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The Role of *c-KIT* in Tumorigenesis: Evaluation in Canine Cutaneous Mast Cell Tumors[1]

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Abstract

The *c-KIT* proto-oncogene has been implicated in the pathogenesis of several neoplastic diseases, including gastrointestinal stromal tumors and mastocytosis in humans, and mast cell tumors (MCTs) in canines. Cutaneous MCTs are common neoplasms in dogs and have a variable biologic behavior. The goal of this study was to define the prognostic significance of *c-KIT* mutations identified in canine MCTs and the associations between *c-KIT* mutations, KIT localization, and KIT expression levels. Microdissection and polymerase chain reaction were performed on 60 MCTs to identify *c-KIT* mutations. Anti-KIT antibodies were used for immunohistochemical evaluation of KIT localization. Forty-two MCTs were included in a tissue microarray, and KIT expression was quantified using immunofluorescence. Canine MCTs with *c-KIT* mutations were significantly associated with an increased incidence of recurrent disease and death. *c-KIT* mutations were also significantly associated with aberrant protein localization; however, the level of KIT expression did not correlate with either *c-KIT* mutations or changes in protein localization. Considering the high prevalence of canine MCTs and the central role of *c-KIT* in the tumorigenesis of certain tumors, canine MCTs are an excellent model for characterizing the role of *c-KIT* in neoplastic diseases and is a potential target for novel therapeutic agents in clinical trials.

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Introduction

The *c-KIT* proto-oncogene encodes the receptor tyrosine kinase KIT, which consists of an extracellular ligand binding domain composed of five immunoglobulin-like loops, a transmembrane domain, a negative regulatory juxtamembrane domain, and a split cytoplasmic kinase domain [1–3]. The ligand for KIT is stem cell factor, which is also known as steel factor, KIT ligand, or mast cell growth factor [4–7]. 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 [8–14]. In addition to these functions, in mast cells, KIT has been shown to be important for fibronectin adhesion, chemotaxis, and degranulation [5,15–19].

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 in humans [20] and in the kinase domain at codon 816 of human mastocytosis patients [21–23]. Additionally, aberrant KIT expression is increasingly being identified in multiple neoplasms, including small cell lung cancer, prostate cancer, and acute myeloblastic leukemia [24–29]. 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 [24,26], and in prostate cancer, where truncated isoforms of KIT that signal through phospholipase C-γ1 have been characterized [27,30]. However, for several other cancers, the significance of this aberrant expression has not been elucidated.

Activating *c-KIT* mutations [31–34] and aberrant KIT expression has also been described in canine cutaneous mast cell tumors (MCTs) [35–39], therefore implicating *c-KIT* in their pathogenesis. Unlike mastocytosis in humans, which is a rather

Abbreviations: MCT, mast cell tumor; LCM, laser capture microdissection; ITD, internal tandem duplication; HR, hazard ratio; OR, odds ratio

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rare condition and usually has a positive prognosis [40–42], canine cutaneous MCTs are one of the most common neoplastic diseases in dogs (accounting for 7–21% of all cutaneous neoplasms) [43–46] and have an extremely variable biologic behavior ranging from a benign mass to a fatal metastatic disease [34,47,48]. 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 [49,50]. 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 [45,51]. 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 [47,48]. The most commonly used system is that proposed by Patnaik et al. [48], which defines grade 1 MCTs as being well-differentiated tumors with good prognosis, grade 3 MCTs as being poorly differentiated tumors with poor prognosis, and grade 2 MCTs as being of intermediate differentiation with 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 [31–34,52–55]. ITD *c-KIT* mutations were identified in 9% of canine MCTs in one study that looked at the mutation status of 88 randomly selected MCTs [33], but these mutations may occur in as many as 30% to 50% of all intermediate- to high-grade MCTs [53]. All, except for one, of the previously described ITDs are in-frame duplications that range from approximately 39 to 69 bp in size [31–34,53–55], and all of the mutations that have been characterized thus far produce a constitutively activated form of KIT in the absence of ligand [31,32,54]. Previous work by our laboratory has shown that *c-KIT* mutations are significantly associated with histologically higher-grade canine MCTs [33]. 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 perimembrane KIT localization [39].

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 *c-KIT*'s juxtamembrane domain were identified in 15% of the MCTs examined, using laser capture microdissection (LCM) and polymerase chain reaction (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 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 the 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 MCT and the histologic grade of each tumor were 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) complete follow-up data from the referring veterinarian were available; and 4) adequate formalin-fixed paraffin-embedded tissues for DNA extraction and immunohistochemistry were 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 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 [56]. Histologic grades represent a consensus of those results.

LCM and DNA Extraction

LCM was used to isolate neoplastic mast cells for DNA extraction and subsequent PCR amplification of *c-KIT* exon 11 and intron 11 to identify ITD *c-KIT* mutations. Five- to 7-μm sections of each formalin-fixed paraffin-embedded MCT were dehydrated and stained with hematoxylin for LCM. A total of 2000 to 4000 neoplastic mast cells was extracted from each tumor sample using the Pixcell LCM system with Macro LCM caps (Arcturus, Mountain View, CA) (Figure 1). Extracted cells that 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, and 1% Tween) and 1.5 μl of 15 mg/ml Proteinase K (Roche, Indianapolis, IN) at 37°C. Samples were centrifuged at 4000 rpm 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*

PCR amplification was performed using a previously described primer pair that flanks exon 11 and the 5′ end of intron 11 [55], which includes the previously described ITD region of the *c-KIT* proto-oncogene in canine MCTs [31–34,53–55].

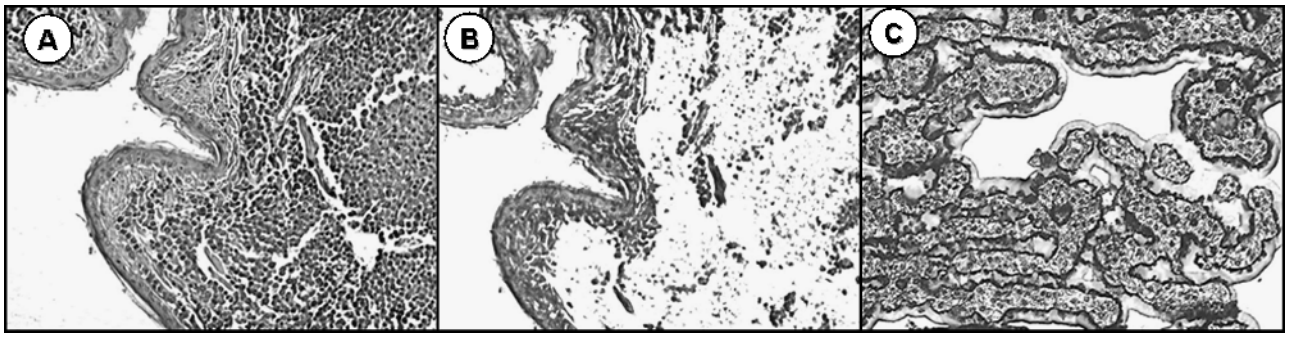

Figure 1. *LCM of neoplastic canine cutaneous MCTs (original magnification, ×10). LCM was performed using archival formalin-fixed paraffin-embedded tissue sections. DNA was extracted from captured cells, and PCR amplification was performed to identify* c-KIT *mutations. (A) Hematoxylin-stained section of MCT prior to microdissection. (B) Section of MCT following microdissection. (C) Laser capture microdissected cells adhered to cap.*

PCRs were prepared in a 25-μl total reaction volume, with 5 μl of LCM-extracted DNA, 5 pmol of each primer, 0.5 U of Taq polymerase (Invitrogen, Carlsbad, CA), and final concentrations of 80 μM deoxynucleoside triphosphate, 2 mM $MgCl_2$, 20 mM Tris–HCl, and 50 μl of KCl. Cycling conditions were as follows: 94°C for 4 minutes; 35 to 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 2).

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) and were subsequently chemically transformed into competent *Escherichia coli* cells according to the manufacturer's protocol. *c-KIT* clones were sequenced either through an automated sequencing technique using fluorescently labeled dideoxynucleotides 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 a Thermo Sequenase Radiolabeled Terminator Cycle Sequencing kit (USB Corporation, Cleveland, OH) and ^{33}P-labeled dideoxynucleotide triphosphates according to the manufacturer's protocol, followed by 48 to 72 hours of exposure to Biomax MR Scientific Imaging Film (Kodak, Rochester, NY).

Immunohistochemistry

Tissue sections of canine cutaneous MCTs were used for the immunohistochemical evaluation of KIT protein localization, as previously described [39]. 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 and Decker, Towson, MD) for 20 minutes, and nonspecific immunoglobulin binding was blocked by incubation of slides for 10 minutes with a protein-blocking agent (Dako). Using an autostainer, slides were incubated for 30 minutes with a rabbit anti–human c-KIT antibody (Dako) at a dilution of 1:100. A streptavidin immunoperoxidase staining procedure (Dako) was used for immunolabeling. The immunoreaction was visualized with 3,3′-diaminobenzidine substrate (Dako). 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 that the 30-minute incubation with primary antibodies was replaced with a 30-minute incubation with the buffer. KIT staining patterns and protein localization for each MCT were characterized as being perimembrane (KIT staining pattern I), focal or stippled cytoplasmic (KIT staining pattern II), or diffuse cytoplasmic protein localization (KIT staining pattern III), as previously described [39] (Figure 3). The evaluation of KIT

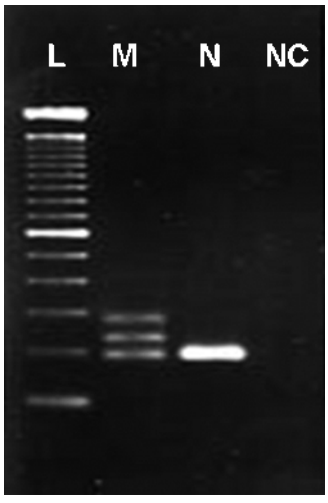

Figure 2. *A 2% agarose gel of PCR-amplified c-KIT exon 11 and intron 11 from LCM-extracted DNA from canine MCTs. L: 100-bp ladder; M: heterozygous for normal allele (191 bp) and mutant allele (250 bp), with an upper band representing heterodimerization of normal and mutant alleles; N: 191-bp homozygous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

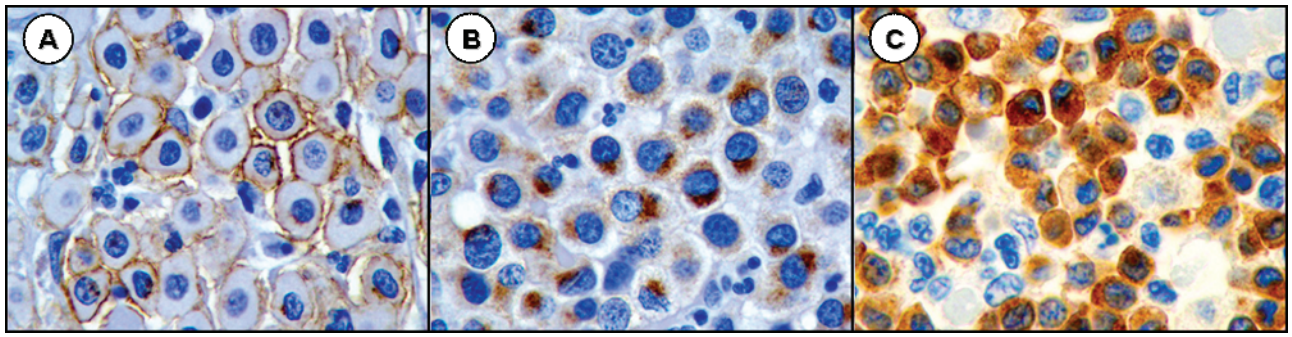

Figure 3. *Sections of canine cutaneous MCTs (skin) stained with anti-KIT antibodies and counterstained with hematoxylin (original magnification, ×100, oil) representing three patterns of KIT localization identified in neoplastic canine mast cells. (A) KIT staining pattern I, consisting of perimembrane protein localization. (B) KIT staining pattern II, consisting of focal to stippled cytoplasmic staining. (C) KIT staining pattern III, consisting of diffuse cytoplasmic staining.*

protein localization was performed by a single investigator (J.D.W.) to eliminate interobserver 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. MCTs included in the tissue array were chosen based on the availability of tissues for transfer 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 to create a cohesive block. The Five-Micron 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) was used for antigen retrieval. Nonspecific antibody binding was performed with 5% donkey serum with blocking buffer. Slides were incubated with primary rabbit anti–human c-KIT (Dako) 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 counterstained with 4′,6-diamidino-2-phenylindole. 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 models were developed for each risk factor for each outcome, and the level of association was assessed through the risk factors' *P* value in the model. Risk factors with $P \leq .20$ were considered for inclusion in the multivariable model, which included the two variables *c-KIT* mutation status and KIT staining pattern.

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, and weight) were included in the multivariable model to account for their effects on model outcome. Results were reported as odds ratio (OR): OR < 1 means that the likelihood of the occurrence of an event is reduced, whereas OR > 1 indicates that the likelihood of an event is increased. OR = 1 indicates that the risk factor neither increases or 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, NC) for survival analysis, using survival times (time-to-event) as the model outcome, and produced point estimates of the hazard ratio (HR; risk ratio) for risk factors in the model. Proportional hazards regression models were developed for the 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, and weight) was 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 HRs. Comparable to OR, HR < 1 indicates that the risk factor increases time to outcome, whereas HR > 1 indicates 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 between 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 to 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 was 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 represented by single dogs. According to the Patnaik histologic grading system for canine MCTs [48], 8 MCTs were grade 1, 45 MCTs were grade 2, and 7 MCTs were grade 3.

c-KIT *Mutations in Canine MCTs*

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 to 8 were similar to previously described ITD *c-KIT* mutations [30–33,52–54]. All of these ITD mutations were in-frame mutations that ranged from 45 to 60 bp in size. In cases 5, 6, 7, and 8, duplications extended by one, two, three, and four 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 2 and five were grade 3 (Table 1).

According to multivariable analysis, patients with MCTs containing ITD *c-KIT* mutations had significantly decreased survival times [P = .0068, HR = 6.23 (1.66–23.4)] and an increased incidence of mortality due to MCT-related disease [P = .0011, OR = 15 (2.95–76.31)] (Figure 4). 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 = .0255, OR = 5.4 (1.23–23.75)] and at sites outside of the original tumor margins [P = .0016, OR = 6.13 (1.99–18.92)] and with a decreased disease-free interval both at the site of the original tumor [P = .0157, HR = 5.78 (1.40–23.99)] and at sites outside of the tumor margin [P = .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.

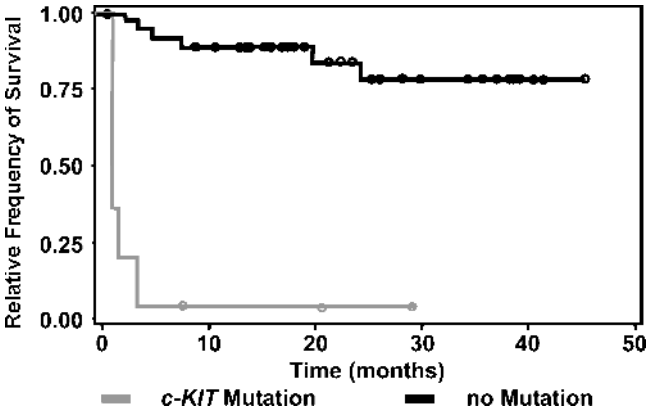

Figure 4. *Kaplan-Meier survival curve: relative frequency of survival* versus *time in months for canine cutaneous MCT patients with and without identified* c-KIT *mutations. The presence of duplication mutation in the* c-KIT *proto-oncogene was significantly associated with a decreased survival duration [P = .0068, HR = 6.23 (1.66–23.40)].*

Twenty-five of the 60 MCTs examined had KIT staining pattern I, which is characterized by perimembrane KIT protein localization, as seen in non-neoplastic (inflammatory) mast cells. Twenty-four of 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 perimembrane protein localization; the remaining 11 MCTs had KIT staining pattern III, which is characterized by diffuse cytoplasmic KIT localization. Seven of nine MCTs (77.8%) with ITD *c-KIT* mutations also had aberrant KIT protein localization (KIT staining pattern II or III). Two of the MCTs with ITD *c-KIT* mutations had KIT staining pattern I, three cases had KIT staining pattern II, and four cases had KIT staining pattern III. A significant trend was identified between the presence of ITD *c-KIT* mutations and an increased cytoplasmic localization of KIT (P = .046) (Figure 5), as evidenced by higher KIT staining patterns.

KIT Protein Expression

The tissue microarray representing 42 of 60 samples was used to quantify KIT immunofluorescence. Relationships between immunofluorescence, and *c-KIT* mutations and KIT

Table 1. Mutation and Case Description for Cases with ITD *c-KIT* Mutations.

Case Number	Duplication Size	Duplication Location	Histologic Grade*	KIT Staining Pattern†	Local Recurrence (months)	Distant Recurrence (months)	MCT-Related Death (months)	Time to Last Follow-Up (If Alive) (months)
1	45	Exon 11	3	3	None	None	0.5	N/A
2	45	Exon 11	2	2	None	None	None	29.1
3	45	Exon 11	3	3	None	0.5	0.5	N/A
4	45	Exon 11	2	2	0.5	0.5	0.5	N/A
5	60	Exon 11/intron 11	3	2	1	1	1	N/A
6	54	Exon 11/intron 11	3	3	2	2	3	N/A
7	60	Exon 11/intron 11	3	3	None	0.6	0.6	N/A
8	57	Exon 11/intron 11	2	1	None	None	None	7.3‡
9	15	Intron 11§	2	1	None	None	None	20.4

*Histologic grading was performed based on the Patnaik histologic grading system for canine cutaneous MCTs [48].
†KIT staining patterns were classified as described by Webster et al. [39].
‡Dog 8 died at 7.3 months due to causes unrelated to mast cell disease.
§Mutation in dog 9 consisted of a 24-bp 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.

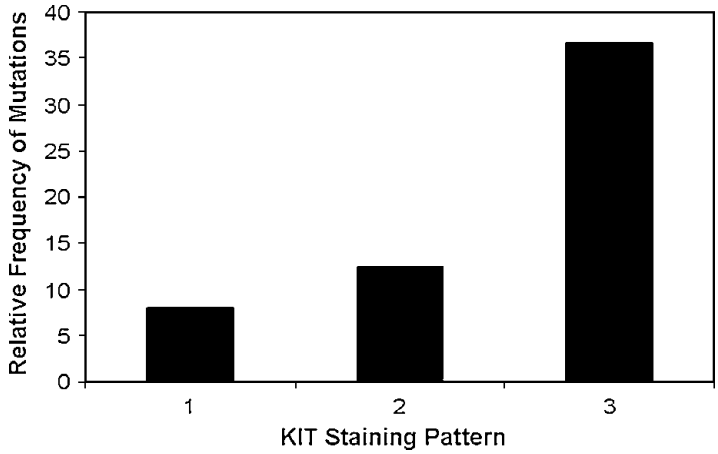

Figure 5. *Correlation between ITD* c-KIT *mutations and KIT protein localization in canine MCTs. A significant association was found between the presence of* c-KIT *mutations and the cellular localization of KIT in canine MCTs (P = .046). Seven of nine (77.8%) MCTs with ITD* c-KIT *mutations had aberrant KIT localization in neoplastic MCTs.*

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 protein levels 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 canine MCTs. These data document the importance of the *c-KIT* proto-oncogene in the tumorigenesis of canine cutaneous MCTs and clearly identify 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* [31,32]. Following the identification of *c-KIT* mutations in canine MCTs, work by our laboratory has shown that the presence of *c-KIT* mutations is significantly associated with higher histologic grade MCTs [33]. 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 grades 2 and 3, whereas 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 with 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 variations in the treatment protocols used in a given study population. 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 [33,53]. The predominance of intermediate- and high-grade tumors in the latter study [53] 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 [47,48,51]. Based on the results of this study and previous studies, the true incidence of ITD *c-KIT* is likely to be between 9% and 15% in all MCTs. However, these mutations may occur in as many as 50% of high-grade canine MCTs [33,53].

Previously, our laboratory has shown that increased cytoplasmic KIT protein localization in neoplastic mast cells is associated with both a decreased disease-free survival and an overall survival of dogs with cutaneous MCTs [39]. In this 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 nine MCTs with *c-KIT* mutations had aberrant KIT protein localization. Although the significance of this relationship is not currently clear, this may suggest 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 = .0052) and time until MCT-related deaths (P = .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* 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 28 MCTs included in this study had aberrant KIT localization without the presence of ITD *c-KIT* mutations. However, these 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 because 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 [32,33]. 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 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 overexpression of KIT and subsequent increases in receptor sensitivity to its ligand. 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 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 [43,45] and, in many cases, share morphologic, clinical, and molecular characteristics similar to those of 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 [57,58]. 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.

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