Prevalence and importance of internal tandem duplications in exons 11 and 12 of c-*kit* in mast cell tumors of dogs

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Objective—To determine the prevalence of activating internal tandem duplications (ITDs) in exons 11 and 12 of c-*kit* in mast cell tumors (MCTs) of dogs and to correlate these mutations with prognosis.

Sample Population—157 formalin-fixed, paraffin-embedded MCTs from dogs in the pathology database of the Veterinary Medical Teaching Hospital at the University of California, Davis.

Procedure—Genomic DNA was isolated from tumor specimens and a polymerase chain reaction procedure was performed to determine whether there were ITDs in exons 11 and 12.

Results—We identified ITDs in 1 of 12 (8%) grade-I, 42 of 119 (35%) grade-II, and 9 of 26 (35%) grade-III tumors (overall prevalence, 52 of 157 [33%]). Logistic regression analysis revealed that the odds of grade-II and -III tumors possessing an ITD were approximately 5 times greater than that for grade-I tumors, although these odds did not differ significantly. Although MCTs possessing an ITD were twice as likely to recur after excision and twice as likely to result in metastasis as those without an ITD, these values also did not differ significantly.

Conclusions and Clinical Relevance—These results provide evidence that ITDs in c-*kit* occur frequently in MCTs of dogs. The high prevalence of c-*kit* activating mutations in MCTs of dogs combined with the relative abundance of mast cell disease in dogs provide an ideal naturally developing tumor in which to test the safety and efficacy of novel small-molecule kinase inhibitors such as imatinib mesylate. (*Am J Vet Res* 2002;63:1718–1723)

Mast cell tumors (MCTs) are the most common neoplasm in the skin of dogs.[1] Course of the disease can be unpredictable, ranging from benign behavior to aggressive progress with metastasis to the regional lymph nodes, liver, spleen, and bone marrow. Biological behavior of MCTs in dogs is most consistently correlated with histologic grade, although tumor location, growth rate, clinical stage, concurrent treatment, and proliferative activity (such as Ki67 and **argyrophilic nucleolar organizing regions** [**AgNOR**]) have also been associated with prognosis.[2-8]

Investigators have studied the potential role of Kit dysfunction in malignant mast cells. Kit is a receptor tyrosine kinase encoded by the proto-oncogene c-*kit*, which was initially identified as the acutely transforming component of the Hardy-Zuckerman 4-feline sarcoma virus.[9] In normal mast cells, Kit signaling is critical for differentiation, maturation, proliferation, cell survival, and function.[10-17] The discovery of point mutations in the catalytic domain of c-*kit* that led to constitutive activation despite the lack of ligand binding in 3 lines of malignant mast cells provided an initial indication that dysregulation of Kit may promote uncontrolled growth or survival of mast cells.[18-20] Following this discovery, similar activating mutations were identified in human patients with aggressive mastocytosis, documenting that such mutations may contribute to naturally developing mast cell disease.[21-27]

Because of the link between mast cell disorders and dysfunctional Kit, several investigators have undertaken studies to determine whether there are mutations in c-*kit* in MCTs of dogs. Interestingly, although c-*kit* derived from tumors of dogs did not contain the previously described activating mutations in the catalytic domain, novel mutations consisting of **internal tandem duplications (ITDs)** in exons 11 and 12 of the gene were identified.[28-30] This region of c-*kit* encodes the negative regulatory juxtamembrane domain that is responsible for preventing receptor dimerization in the absence of ligand binding.[31] The duplications in exons 11 and 12 were associated with constitutive phosphorylation of Kit despite a lack of ligand binding, suggesting that activating mutations in c-*kit* may play a role in the pathogenesis of MCTs in dogs.

Other studies that investigated MCTs of dogs for c-*kit* mutations involved small numbers of specimens. Therefore, the purpose of the study reported here was to identify the prevalence of ITDs in a larger population of dogs with MCTs and to correlate these mutations with histologic grade and biological behavior of the tumors.

Received Mar 19, 2002.
Accepted Jun 25, 2002.
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Supported by grants from the American Kennel Club Canine Health Foundation (No. 2010) and the American Cancer Society (No. IRG-95-125-04).
The authors thank Dr. Bruce Madewell for technical assistance.
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Materials and Methods

Source of MCTs—Medical records were reviewed for dogs in which a MCT was diagnosed at the Veterinary Medical Teaching Hospital at the University of California, Davis. Those dogs for which there was adequate information including signalment; location of tumor; staging of tumor; dates of local recurrence, metastasis, death, or last follow-up examination; prior or subsequent occurrence of MCTs; prior

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surgery or adjuvant therapy; and cause of death were eligible for inclusion in the study. Follow-up information was obtained via direct communication with owners or referring veterinarians. After final review, MCTs from 165 dogs were deemed appropriate for inclusion in the study. New H&E sections were obtained for each tumor sample, and a single pathologist (PFM) reviewed each by means of light microscopy. Tumors were graded in accordance with the Patnaik scheme.[8]

Source of malignant mast cell lines—The BR canine mastocytoma cell line (possessing a point mutation in the juxtamembrane domain, Leu575Pro) and the C2 canine mastocytoma cell line (possessing a 48-**base pair (bp)** tandem duplication in the juxtamembrane domain) were obtained[a] for use in the study.

Genomic DNA extraction from paraffin-embedded sections and cell lines—Sections of each formalin-fixed, paraffin-embedded tumor sample were cut at a thickness of 25 µm and placed in microcentrifuge tubes. Paraffin was removed by the use of xylene treatment and ethanol washes. Samples were dried, 500 µL of a proteinase K buffer (20 µg of proteinase K/mL) was added, and digestion was performed overnight at 37°C. The following day, supernatant was transferred to 2-mL phase lock gel tubes,[b] and phenol-chloroform extraction was performed. After precipitation with isopropanol, the DNA pellet was washed, dried, and re-suspended with 30 to 50 µL of tris-buffered EDTA, and the concentration of genomic DNA was determined. For the C2 and BR canine mast cell lines, 5×10^6 cells were collected from each, and genomic DNA was extracted by use of a commercially available tissue extraction kit.[c]

Polymerase chain reaction (PCR) procedure for intron 11 and detection of ITDs—Because we used formalin-fixed, paraffin-embedded tissue in this study, it was necessary to develop a PCR assay that could be reliably used on genomic DNA obtained from these tissues. Such DNA is often fragmented, and it can be difficult to obtain reproducible results by use of primer pairs that span long distances. Because all of the previously identified ITDs in MCTs of dogs have been located in the 3′ region of exon 11, we wanted to use a forward primer based in the 5′ end of exon 11 and a reverse primer based in intron 11 to screen the MCT specimens, which would thereby generate short PCR products (< 200 bp).

Genomic DNA derived from the C2 and BR canine mast cell lines was used to perform a PCR assay for intron 11 that used specially designed primers (**Appendix**). The PCR reaction was performed with primers P1 and P5 for 30 cycles consisting of 94°C for 45 seconds, 58°C for 45 seconds, and 72°C for 45 seconds. The PCR product was analyzed by use of agarose gel electrophoresis and sequenced directly. To screen for ITDs in exons 11 and 12, 2 primer pairs were used. Primer PE1 was based in the 5′ end of exon 11, and 2 reverse primers (PE2 and PE3) were based in the 5′ end of intron 11. The PCR assay was performed by use of pairs PE1 and PE2 (PCR product of 190 bp) or PE1 and PE3 (PCR product of 170 bp) for 40 cycles consisting of 94°C for 1 minute, 59°C for 1 minute, and 72°C for 1 minute. Resulting PCR products were analyzed by use of electrophoresis on a 4% agarose gel.

Statistical analysis—Exact logistic regression analysis was used to evaluate the association of ITDs in c-*kit* with each of several factors, including tumor grade, development of local recurrence, development of metastatic disease, and overall survival. Conditional exact logistic regression was selected for the statistical analysis, because it does not require large sample (asymptotic) approximations for estimation of variables.

Results

Development and validation of PCR-based assay—To develop primers based in intron 11, we sequenced a portion of c-*kit*, using genomic DNA derived from 2 canine mast cell lines (C2 and BR). A forward primer based in exon 11 and a reverse primer based in exon 12 generated PCR products that spanned intron 11 (**Fig 1**). This intron was found to be 285 bp, compared to 280 bp in humans and 300 bp in mice.[32,33]

We then designed primers to be used for screening of the MCT specimens for ITDs that would result in predicted PCR products of 170 bp (PE1 and PE3) or 190 bp (PE1 and PE2). Two sets of primers were used so that any positive result obtained with 1 set could be confirmed with the other set, thus ensuring that false-positive results would be less likely to occur. These

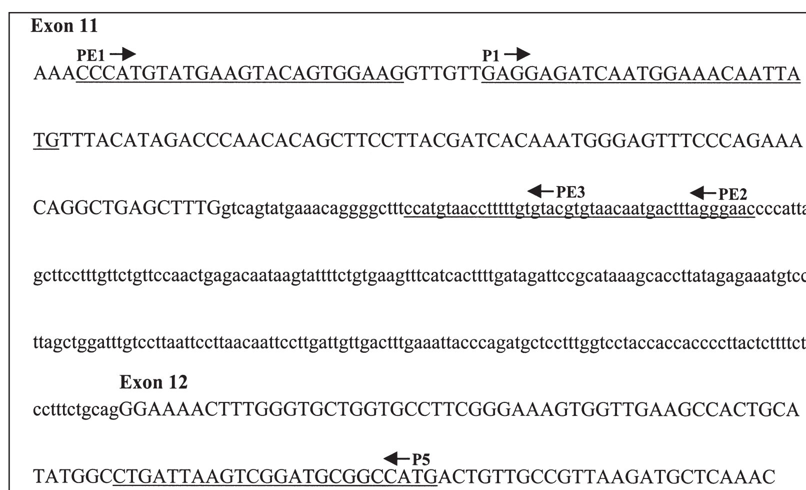

Figure 1—Sequence of intron 11 and location of primers used in polymerase chain reaction (PCR) analysis. The sequence of intron 11 is indicated by the nucleotides listed in lower-case letters. Location of each primer used for intron 11 sequencing (P1 and P5) and detection of c-*kit* internal tandem duplications (ITDs) for PE1, PE2, and PE3 are indicated (underlined).

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primer pairs were initially tested on genomic DNA obtained from the C2 cell line (which possesses a 48-bp ITD but without a **wild-type [WT]** c-*kit* allele) and the BR cell line (which does not contain an ITD), and they provided the expected products **(Fig 2)**. The primer pairs were then tested on fresh genomic DNA derived from MCT specimens used in another study in which our laboratory group first identified the ITD in c-*kit*.[34] All ITDs were reliably detected by use of either pair of primers (ie, PE1 and PE2 or PE1 and PE3; data not shown). The primer pairs PE1-PE2 and PE1-PE3 were found to be reliable for the detection of ITDs in c-*kit* in MCTs of dogs.

Prevalence and prognostic importance of ITDs in MCTs of dogs—The pathology database for our institution was used to enable us to identify MCT specimens for inclusion in the study reported here. After final review, 165 MCTs were deemed appropriate, and new H&E sections were obtained and reviewed by means of light microscopy. Eight of the MCT specimens were subsequently disqualified because of a lack of sufficient information or an incorrect diagnosis, and several of the remaining tumors were reclassified, which resulted in a final distribution for the 157 MCTs of 12 grade-I tumors, 119 grade-II tumors, and 26 grade-III tumors.

The MCT specimens were obtained from dogs that ranged from 0.4 to 16.5 years of age (mean, 8.3 years; median, 8.7 years). The most common breeds from which MCTs were obtained were Labrador Retriever (n = 39), Boxer (16), Golden Retriever (12), Cocker Spaniel (10), Staffordshire Bull Terrier (7), Boston Terrier (6), Chinese Shar-Pei (5), Miniature Schnauzer (5), German Shepherd Dog (4), and Bernese Mountain Dog (4). The remaining 49 dogs comprised various breeds.

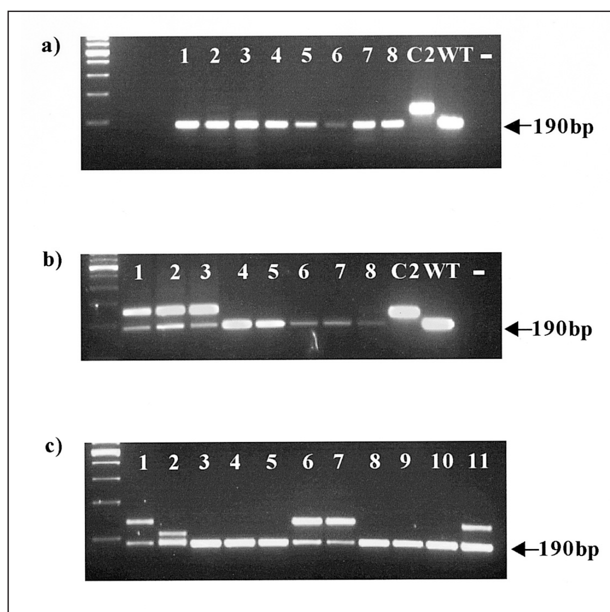

Figure 2—Representative results of agarose gel electrophoreses of PCR products from various paraffin-embedded mast cell tumors (MCTs) obtained from dogs and assayed for c-*kit* ITDs by use of the primer pair PE1 and PE2. Control samples consisted of PCR products derived from C2 genomic DNA (a canine MCT cell line that possesses a 48-base pair [bp] ITD in exon 11 and does not contain wild-type [WT] c-*kit*; lane C2), and the expected WT product derived from BR genomic DNA (a canine MCT cell line that does not possesses an ITD; lane WT). Lanes on the far right side (lane –) represent PCR products from reactions in which no DNA was added (negative-control sample). aPanel a:—Results for 8 grade-I MCTs. Notice that no ITDs are detected. bPanel b: —Results for 8 grade-II MCTs. Notice that ITDs are evident in lanes 1, 2, and 3. Panel c: cC—Results for 11 grade-III MCTs. Notice that ITDs are evident in lanes 1, 2, 6, 7, and 11.

To determine the prevalence of c-*kit* ITDs in this sample of MCTs, genomic DNA was isolated from the MCT specimens, and PCR assay was performed by use of primers PE1 and PE2. We detected ITDs in 1 of 12 (8%) grade-I, 42 of 119 (35%) grade-II, and 9 of 26 (35%) grade-III tumors **(Table 1**; Fig 2), for an overall prevalence of 52 of 157 (33%) in the population examined. Each ITD was confirmed by repeat of the PCR procedure with both pairs of primers (ie, PE1-PE2 and PE1-PE3). The ITDs derived from 4 MCT specimens were purified on agarose gels and directly sequenced **(Fig 3)**. Each of these ITDs was unique, although they all occurred in the 3′ end of exon 11 and incorporated similar portions of a duplicated sequence. Size of these ITDs varied from 39 to 63 bp, and each was an in-frame duplication. Interestingly, 1 of the ITDs incorporated 4 bp of intron 11; this phenomenon has been reported[34] for c-*kit* ITDs in MCTs of dogs.

Statistical analysis revealed that detection of c-*kit* ITDs was not associated with a particular breed of dog, sex, neutering status, age at time of examination, tumor location, or survival. Odds of grade-II and -III tumors possessing ITDs were approximately 5 times greater than the odds for grade-I tumors; however, the odds for grade-II and -III tumors did not differ significantly (P = 0.10 and 0.18, respectively) from the odds for grade-I tumors (Table 1). Although those MCTs that possessed an ITD were twice as likely to recur after excision and twice as likely to result in metastasis as those MCTs that did not possess an ITD, these values did not differ significantly (P = 0.22 and 0.13, respectively).

Discussion

Dysregulation of Kit signaling occurs in various neoplastic conditions in humans, including mastocytosis, gastrointestinal stromal tumors, small-cell lung

Table 1—Exact logistic regression analysis of variables potentially associated with c-*kit* internal tandem duplications (ITDs) in 157 mast cell tumors obtained from dogs

Variable	Results of ITD analysis		Odds ratio	95% confidence interval	*P*
	Positive	Negative			
Died					
No	39	88	1.00	NA	NA
Yes	13	17	1.72	0.69–4.19	0.27
Tumor grade					
I	1	11	1.00	NA	NA
II	42	77	5.94	0.75–48.09	0.10
III	9	17	5.61	0.62–278.04	0.18
Local recurrence					
No	44	97	1.00	NA	NA
Yes	8	8	2.19	0.67–7.19	0.22
Metastasis					
No	38.8	9	1.00	NA	NA
Yes	14	16	2.04	0.83–4.97	0.13

NA = Not applicable.

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Exon 11
→
AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAGA
CCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTG***ACACAGCTTCCTTACGA***
TCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTG **[48 bp ITD]**

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAGA
CCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGA***ACACAGCTTCCTTACGATCACAAATGG***
GAGTTTCCCAGAAACAGGCTGAGCTTTG **[39 bp ITD]**

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAGA
CCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAAC***CCAACACAGCTTCCTTACGATCA***
CAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTG **[45 bp ITD]**

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAGA
CCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTG***GTCACCCAAC***
ACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTG **[63 bp ITD]**

Figure 3—Sequence of c-*kit* ITDs. The larger PCR products possessing presumed c-*kit* ITDs were extracted from agarose gels and sequenced by use of primers PE1 and PE3 for 4 representative MCT specimens. Each ITD is denoted by nucleotides listed in bold and italics. Size of each ITD is indicated in brackets). Notice the 4 bp of intron 11 (box) that are included in the 63-bp ITD of the fourth specimen.

carcinoma, glioblastoma, and ovarian tumors.[24,26,27,35-43] In dogs, c-*kit* mutations consisting of ITDs leading to constitutive activation of Kit have been identified.[28-30] Because other investigations evaluated only a small number of MCTs of dogs, the purpose of the study reported here was to determine the true prevalence of c-*kit* ITDs in MCTs and to correlate this information with tumor grade and biological behavior of disease.

To screen a large number of tumor specimens for c-*kit* ITDs, we sequenced intron 11 of c-*kit* and developed a PCR-based assay capable of reliably detecting ITDs involving exons 11 and 12 in genomic DNA derived from paraffin-embedded samples. The ITDs were subsequently detected in 35% of grade-II and -III tumors, with an overall prevalence of 33% in the 157 MCTs evaluated in this study. We had predicted that c-*kit* ITDs would not be associated with benign MCTs (all grade-I and many grade-II tumors). Whereas only 1 grade-I tumor was found to possess an ITD, the correlation between detection of an ITD and tumor grade was not significant. This may have been attributable to the small number of grade-I MCTs (n = 12) available for analysis. In other studies,[4,8] investigators have reported that 30 to 40% of all MCTs of dogs are classified as grade-I tumors, but given that tissue samples for the study reported here were collected from submissions to a referral institution, it is probable that more aggressive clinical cases were likely to be represented in our population of MCTs. We are currently collecting fresh MCT samples from patients at local veterinary practices for ITD analysis to address this concern. Interestingly, none of the fresh grade-I tumors collected for that study have possessed an ITD (data not shown).

We had hypothesized that detection of an ITD would be associated with a higher likelihood of local recurrence and the development of metastasis. However, we did not find a significant difference in the rates of recurrence and metastasis in dogs with MCTs that had an ITD, compared with those without an ITD (Table 1). Therefore, it is possible that an ITD does not differentiate those grade-II and -III MCTs more likely to behave in a biologically aggressive manner. One potential explanation for this finding is the fact that PCR assay of genomic DNA from the tumor specimens was performed on whole tumor specimens, and, thus, it was not possible to determine whether the MCTs that possessed an ITD retained or lost the WT allele. This is important, because a study[44] of ITDs in the closely related Flt3 gene in human patients with acute myelogenous leukemia revealed that that those patients with loss of the WT allele (Flt3 ITD/–) had a worse prognosis than those patients that maintained heterozygosity (Flt3 ITD/WT). Therfore, it is possible that such a scenario applies to MCTs of dogs (ie, dogs with MCTs that possess an ITD but have lost the corresponding WT allele fare worse than those with MCTs that remain heterozygous). To test this hypothesis, it would be necessary to perform laser-capture microdissection on tumor samples to ensure that DNA was collected only from tumor cells and not stromal tissue. The need for this type of analysis is supported by the fact that all MCTs with an ITD also had the WT product, as determined by PCR analysis.

Another possible explanation for the lack of prognostic importance is that MCTs may be heterogeneous among animals, and specific animals may have various, or even multiple, molecular events associated with the progression of these tumors. If so, a c-*kit* ITD may simply represent one important event in the early development of MCTs, and other mutations may occur later that are responsible for the progression of these tumors into more aggressive forms.

In the study reported here, we documented c-*kit* activating ITDs in approximately 35% of grade-II and

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-III MCTs of dogs. The small-molecule kinase inhibitor STI571 (imatinib mesylate) reportedly can induce clinical responses in human patients with gastrointestinal stromal tumors that possess similar activating mutations in c-*kit*.[45-47] Clinical trials investigating the efficacy of STI571 in the treatment of gastrointestinal stromal tumors have been limited by the fact that such tumors are infrequent in the human population. The high prevalence of c-*kit* ITDs combined with the relative abundance of mast cell disease in dogs provides an ideal naturally developing tumor in which to test the safety and efficacy of such novel small-molecule kinase inhibitors.

[a]Provided by Dr. Warren Gold, Department of Pulmonary Medicine, Cardiovascular Research Institute, University of California, San Francisco, Calif.
[b]Phase lock gel, Eppendorf, Westbury, NY.
[c]DNeasy, Qiagen, Valencia, Calif.

Appendix

Primer design for polymerase chain reaction assay of intron 11 and detection of c-*kit* internal tandem duplications in exons 11 and 12 in mast cell tumors obtained from dogs

Primer	Sequence	Location
P1	5'GAG GAG ATC AAT GGA AAC AAT TAT G–3'	bp 1,687 to 1,711 of exon 11
P5	5'CAT GGC CGC ATC CGA CTT AAT CAG–3'	bp 1,860 to 1,837 of exon 12
PE1	5'CCC ATG TAT GAA GTA CAG TGG AAG–3'	bp 1,657 to 1,680 of exon 11
PE2	5'GTT CCC TAA AGT CAT TGT TAC ACG–3'	bp 43 to 66 of intron 11
PE3	5'CAC GTA CAC AAA AAG GTT ACA TGG–3'	bp 23 to 46 of intron 11

bp = Base pair.

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