in graded ethanol, and rinsed in distilled

water. Antigen retrieval was performed using the Ventana medium cell conditioner protocol (Ventana, Tucson, AZ). Sections were subsequently incubated with mouse monoclonal anti-Ki67 primary antibodies (MIB1, Dako Cytomation, Carpenteria, CA) at a 1:50 dilution for 32 minutes. The immuno-reaction was detected using a commercial alkaline phosphatase-based enhanced strepavidin-biotin secondary antibody system (Ventana, Tucson, AZ), and slides were then counter stained with Mayer's hematoxylin. Negative controls, consisting of canine cutaneous MCTs that were treated identical to the other tissue sections except buffer was used in place of primary antibody, were included in each run. The basal layer of the epidermis served as an internal positive control for Ki67 immunohistochemical staining.

AgNOR histochemical staining

AgNOR histochemical staining was performed using a previously described modified one-step silver staining technique[169]. In brief, 5 µm sections of formalin-fixed paraffin-embedded tissue were cut, deparaffinized in xylene, rehydrated in graded ethanol, and rinsed in distilled water. Slides were incubated for 30 minutes at room temperature in the dark with freshly made AgNOR staining solution consisting of 0.02 g gelatin in 1 mL of

1% formic acid and 1 g silver nitrate in 2 mL of distilled water. Following AgNOR staining, slides were rinsed with distilled water, dehydrated with graded ethanol and xylene and coverslipped.

Evaluation of KIT immuno-staining Patterns

KIT immunohistochemical staining was evaluated as previously described for canine cutaneous MCTs[159]. In brief, 3 patterns of KIT protein localization were identified: 1. KIT pattern I, which consisted of a predominately perimembrane pattern of KIT protein localization with minimal cytoplasmic KIT protein localization; 2. KIT pattern II, which consisted of focal to stippled cytoplasmic KIT protein localization; and 3. KIT pattern III, which consisted of diffuse KIT cytoplasmic KIT protein localization. Each MCT was classified as having one of these 3 immuno-staining patterns based on the highest staining pattern present in at least 10% (estimated based on 100 neoplastic cells in a high power field) of the neoplastic cell population or being present in large clusters of neoplastic cells within the tumor. Cells on the margins of the tissue sections were not considered for classification due to possible artifactual staining.

Evaluation of tryptase immuno-staining

Tryptase expression was evaluated in canine MCTs as previously described[159]. MCTs were classified into 3 groups based on their different tryptase staining patterns. Tryptase staining pattern I was characterized by neoplastic mast cells that were diffusely positive for tryptase throughout the cytoplasm, obscuring all other cytoplasmic features. MCTs with tryptase staining pattern II consisted of neoplastic mast cells with moderate amounts of stippling throughout the cytoplasm. In MCTs with tryptase staining pattern III, neoplastic mast cells had weak cytoplasmic stippling. Due to the presence of occasional degranulated cells within MCTs, each MCT was assigned to 1 of these 3 tryptase staining patterns based on the predominate staining found in the majority of neoplastic cells throughout the section, instead of being classified based on the highest staining pattern present. Cells on the margins of the sections were not considered for classification to avoid possible artifactual staining.

Evaluation of PCNA and Ki67 *immuno-staining*

In order to evaluate PCNA and Ki67 immunohistochemical staining areas with the highest proportion of immuno-positive neoplastic mast cells were identified at 100X magnification using an American Optical light microscope. Upon identification of highly proliferative areas the

number of immuno-positive cells present in a 10 mm x 10 mm grid area was counted using a 1 cm^2 10 x 10 grid reticle at 400X magnification. The number of immuno-positive cells per grid area was evaluated over 5 high powered fields and subsequently averaged in order to obtain an average S-phase index in the case of PCNA immuno-staining, and the proliferation index in the case of Ki67 immuno-staining.

Evaluation of AgNOR histochemical staining

In order to determine the average AgNOR count/cell in each tumor, AgNORs were counted in 100 randomly selected neoplastic mast cells throughout the tumor at 1000x magnification. Individual AgNORs were resolved by focusing up and down while counting within individual nuclei. Average AgNOR counts/cell was then determined based on averaging the counts within these 100 random neoplastic cells.

Laser capture microdissection and analysis of c-KIT mutations

Laser capture microdissection (LCM) was used to isolate neoplastic mast cells for DNA extraction and subsequent PCR amplification of c-*KIT* exon 11 and intron 11 in order to identify ITD c-*KIT* mutations as previously described[162]. Five to 7 µm sections of each formalin-fixed, paraffin-embedded MCTs were stained with hematoxylin and

dehydrated in graded alcohol for laser capture microdissection. Two-thousand to four-thousand neoplastic mast cells were extracted from each tumor sample using the Pixcell laser capture microdissection system with Macro LCM caps (Arcturus, Mountain View, CA). Extracted cells adhered to the Macro LCM caps were incubated overnight in 50 µl of DNA extraction buffer (10 mM Tris pH 8.0, 1 mM EDTA, 1% Tween) and 1.5 µl of 15 mg/ml Proteinase K (Roche, Indianapolis, IN) at 37°C. Samples were centrifuged at 1,306 x g rpms for 5 minutes, and Proteinase K was inactivated by heating at 95°C for 8 minutes.

PCR amplification of c-KIT exon 11 and intron 11

Polymerase chain reaction (PCR) amplification was performed using a previously described primer pair that flanks exon 11 and the 5′ end of intron 11[146], which includes the previously described ITD region of the c-*KIT* proto-oncogene in canine MCTs[91,95,96]. Polymerase chain reactions were prepared in a 25 µl total reaction volume, with 5 µl LCM extracted DNA, 5 pmol of each primer, 0.5 units of Taq polymerase (Invitrogen, Carlsbad, CA), and final concentrations of 80 µM dNTPs, 2 mM $MgCl_2$, 20 mM Tris-HCl, and 50 µl KCl. Cycling conditions were as follows: 94°C for 4 minutes; 35-45 cycles at 94°C for 1 minute, 55 C for 1 minute, and 72°C for 1 minute; 72°C for 5 minutes.

Amplified products and ITD mutations were visualized by agarose gel electrophoresis on a 2% agarose gel after ethidium bromide staining.

Statistical Analysis

Associations between categorical prognostic markers, and treatment, and time to treatment failure and survival times were evaluated graphically using Kaplan-Meier survival curves, and levels of each categorical variable were compared using log rank survival analysis. Associations between continuous variables and time to treatment failure and survival time were evaluated using Cox proportional hazards analysis.

Results

Twenty-eight canine cutaneous mast cell tumors from 28 dogs were included in this study. Fourteen of the 28 dogs included in this study were female and 14 were male. These dogs ranged in age from 2 to 14 years of age, with an average age of 9.28 years, and represented 16 distinct breeds including mixed breed dogs (9), Labrador retrievers (4), Gordon setters (2), and 13 other breeds which were represented by individual animals.

Dogs were considered to have obtained adequate local therapy when tumors were completely excised with no evidence of neoplastic mast cells at the surgical margins

or when surgical excision was performed with subsequent local radiation therapy. According to these standards, adequate local therapy was obtained in 24 of the 28 dogs including in this study. Four dogs had evidence of microscopic disease at the time that chemotherapy was initiated. Adjunct radiation therapy was performed in 19 patients, and regional lymph nodes were also irradiated in 10 of these patients.

According to univariate survival analyses, increased cytoplasmic KIT protein localization (0.009), increased tryptase expression (p=0.0286), increased histologic grade (p=0.0062), and increased rate of cellular proliferation as measured by AgNOR staining (p=0.0161) and an increase S-phase index as measured by PCNA (p=0.0293) were significantly associated with a decreased time to treatment failure. Mast cell tumors with *c-KIT* mutations were not associated with a significantly decreased time to treatment failure, however a trend was present (p=0.0576) (Figure 21, A-D). The proliferation index as measured by Ki67 was not associated with a decreased time to treatment failure.

Similarly, histologic grade III MCTs (p=0.004), the presence of ITD *c-KIT* mutations (p=0.0179), an increased proliferation index as measured by Ki67 (p=0.034), and an increased rate of cellular proliferation as measured by

AgNOR staining (p=0.0019) were significantly associated with a decreased survival time in canine MCT patients that were treated with vinblastine and prednisone. Increased cytoplasmic KIT protein localization was also associated with decreased survival times, although this association was of borderline significance (p=0.0502) (Figure 22 A-D).

Although previous studies have described patient survival following treatment with vinblastine and prednisone[26], no studies have compared the outcome of treatment with vinblastine and prednisone to that of surgery alone in similar groups of dogs. In order to retrospectively evaluate the efficacy of vinblastine and prednisone in treating canine cutaneous MCTs, survival curves representing time to treatment failure and survival times were compared between dogs treated in this study and dogs treated with surgery alone that were evaluated as part of a previous study[162]. As a means of ensuring that similar populations of animals were compared, comparisons were made between groups of animals that had similar pre-treatment prognoses based on histologic grade, KIT staining patterns, or the presence of ITD *c-KIT* mutations. Histologic grade III MCTs treated with surgery alone had a significantly shorter time to treatment failure (p=0.002) and survival time (p=0.009) as compared to MCTs treated with vinblastine

and prednisone (Figure 23). No significant differences were found between histologic grade II MCTs treated with surgery alone and those treated with vinblastine and prednisone, or between MCTs treated with surgery alone with KIT staining patterns II or III, or with *c-KIT* mutations and those with similar pre-treatment prognostic profiles treated with vinblastine and prednisone.

Discussion

The results of this study demonstrate the utility of histologic grade, KIT staining patterns, the evaluation of *c-KIT* mutations, tryptase staining patterns, and the proliferation markers Ki67, PCNA, and AgNOR in the prognostication of canine MCTs treated with vinblastine and prednisone. Only histologic grade and AgNOR counts were significantly associated with both the time to treatment failure and the survival time of the dogs included in this study, suggesting that these may be the best markers for predicting the response to treatment with vinblastine and prednisone. Increased cytoplasmic KIT protein localization was significantly associated with a decreased time to treatment failure and was of borderline significance in terms of its association with survival. Similarly, the presence of *c-KIT* mutations was significantly associated with a decreased time to treatment failure, and although

there was a strong trend, the presence of *c-KIT* mutations was not significantly associated with a decreased time to treatment failure. In both cases, this borderline significance is likely to be due to the low case numbers included in this study, and it is likely that statistical significance could be gained if more cases were added to this study. Based on these results and the results of previous studies histologic grade, the evaluations of KIT protein localization and *c-KIT* mutations, and the assessment of cellular proliferation using Ki67 immunostaining and AgNOR histochemical staining should be used in concert to prognosticate canine MCTs especially when considering vinblastine and prednisone as adjunct chemotherapy[26,159,162]. Although each of these markers is associated with the progression of disease, no single marker has 100% specificity or 100% sensitivity in terms of identifying aggressive MCTs, therefore these markers should be used in combination in order to gain a complete picture of the disease.

In this study we have shown that histologic grade III MCTs treated with vinblastine and prednisone have significantly longer survival durations as compared to those treated with surgery alone. These results clearly show that histologic grade III MCTs may benefit from

adjunct chemotherapy with vinblastine and prednisone, and that in the case of grade III MCTs histologic grading alone may be sufficient for therapeutic determinations. However, the primary issue of treating histologic grade II MCTs still remains, and it is for these tumors that additional prognostic markers are needed. Therefore, in the face of intermediate grade MCTs, additional prognostic markers such as KIT staining patterns, the presence of *c-KIT* mutations, and proliferation markers such as Ki67 and AgNORs should be used in combination in order to better predict the biologic behavior of a given MCT. None of these markers alone can definitively differentiate benign vs. malignant tumors, but together they can help to clarify the behavior of a given tumor.

According to the Kaplan-Meier survival curves, there appears to be an increased survival duration and an increased disease free interval in the first 10-12 months following treatment of MCTs with KIT staining pattern III or with ITD *c-KIT* mutations treated with vinblastine and prednisone as compared to those treated with surgery alone. These differences suggest that KIT immuno-staining patterns and the presence of ITD *c-KIT* mutations may also be used to identify patients that are likely to benefit from adjunct chemotherapy with vinblastine and prednisone, especially in

terms of improving the immediate survival. The lack of statistically significant differences between those treated with surgery alone and those treated with vinblastine and prednisone may be attributed to the small sample sizes used in this study or to the fact that minimal differences in survival can be seen after 1 year post-treatment.

Together the results of this study clearly demonstrate that utility of vinblastine and prednisone as an adjunct chemotherapy in the treatment of a subpopulation of malignant canine cutaneous MCTs. However, there is still much room for improvement, and a larger proportion of canine MCTs may benefit from more targeted therapies, such as receptor tyrosine kinase inhibitors. In the future novel therapeutic protocols should be evaluated in order to improve the current standards of care for the treatment of canine cutaneous MCTs.

In summary, the results of this study suggest that histologic grade, KIT staining patterns, and the presence of ITD *c-KIT* mutations can be used to identify patients that are likely to benefit from adjunct chemotherapy with vinblastine and prednisone. Additionally, these markers, in combination with the evaluation of cellular proliferation using Ki67 immuno-staining and AgNOR histochemical staining may be used to predict a given

patient's response to treatment. However, in order to truly define the prognostic significance of these markers and the efficacy of vinblastine and prednisone as an adjunct treatment of canine MCTs, a larger prospective study is needed.

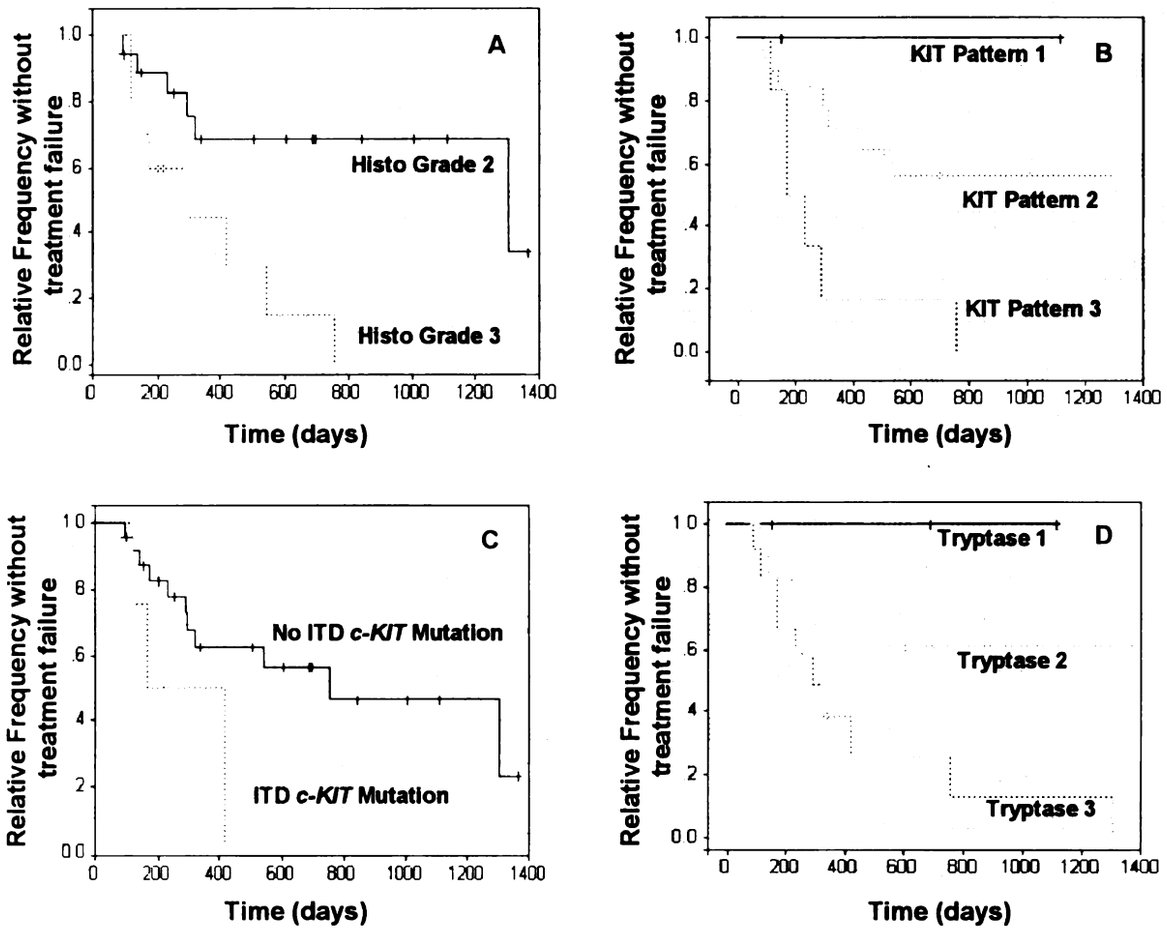

Figure 21: Kaplan-Meier survival curves of time to treatment failure of canine MCTs classified based on prognostic markers. A: Histologic Grade (p=0.0062); B: KIT staining patterns (p=0.009); C: ITD *c-KIT* mutations (p=0.0576); D: Tryptase (p=0.0286).

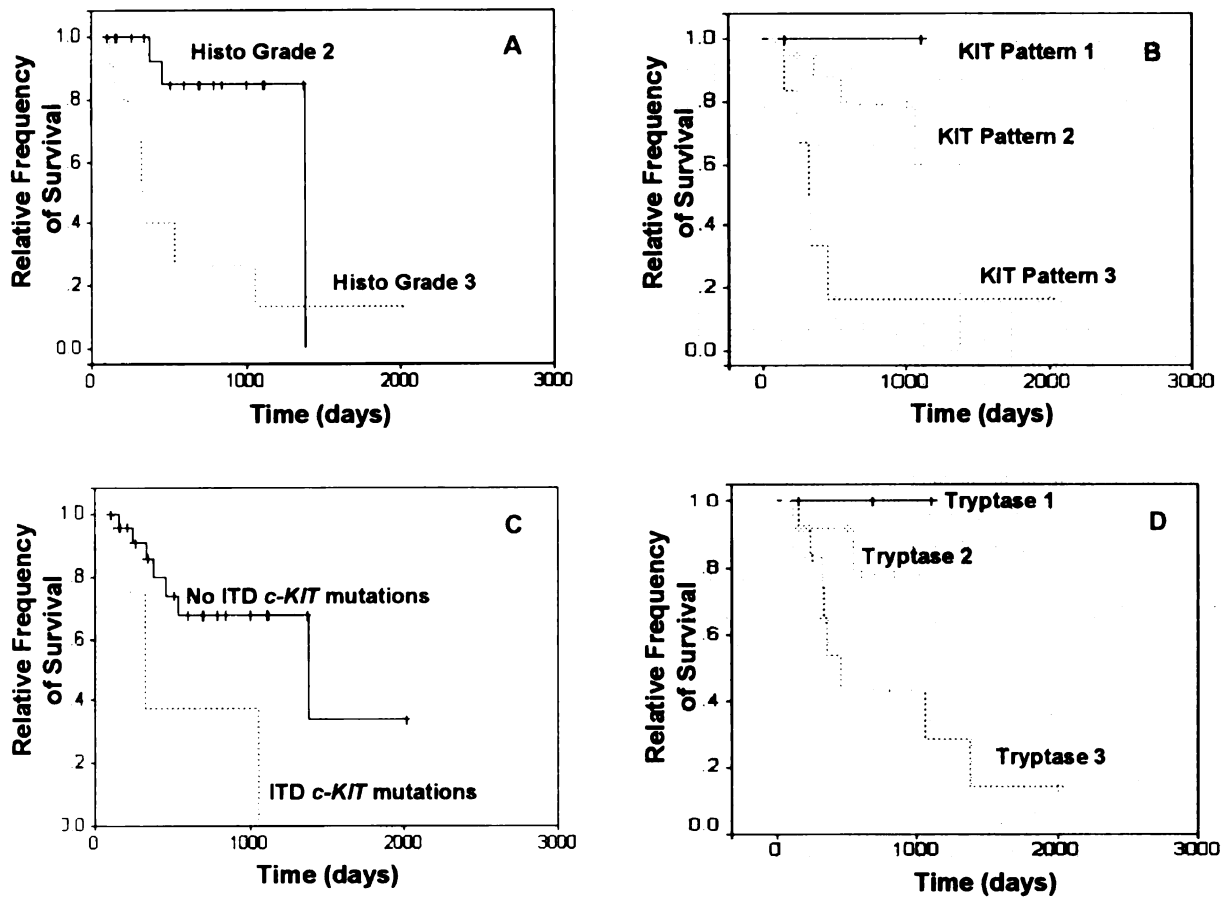

Figure 22: Kaplan-Meier survival curves of survival times of canine MCTs classified based on prognostic markers. A: Histologic Grade (p=0.0004); B: KIT staining patterns (p=0.0502); C: ITD *c-KIT* mutations (p=0.0179); D: Tryptase (p=0.0924).

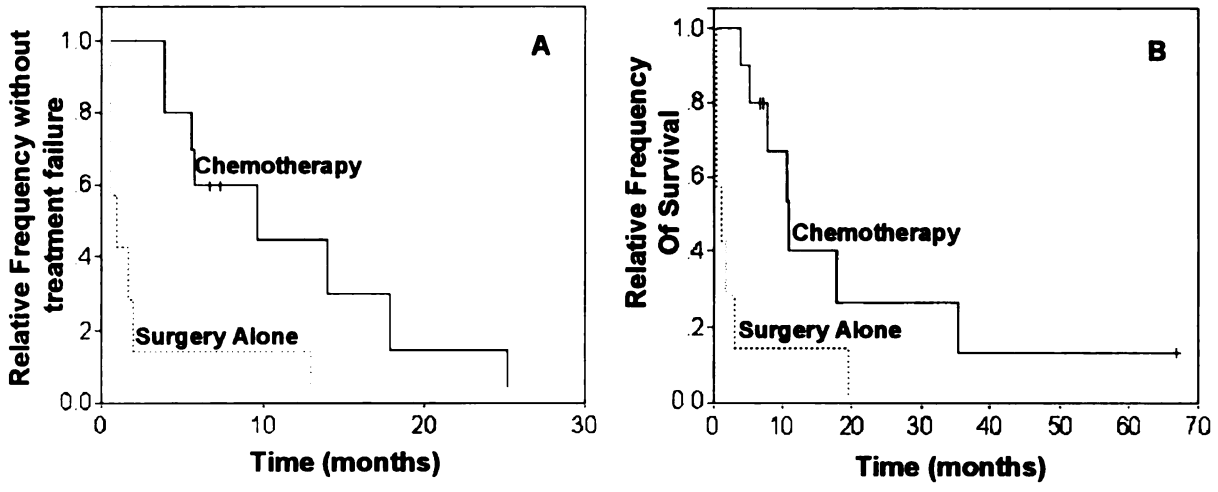

Figure 23: Kaplan-Meier survival curves of time to treatment failure (A) and survival time (B) of grade III MCTs treated with either surgery alone. Canine mast cell tumors treated with vinblastine and prednisone had a significantly increased time to treatment failure ($p=0.002$) and survival time ($p=0.009$) as compared to MCTs treated with surgery alone.

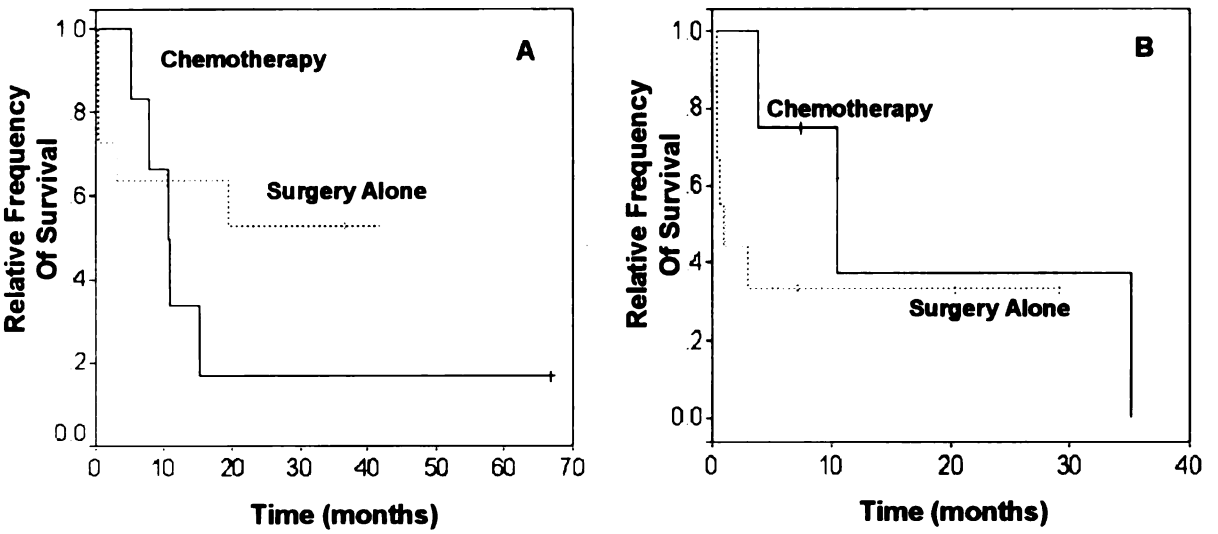

Figure 24: Kaplan-Meier survival curves showing percent survival of KIT pattern III MCTs (A), and MCTs with ITD *c-KIT* mutations (B) treated with vinblastine and prednisone compared to those treated with surgery alone.

CHAPTER 7

IDENTIFICATION OF CANDIDATE GENES ASSOCIATED WITH THE PROGRESSION OF CANINE MAST CELL TUMORS: A PILOT STUDY

Introduction

Canine cutaneous mast cell tumors accounting for 7-21% of all cutaneous neoplasms[1-4]. Canine MCTs have an extremely variable biologic behavior ranging from a solitary benign mass to a potentially fatal metastatic disease[5,7,12,14,96]. Currently, histologic grading is the primary prognostic and therapeutic determinant for canine cutaneous MCTs[12,14,30]. However, despite the significant associations that have been found between histologic grade and patient survival, the predominance of intermediate grade MCTs, the variable behavior associated with intermediate tumors, and the marked degree of inter-observer variation has led many to questions the relevance and utility of these classification systems[17,20-22,24].

Complete surgical excision is the primary treatment modality for canine cutaneous MCTs. In the event of incomplete surgical excision or a tumor that is unresectable, radiation is commonly employed as an adjunct local therapy, offering some benefit for local disease. In addition to surgery and radiation therapy, several

chemotherapeutic protocols have been employed for the treatment of systemic or non-resectable MCTs, with variable degrees of success[5,7]. In the absence of accurate prognostic tools and a thorough understanding of the underlying biology of MCTs, these treatment modalities are of variable use.

Previous work by our laboratory and by others has implicated the *c-KIT* proto-oncogene in the pathogenesis of canine MCTs[16,91,95,96,158,159,162]. The *c-KIT* proto-oncogene encodes the receptor tyrosine kinase KIT[97], which, in conjunction with its ligand stem cell factor (SCF, also known as mast cell growth factor or steel factor)[106,114,115], plays an important role in mast cell proliferation, differentiation, survival, and chemotaxis[100,107-111]. Internal tandem duplications and deletions have been described in the juxtamembrane domain of *c-KIT* in canine MCTs[16,91,95,96,162], and those mutations that have been evaluated have been shown to lead to a constitutively activated form KIT in the absence of ligand[91,95,145]. Additionally, aberrant cytoplasmic localization of the KIT protein has also been described in canine MCTs[94,159]. Previous work by our laboratory has shown that canine MCTs with ITD *c-KIT* mutations or aberrant KIT protein localization are significantly associated with a worse prognosis, as compared to those MCTs that lack ITD *c-*

KIT mutations or aberrant KIT localization[159,162], respectively, and are also significantly associated with an increased rate of cellular proliferation in canine MCTs (Webster et al, unpublished data).

Cancer is the result of a series of several genetic and epigenetic changes[51]. While the significance of *c-KIT* in the pathogenesis of canine MCTs is clear based on the results of the above mentioned studies, it is likely that many more genes play a role in the progression this disease. In order to identify genes associated with the progression of canine MCTs, gene expression profiles of high grade and intermediate grade MCTs were compared using a custom designed canine oligonucleotide microarray. The results of this study identify several candidate genes that may play a role in the progression of canine cutaneous MCTs.

Materials and Methods

Microarray Design and production

A targeted canine oligonucleotide microarray was designed to include genes associated with cancer, inflammation, embryonic and adult stem cells, and bone. Unique 60bp sequences with approximately equal melting temperatures were identified in 851 canine genes coding regions associated with the above biologic systems by the

Bioinformatics Core Facility of Michigan State University. Additionally, 60mers unique to 5 housekeeping genes, beta actin, beta-two microglobulin, glyceraldehyde 3-phosphate dehydrogenase, cyclophilin A, and ribosomal protein L13a were also identified to be used as positive controls in all microarray experiments. Oligonucleotides (60mer) were selected to give unique and specific hybridization signals using the program OligoArray 2.0 developed specifically for design of oligonucleotide probes for microarrays using a thermodynamic approach[171]. Oligonucleotides were then synthesized at Michigan State University's Macromolecular Structural Facility for microarray printing. Microarrays were printed on UltraGaps, Gamma Amino Propyl Silane coated slides (Corning, Corning, NY), at Michigan State University's Genomics Core Facility. Arrays were printed in duplicate on each slide, and each array consisted of 2 rows of 4 blocks, containing 120 features per block with a total of 960 features per array, and 1920 features per slide. Negative controls consisting of either empty features or random oligonucleotides were included in each block, with a total of 73 empty features and 16 random oligonucleotides included in each array (146 and 32 per slide, respectively). Positive controls consisting of the 5 housekeeping genes were included in alternating blocks in

the last 5 positions of these blocks, with each housekeeping gene being printed a total of 4 times per array.

Cases

The tissue collection protocol used in this study was reviewed by the All University Animal Care and Use Committee (AUACUC) at Michigan State University, and this protocol was declared exempt as all tissues were obtained as part of the standard treatment of canine patients at the Veterinary Teaching Hospital at Michigan State University (MSU-VTH). All canine cutaneous MCTs presented to MSU-VTH for surgery or necropsy between July 2004 and December 2006 that were at least 2 cm in diameter were collected for this study. No criteria other than sample quantity and quality were used for inclusion into this study. All tissues obtained for this project were either obtained post-mortem or during surgical removal as part of the standard care for the treatment of each animal. Surgical samples of MCTs were obtained from the center of each tumor with scalpel and thumb forceps or with a punch biopsy, avoiding tumor margins in order to facilitate histopathologic evaluations. Tissue samples collected for this study ranged from 40-500 mg. Each sample was snap frozen in liquid nitrogen and stored at -80°C until the time of RNA isolation.

RNA isolation

For RNA isolation tissues were ground to a fine powder in liquid nitrogen using a mortar and pestal and cell lysis and RNA extraction was performed using the Versagene Fibrous Tissue RNA extraction KIT (Gentra Systems, Mineapolis, MN) with on column DNase treatment according to the manufacturer's protocol. RNA was initially quantified using the Nanodrop spectrophotometer (Wilmington, DE) and RNA quality was assessed using the Agilent Bioanalyzer (Palo Alto, CA).

Histopathology

Sections of each tumor were routinely fixed in 10% neutral buffered formalin and paraffin embedded. The diagnosis of each canine cutaneous MCT was confirmed histologically on 5 µm sections stained with hematoxylin and eosin and each tumor was histologically graded by a single investigator (JDW) using the Patnaik histologic grading system[14].

Identification of c-KIT mutations

In order to identify ITDs and deletions in the juxtamembrane domain of the *c-KIT* proto-oncogene of canine cutaneous MCTs, laser capture microdissection was preformed and genomic DNA was extracted from 5-7 µm sections of formalin-fixed paraffin-embedded of canine MCTs as

previously described[162]. When paraffin-embedded tissues were not available, DNA was extracted from tissue and RNA lysis buffer left over from RNA extractions. Specifically, 100 µl of tissue and lysis solution was combined with 100 µl of 4 M ammonium acetate, 1 µl of glycogen, and 500 µl of 100% ethanol and incubated for 10 minutes at -20°C and centrifuged at 1,306 x g for 10 minutes. Following centrifugation, the supernatant was decanted, and the pellet was rinsed with 70% ethanol and centrifuged again for 30 seconds at 14,000rpms. The supernatant was again decanted and the pellet was dried at room temperature. When dry, DNA was eluted in 30 µl of distilled, de-ionized water and 2 µl of eluted DNA was used in each 25 µl PCR reaction.

Polymerase chain reaction amplification of the juxtamembrane domain of the *c-KIT* proto-oncogene was preformed using forward (CATTTGTTCTCTACCCTAAGTGCT) and reverse (GTTCCCTAAAGTCATTGTTACACG) primers flanking exon 11, including the regions of where ITDs and deletions have been previously characterized in canine MCTs[91,95,96,162]. Polymerase chain reactions were prepared in a 25 µl total reaction volume, with 5 µl LCM extracted DNA, 5 pmol of each primer, 0.5 units of Taq polymerase (Invitrogen, Carlsbad, CA), and final concentrations of 80 µM dNTPs, 2

mM $MgCl_2$, 20 mM Tris-HCl, and 50µl KCl. Cycling conditions were as follows: 94°C for 4 minutes; 35-45 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; 72°C for 5 minutes. Amplified products and ITD and deletion mutations were visualized by agarose gel electrophoresis on a 2% agarose gel after ethidium bromide staining.

Reverse Transcription, labeling, and hybridization

Five to fifteen micrograms of total RNA were concentrated to a final concentration of 312.5-937 ng/µl in 16 µl of DECP-treated water using vacuum centrifugation. cDNA was synthesized with the Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) using anchored oligo-dT primers and amino modified deoxynucleotides and subsequently labeled with either Cy3 or Cy5 fluorescent dyes as part of the Superscript Indirect cDNA Labeling System (Invitrogen, Carlsbad, CA). Cy3 and Cy5 labeling efficiencies were subsequently quantified using the Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE), and equal picomoles of Cy3 and Cy5 labeled probes were hybridized to each slide in order to minimize dye bias. Twenty to forty-five picomoles of each labeled probe (Cy3 and Cy5) were concentrated using a Microcon tube (Millipore, Billerica, MA), and subsequently brought up to a final volume of 110 µl of Ambion Hybridization buffer 3

(Ambion, Austin, TX) for hybridization. Prior to hybridization labeled probes were heated to 70°C for 5 minutes in order to denature cDNA. All hybridization reactions were performed on the GeneTac HybStation (Genomic Solutions, Ann Arbor, MI) using a step down hybridization protocol consisting of 6 hr at 42°C, 6 hr at 35°C, and 6 hr at 30°C. Following the 18 hour hybridization step down, microarrays were washed in 2X SSC and 0.1% SDS at 37°C, followed by a high stringency wash consisting of 0.2X SSC and 0.1% SDS at 25°C, and were finally rinsed with a post wash buffer consisting of 0.2X SSC at 25°C.

Microarray analysis

Slides were scanned using the GeneTAC LS IV microarray scanner (Genomic Solutions, Ann Arbor, MI), and quantification of raw fluorescence and initial spot analyses were using GenePix microarray analysis software (Molecular Devices, Downington, PA). Statistical analyses of the microarray data was preformed using the Limma package for differential expression analsysis with the R package[172,173]. Using Limma, subtractive background corrections were preformed and weighted scores of 0.1 were given to any spots that were flagged bad, missing, or absent. Normalization was performed within each array using global loess normalization, and statistical analyses

of the differential expression of each features was preformed using the Empirical Bayes method. Only spots whose differential expression were statistically significant at a level of $p < 0.05$ and had a minimum of 1.5 fold change in expression were considered to be significant.

Results

Following evaluation of total RNA quantities and qualities, 9 samples were removed from the study due to low RNA yields, and an additional 4 samples were discarded due to poor quality. The remaining 15 dogs were composed of 7 males, and 8 females that ranged from 1.5 to 16 years of age with a mean age of 8.5 years, and these 16 dogs represented 4 breeds including 5 golden retrievers, 3 mixed breed dogs, 6 Labrador retrievers, and 1 rotweiller.

Tumors with available archival paraffin-embedded materials were histologically graded according to the Patnaik histologic classification system for canine cutaneous MCTs[14]. Nine of the 15 MCTs included in this study were histologic grade II, 6 MCTs were histologic grade III. Each tumor was also characterized in terms of the presence of ITDs or deletions in exon 11 of the *c-KIT* proto-oncogene. Internal tandem duplications were identified in 3 of the 15 MCTs included in this study.

Deletions were suspected in 2 MCTs. In one mast cell tumor a 6bp deletion was confirmed by automated sequencing, however a larger deletion was suspected in a second MCTs based on agarose gel electrophoresis, but was never confirmed. No mutations were found in the remaining 10 tumors included in this study.

In order to identify candidate genes that might be involved in the progression of canine cutaneous MCTs, the gene expression profiles of five high grade MCTs were compared to the gene expression profiles of five intermediate grade MCTs. Four of the five high grade MCTs were histologic grade III MCTs with known or suspected mutations in the *c-KIT* proto-oncogene. The remaining high grade MCT was a histologic grade II MCT with a *c-KIT* mutation that had an extremely aggressive biologic behavior resulting in death within 1 year of the original diagnosis. Intermediate grade MCTs consisted of histologic grade II MCTs, in which no mutations were found in the juxtamembrane domain of *c-KIT* (Table 16).

Seventeen genes were identified as having at least 1.5 fold differential expression at a 0.05 level of significance. Sixteen of these 17 genes were significantly down-regulated in high grade MCTs and one gene was significantly up-regulated in high grade. A list of these

genes and the ontogeny of each differentially expressed gene was determined based on the GeneCards database[174] is listed in Table 17.

Discussion

In this study we have identified 17 genes that are significantly differentially expressed in high grade MCTs compared to intermediate grade MCTs. Sixteen of these genes were significantly down-regulated while only one gene was significantly up-regulated. The genes identified in this study fall into a broad range of functional classes. Among these genes, two groups are of particular interest, namely death receptor associated genes and cytokine/chemokine signaling associated genes.

Three of the seventeen genes identified in this study are associated with death receptor signaling and apoptosis, specifically death receptor 6 and RIPK1, which were significantly down-regulated in high grade MCTs, and inhibitor of apoptosis protein 2 (c-IAP2), which was up-regulated. Death receptor 6 is member of the tumor necrosis factor receptor superfamily and ectopic expression of this receptor has been shown to lead to induce apoptosis and NF-κB signaling[175]. RIPK1 is a serine-threonine kinase that interacts with TRADD downstream of TNF receptor 1, and has also been shown to be important for TNF apoptotic and

NF-κB signaling[176]. c-IAP2 is a member of the inhibitor of apoptosis family of proteins with ubiquitin ligase activity[177]. c-IAP2 has been shown to monoubiquitinate caspase 7 and mono- and polyubiquitinate caspase 3[178]. Additionally, c-IAP2 has been shown to be essential for NF-κB mediated inhibition of TNF-induced apoptosis[179]. Up-regulation of c-IAP2 suggests a potential mechanism by which neoplastic cells might suppress apoptotic signaling in canine MCTs (Figure 25). Additional genes involved in death receptor apoptotic signaling were also significantly down-regulated in high grade MCTs. These genes include death associated protein 6 (DAXX), caspase 6, and caspase 8. However, the differences in the expression of these genes were more subtle (data not shown).

The differential expression of DR6, RIPK1, and c-IAP2 in high grade MCTs described in this study provides the first evidence of changes in apoptotic signaling in canine MCTs. The identification of these changes provide the first step towards understanding the regulation of apoptosis in canine MCTs, which is critical for the development of better treatments for these tumors.

An additional interesting finding in this study was that several genes associated with cytokine/chemokine signaling were down-regulated in high grade MCTs compared

to intermediate grade MCTs (Table 17). Mast cells are bone marrow derived inflammatory cells that are critical for early chemokine and cytokine signaling in inflammatory reactions. The decreased expression of several cytokine signaling-associated genes in high grade MCTs could reflect the undifferentiated phenotype of high grade MCTs, and might coincide with a larger proportion of undifferentiated or progenitor cells in high grade MCTs.

In this study we have identified novel candidates for the progression of canine MCTs. At this time these results are only preliminary, as further studies are needed to confirm the differential expression of these genes. Initially, this differential expression needs to be confirmed at the mRNA level using quantitative PCR. Subsequently, these changes need to be confirmed at the protein level using immunohistochemistry and western blotting. Furthermore, the biologic significance of these changes should be examined with additional *in vivo* studies to characterize the relationship between these markers and disease progression. Additionally, further in vitro studies should be performed in order to identify the functional consequences of over-expressing and the knocking down these genes. However, the results of this study identify exciting, new candidate genes to explore in hopes

of better understanding the molecular pathogenesis of canine MCTs.

Table 16: Signalment, histologic grade, and KIT mutation status of cases included in this study.

Slide No.	Case No.	Channel	Age (years)	Breed	Gender	Grade	Mutation
1	A	Cy3	3.6	Rottwieller	Female	3	Duplication
	B	Cy5	8.7	Golden	Male	2	None
2	C	Cy3	9.9	Golden	Female	3	Duplication
	D	Cy5	5.9	Labrador	Female	2	None
3	E	Cy3	10.8	Labrador	Female	2	None
	F	Cy5	11	Mixed	Male	2	Deletion
4	G	Cy3	5.67	Golden	Male	2	None
	H	Cy5	1.53	Labrador	Female	3	Suspected Deletion
5	I	Cy3	9	Mixed	Male	3	Duplication
	J	Cy5	11.7	Labrador	Female	2	None

Table 17: Differentially expressed genes in malignant vs. intermediate canine mast cell tumors.

Name	Function	Fold Change	P.Value
Chek-1	Cell cycle regulation	0.497	0.038261
Bone morphogenic protein 6	Cartilage and Bone Formation	0.567	0.005747
Vascular endothelial growth factor receptor	Angiogenesis	0.573	0.001898
Mitogen-activated protein kinase kinase kinase 7 interacting protein 2	MAPK, NF-κB, WNT signaling	0.61	0.045399
Heat shock 70kDa protein 5	Apoptosis inhibition	0.618	0.019953
Interleukin 15 receptor; alpha	STAT/JAK signal transduction	0.625	0.045846
CDC7 cell division cycle 7	G1/S cell cycle transition; DNA replication	0.628	0.000992
Retinoid X receptor; beta (RXRB)	Transcriptional regulation	0.639	0.007438
Death receptor 6	Apoptosis; NF-κB signaling	0.656	0.003862
FK506 Binding Protein 9	Isomerase; Ion binding	0.657	0.004945
Thrombospondin 1	Extra-cellular matrix; Cell-cell/cell-matrix interactions	0.657	0.014388
RIPK1	Apoptosis; NF-κB signaling	0.658	0.002233
Chemokine (C-C motif) receptor 9 (CCR9); transcript variant B	Cytokine receptor	0.663	0.004733
Integrin; alpha M	Cell-cell/ cell-matrix interactions	0.663	0.003441
Chemokine (C motif) receptor 1	Chemokine receptor	0.664	0.004105
CD40 ligand	B-cell proliferation; Immunoglobulin class switch	0.666	0.008779
Inhibitor of apoptosis protein 2	Apoptosis inhibition; TNF signaling inhibition	1.944	0.01112

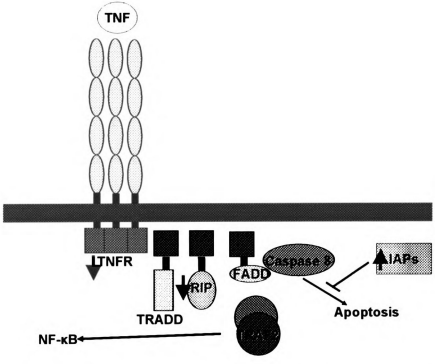

Figure 25: Diagram of tumor necrosis factor (TNF) signaling. Upon ligand binding, tumor necrosis factor receptors aggregate allowing for downstream signaling through both apoptotic and NFκB pathways. TNF-receptor associated death domain (TRADD) and RIP are important for both signaling pathways. NFκB signaling is activated through their interactions with TNF-receptor associated factor 2 (TRAF2). The death receptor apoptotic pathway is activated through interactions with Fas associated death domain (FADD), which is involved in caspase 8 activation. The inhibitors of apoptosis proteins c-IAP2 has been shown to inhibit TNF induced caspase 8 activation. Arrows indicated differential expression seen in high grade MCTs.

CONCLUSIONS

Due to the variable and potentially aggressive behavior of canine MCTs; the undesirable side effects associated with current therapeutics; and the emotional and financial stresses faced by owners, accurate prognostication of canine MCTs is of critical importance[5,7,13-14]. Although the current Patnaik histologic grading system has been shown to be correlated with survival when evaluated within a large population[14], the marked degree of inter-observer variation and the predominance of intermediate grade MCTs has led many to question the relevance of this system[17,20-22,24]. In light of these limitations, novel markers are needed for canine MCT prognostication. The studies described in this dissertation further identify and characterize multiple prognostic markers for canine cutaneous MCTs. Specifically, the results of this dissertation demonstrate the utility of KIT staining patterns[159], *c-KIT* mutations[162], Ki67 immunostaining, and AgNOR histochemical staining in the routine prognostication of canine cutaneous MCTs treated with surgery alone, or in combination with vinblastine and prednisone. Furthermore, the results of these studies demonstrate the inadequacies of tumor

depth[158], tryptase immunostaining[159], and S-phase evaluations in canine MCT prognostication.

In these studies several markers have been shown to be significantly associated with disease progression. However, no single marker can clearly predict the biologic behavior of canine MCTs. Based on the results of these studies, we propose that a panel of markers including histologic grade, the evaluation of KIT staining patterns, the assessment of cellular proliferation using Ki67 immunostaining and AgNOR histochemical staining, and the evaluation of *c-KIT* mutations, should be routinely used in the prognostication of canine MCTs. In the future, prospective studies should evaluate the use of these markers in combination, in order to maximize their utility in a diagnostic setting. A prospective evaluation of these markers will allow for a more accurate assessment of each marker individually and a better understanding of how these markers can best be used in combination in a routine diagnostic setting. Additionally, as novel therapeutic protocols are developed the prognostic value of these markers should be re-evaluated, as these markers may vary in their association with the outcome of specific treatments.

The studies described in this dissertation further define the role of the *c-KIT* proto-oncogene in the progression of canine MCTs. Specifically, these studies have shown that increased cytoplasmic localization of KIT and ITD *c-KIT* mutations are significantly associated with decreased disease-free survival and survival duration[159,162]. These results strongly support the hypothesis that changes in the *c-KIT* proto-oncogene and the KIT protein play important roles in the progression of canine cutaneous MCTs.

In our studies, ITD *c-KIT* mutations and aberrant KIT protein localization were associated with an increased rate of cellular proliferation as measured by AgNOR counts, and an increased proliferation index as measured by Ki67 immunostaining. These results suggest that one way KIT may promote the progression of canine MCTs is by increasing cellular proliferation. Together, the results of these studies suggest that changes in KIT protein localization and the presence of ITD *c-KIT* mutations may not only be useful as prognostic markers, but KIT may also be the best therapeutic target for canine MCTs.

Despite the significant association between aberrant KIT localization and the progression of canine MCTs, little is known in terms of the true biologic significance of this

change. Although we have found a significant association between aberrant KIT protein localization and the presence of ITD *c-KIT* mutations, we have also found a substantial number of canine MCTs with aberrant KIT localization which lack ITD *c-KIT* mutations[162]. In these cases, aberrant KIT localization may result from additional mutations in *c-KIT*, although no mutations were found in the phospho-transferase region of the kinase domain in 32 MCTs[181]. Other potential explanations for this change in KIT protein localization include the possible presence of aberrant truncated isoforms of KIT, as seen in human prostate cancer[130]; increased recycling from the cytoplasmic membrane as a result of autocrine or paracrine signaling loops; or simply, defective post-translation modifications or intracellular transportation. In the future, additional *in vitro* and *in vivo* studies are needed not only to identify the cause of this aberrant KIT protein localization, but also to determine the downstream consequences of these changes. Recently, our laboratory has cloned the canine *c-KIT* transcript in a GFP-tagged expression vector in order to begin these investigations.

Since cancer is the result of the accumulation of genetic and epigenetic changes several additional genes aside from *c-KIT* must be involved in the progression of

canine cutaneous MCTs. In order to identify additional genes involved in the progression of canine MCTs, the final project of this dissertation was to conduct a pilot microarray experiment comparing the gene expression profiles of a series of high grade MCTs to the expression profiles of a series of intermediate grade MCTs. In this study, we identified 17 candidate genes that were differentially expressed between these two populations of MCTs. Although the potential role of these candidate genes in the progression of canine MCTs is intriguing, these results are only the starting point for future studies. Differential expression of these genes needs to be confirmed first using quantitative reverse transcriptase PCR. Subsequently, the significance of this differential expression needs to be evaluated on the protein level using immunohistochemistry and western blotting. These studies need to be followed by functional studies in order to clearly delineate the biologic significance of these changes.

The results of the studies presented in this dissertation significantly add to our current body of knowledge of canine cutaneous MCTs, both in the diagnostic setting and in our understanding of the biology of these tumors. Specifically, we have identified novel prognostic

factors, such as KIT staining patterns and *c-KIT* mutations, and have clarified the utility of previously identified prognostic factors, such as tumor depth and proliferation markers in the routine prognostication of canine MCTs. These studies have also added to our understanding of the molecular biology of canine cutaneous MCTs, as the data provided in these studies further characterize the role of *c-KIT* in the progression of canine MCTs. Additionally, our pilot microarray study has identified several genes that may also be involved in the progression of canine MCTs. The results of these studies will serve as the foundation for new hypotheses and future studies that will further clarify the biology of canine MCTs.

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