

Gain-of-Function Mutations in the Extracellular Domain of KIT Are Common in Canine Mast Cell Tumors

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Abstract

In the current study, we examined the types and frequency of KIT mutations in mast cell tumors from 191 dogs. Sequencing of reverse transcription-PCR products revealed alterations in 50 (26.2%) of the dogs. Most mutations were in exon 11 ($n = 32$), and of these, most were internal tandem duplications ($n = 25$) between residues 571 and 590. Within exon 11, there were two hotspots for mutations at codons 555-559 and 571-590. In addition, nine dogs had mutations in exon 8 and eight had mutations in exon 9. We selected the two most common mutants and two representative exon 11 mutants for further analysis. When expressed in Ba/F3 cells, they were constitutively tyrosine phosphorylated and induced growth factor-independent cell proliferation. AG1296, a tyrosine kinase inhibitor, dose dependently inhibited both the tyrosine phosphorylation of these mutants and their induction of growth factor-independent proliferation. This study shows that activating mutations in not only exon 11 but also exons 8 and 9 are common in canine mast cell tumors. These results also show that Ba/F3 cells can be used for the direct characterization of canine *KIT* mutants, eliminating the need to make equivalent mutations in the mouse or human genes. (Mol Cancer Res 2008;6(7):1137-45)

Introduction

The *KIT* gene encodes a transmembrane type III tyrosine kinase that is the receptor for stem cell factor (SCF; ref. 1). The protein includes an extracellular domain composed of five immunoglobulin-like domains (encoded by exons 1-9), a transmembrane domain (exon 10), and an intracellular domain (exons 11-21). The intracellular domain is further divided into a negative regulatory juxtamembrane domain (exons 11 and 12) and a cytoplasmic tyrosine kinase domain that is split by an insert into ATP-binding (exon 13) and phosphotransferase lobes (exon 17). As in many other receptor tyrosine kinases, ligand binding induces receptor dimerization, which activates the tyrosine kinase, resulting in autophosphorylation and the phosphorylation of exogenous substrates. These phosphorylations then lead to downstream signal transduction.

Gain-of-function mutations of *KIT* (i.e., mutations that cause constitutive activation of the *KIT* tyrosine kinase) are associated with gastrointestinal stromal tumors, acute myelogenous leukemia, sinonasal T-cell lymphomas, and seminomas/dysgerminomas in humans (1). Such activating mutations, especially the well-known D⁸¹⁶V substitution, which lies in exon 17, have also been implicated in mastocytosis, a relatively rare multidimensional disease associated with mast cell infiltration into cutaneous and sometimes noncutaneous tissues, including bone marrow, spleen, and liver (2).

Previous studies have also identified *KIT* mutations in canine mast cell tumors. Mast cell neoplasms are one of the most common tumors in dogs, accounting for ~20% of all tumors (3). Mast cell tumors tend to occur in middle-aged to elderly dogs (mean age of ~9 years), with a predominance in Boxers, Terriers, and Labrador Retrievers (4). The clinical course of the disease varies from benign to highly malignant. Most tumors are benign, develop slowly, and persist for years without increasing in size or metastasizing. However, a large number are highly aggressive and present a significant threat to canine health.

Known *KIT* mutations associated with canine mast cell tumors include internal tandem duplications (ITD) and small insertions and deletions in the intracellular juxtamembrane region (exons 11 and 12; refs. 5-8). In support of the idea that activating *KIT* mutations play a causative role in canine mast cell tumors, a phase I trial published in 2003 showed that SU11654 (Sugen), an inhibitor of several receptor tyrosine

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kinases including KIT, causes significant shrinkage or stabilization of mast cell tumors (9). A more recent study by Kobie et al. (10) also found that the tyrosine kinase inhibitor STI571 (Glivec; Novartis) induces the regression of xenografted canine mast cell tumors in severe combined immunodeficient mice. Finally, Gleixner et al. (11) very recently reported that a variety of tyrosine kinase inhibitors that can inhibit KIT suppress the proliferation of canine mastocytoma cell lines.

In the current study, we examined the types and frequency of *KIT* mutations in canine mast cell tumors. We also examined the effects of the identified mutations on KIT function to gain insight into the pathogenesis of the different mutations and to clarify the role of KIT in mast cell and other diseases. This was the largest study of this type to date, involving 191 dogs. Although a majority of the mutations were found in exon 11, we show for the first time that mutations in exons 8 and 9, which encode the fifth immunoglobulin-like domain, are common in canine mast cell tumors. We tested the four most common of these mutations, and all activated the KIT tyrosine kinase. Finally, as part of this study, we developed and validated a system that allows the direct expression and analysis of canine *KIT* mutants, eliminating the need to make and analyze equivalent mutations in the mouse or human *KIT* genes.

Results

Alterations in *KIT* in Canine Mast Cell Tumors

Previous studies in a variety of species, including dog, have identified mutations in exons 8, 9, 11, and 17 of *KIT* (1). We therefore examined the sequences of exons 8 to 13 and 17 to 19 of *KIT* in canine mast cell tumors. A total of 202 dogs were enrolled, and sequences were obtained from 191. The majority of the dogs were mixed breeds, and the remainder included Labrador, Golden Retriever, Boxer, Rhodesian Ridgeback, Weimaraner, and other breeds (data not shown). Biopsies were taken from an enlarged mass (previous tumor, cutaneous metastasis, or internal organ) previously confirmed to be a mast cell tumor. We first cloned and sequenced exons 8 to 13 and 17 to 19 using reverse transcription-PCR. All genetic alterations were confirmed by sequencing of an independent reverse transcription-PCR. Genetic alterations were found in 50 (26.2%) of the 191 dogs (Table 1; Fig. 1). Of the 50 dogs with mutations, most ($n = 32$) had mutations in exon 11. Mutations were also frequently found in exons 8 ($n = 9$) and 9 ($n = 8$), and a single dog had a mutation in exon 17.

Within exon 11, rare in-frame mutations and deletions (Del⁵⁵⁵⁻⁵⁵⁷ InsV, Del⁵⁵⁶⁻⁵⁵⁷, K⁵⁵⁷ InsF, K⁵⁵⁷N InsP, and K⁵⁵⁷R Del⁵⁵⁸⁻⁵⁵⁹) were identified in the proximal (5') region. Most of the alterations ($n = 25$) were ITDs located in the distal part (3') of exon 11 between residues 571 and 590 (ITD⁵⁷¹⁻⁵⁹⁰). The mutations in exon 8 included a frequent ($n = 8$) ITD (12-bp duplication), resulting in the insertion of the amino acid sequence QILT at residue 421 (ITD⁴¹⁷⁻⁴²¹) and a single case of a Q⁴³⁰R substitution. Mutations in exon 9 included five cases of a S⁴⁷⁹I substitution and three of a N⁵⁰⁸I substitution. The single mutation found in exon 17 was a deletion of residues 826 to 828 coupled with an insertion of a DT sequence (Del⁸²⁶⁻⁸²⁸ InsDT).

In addition to these mutations, we found that all 191 dogs had three nucleotides that differed from the reported sequence

Table 1. Summary of Mutations in the *KIT* Gene Found in Canine Mast Cell Tumors

Mutation	Equivalent Residues in Human c-Kit Protein	No. Dogs (% of Total)*
Exon 8		
ITD ⁴¹⁷⁻⁴²¹	418	8
Q ⁴³⁰ R	427	1
Total	—	9 (4.7%)
Exon 9		
S ⁴⁷⁹ I	476	5
N ⁵⁰⁸ I	505	3
Total	—	8 (4.2%)
Exon 11		
Del ⁵⁵⁵⁻⁵⁵⁷ InsV	556-558	1
Del ⁵⁵⁶⁻⁵⁵⁷	556	1
K ⁵⁵⁷ InsF	558	1
K ⁵⁵⁷ N InsP	558	3
K ⁵⁵⁷ R Del ⁵⁵⁸⁻⁵⁵⁹	558	1
ITD ⁵⁷¹⁻⁵⁷⁹	573-581	1
ITD ⁵⁷¹⁻⁵⁸¹	573-583	1
ITD ⁵⁷¹⁻⁵⁸³	573-585	2
ITD ⁵⁷¹⁻⁵⁸⁵	573-587	1
ITD ⁵⁷¹⁻⁵⁸⁹	573-591	2
ITD ⁵⁷²⁻⁵⁸³	574-585	1
ITD ⁵⁷²⁻⁵⁸⁵	574-587	1
ITD ⁵⁷²⁻⁵⁸⁶	574-588	2
ITD ⁵⁷²⁻⁵⁸⁷	574-589	1
ITD ⁵⁷²⁻⁵⁸⁸	574-590	4
ITD ⁵⁷²⁻⁵⁸⁹	574-591	1
ITD ⁵⁷²⁻⁵⁹⁰	574-592	1
ITD ⁵⁷³⁻⁵⁸⁵	575-587	1
ITD ⁵⁷³⁻⁵⁹⁰	575-592	1
ITD ⁵⁷³⁻⁵⁹¹	575-593	1
ITD ⁵⁷⁵⁻⁵⁸²	577-584	1
ITD ⁵⁷⁶⁻⁵⁹⁰	578-592	3
Total	—	32 (16.8%)
Exon 17		
Del ⁸²⁶⁻⁸²⁸ InsDT	827	1 (0.5%)
All mutations	—	50 (26.2%)

*The cDNA for *c-kit* was cloned and sequenced from a total of 191 dogs.

for canine *KIT* in Genbank (Table 2). Two of these changes, c.1818C>T p. and c.2007A>T p., were conservative, and the third, c.1325G>A p., resulted in an amino acid change at codon 442 (G⁴⁴²D). Because our study included a large number of dogs and a variety of species, we suspect that these differences are errors in the published sequence or that there are polymorphisms at these sites. We also found evidence for common (>20% of the dogs) conservative polymorphisms at three additional codons (c.1275G>A p., c.1731C>T p., and c.2355G>A p.).

Expression of Canine *KIT* in Mouse Ba/F3 Cells

Activating mutations in *KIT* have previously been identified in canine mast cell tumors; however, demonstration that the mutations are activating has required the generation and analysis of equivalent mutants in human or mouse *KIT* (6, 12). To avoid this time-consuming step and to directly analyze the effect of the mutations identified in this study, we searched for a system in which cDNAs encoding canine wild-type (WT) and mutant forms of *KIT* could be functionally expressed. For this purpose, we focused on mouse Ba/F3 cells, an interleukin-3 (IL-3)-dependent hematopoietic cell line that has been previously used to examine the function of human *KIT* mutants (13). This cell line is becoming an increasingly important tool for characterizing protein tyrosine kinases and for the discovery of tyrosine kinase inhibitors (14).

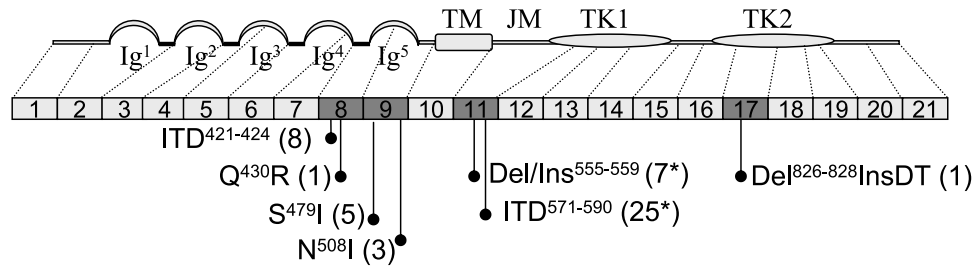


FIGURE 1. Location and frequency of KIT mutations identified in canine mast cell tumors. Domain structure of canine KIT is shown at the top, and the respective exons of the *KIT* gene are shown in the shaded boxes below. The number of dogs with each mutation is shown in parentheses. Asterisks (*) indicate that the category includes several individual mutations (see Table 1). Ig, immunoglobulin-like domain; TM, transmembrane domain; JM, juxtamembrane domain; TK1, tyrosine kinase domain lobe 1; TK2, tyrosine kinase domain lobe 2.

For all verified mutants, we cloned the entire coding sequence using reverse transcription-PCR and the Gateway cloning system. The cloned cDNAs were then inserted into the L_{XSN}^{GW} vector for expression in mammalian cells. We selected the two most common mutants (ITD⁴¹⁷⁻⁴²¹ and S^{479I}) and two representative exon 11 mutants for further analysis. The exon 11 mutants included one from the proximal region (Del⁵⁵⁵⁻⁵⁵⁷ InsV) and one from the distal region (ITD⁵⁷²⁻⁵⁸⁶) because mutations in these regions have been shown to have different functional effects in human gastrointestinal stromal tumor (15). These mutants were expressed in Ba/F3 cells, and after selection of transfected cells with G418, cell surface expression of KIT was verified by fluorescence-activated cell sorting (FACS) using ACK45, a monoclonal antibody to murine KIT that cross-reacts with canine KIT (Fig. 2; ref. 16). We also confirmed expression of KIT on C2 canine mastocytoma cells, which have been previously shown to express a mutant of KIT (6). In contrast, untransfected Ba/F3 cells (Fig. 2, dotted lines) did not express KIT.

Tyrosine Phosphorylation of Canine KIT in Ba/F3 Cells

Binding of SCF by KIT causes receptor dimerization, which then activates the tyrosine kinase domain, leading to receptor autophosphorylation. Oncogenic mutations in *KIT* act by enhancing the sensitivity to SCF or by constitutively activating the tyrosine kinase domain of KIT (1). Therefore, to examine the activity of the four selected mutants, we immunoprecipitated KIT from Ba/F3 cells and analyzed its tyrosine phosphorylation by Western blotting. Western blotting of the immunoprecipitates with a murine KIT antibody confirmed that the transfected Ba/F3 cells contain KIT protein, whereas KIT was not detected in untransfected Ba/F3 cells (Fig. 3A, bottom).

Western blotting with an antibody to phosphotyrosine confirmed that WT KIT was tyrosine phosphorylated only in the presence of SCF (Fig. 3A, top), whereas all four of the mutant KIT proteins were maximally phosphorylated in the absence of SCF. Similarly, in C2 cells, KIT was constitutively phosphorylated in the absence of SCF. Finally, these results show that mouse SCF activates canine KIT.

Effect of the Canine KIT Mutants on the Proliferation of Ba/F3 Cells

Ba/F3 cells are an IL-3–dependent cell line, and previous studies have shown that expressing activated mutants of *KIT* in these cells induces growth factor–independent proliferation (12, 13). Therefore, we next examined the growth of the transfected Ba/F3 cells in the presence and absence of SCF and IL-3 (Fig. 3B). In all cases, IL-3 induced maximal cell proliferation. As expected, Ba/F3 cells transfected with WT canine *KIT* did not proliferate in the absence of added growth factors but rapidly proliferated in the presence of SCF. In contrast, all four of the tested mutants induced growth factor–independent proliferation that was not enhanced by SCF. Of the tested *KIT* mutants, Del⁵⁵⁵⁻⁵⁵⁷ InsV caused the most rapid cell proliferation. C2 cells also underwent rapid growth factor–independent proliferation that was not enhanced by SCF.

The Tyrosine Kinase Inhibitor AG1296 Inhibits Growth Factor–Independent Proliferation of Ba/F3 Cells Expressing Canine KIT Mutants

Our results revealed that canine *KIT* mutants common in mast cell tumors can be functionally expressed in murine Ba/F3 cells. The results further suggest that these mutations cause growth factor–independent proliferation by constitutively acti-

Table 2. Polymorphisms Identified in the Canine *KIT* Gene

Codon	Published Sequence*	Identified Sequence	Nomenclature	Amino Acid Alteration	Frequency (n = 191)
G442D	GGT	GAT	c.1325G>A p.	Gly442Asp	100%
T606T	ACC	ACT	c.1818C>T p.	Thr606Thr	100%
T669T	ACA	ACT	c.2007A>T p.	Thr669Thr	100%
T425T	ACG	ACA	c.1275G>A p.	Thr425Thr	64%
Y577Y	TAC	TAT	c.1731C>T p.	Tyr577Tyr	24%
K785K	AAG	AAA	c.2355G>A p.	Lys785Lys	18%

*According to Genbank accession number NM001003181.

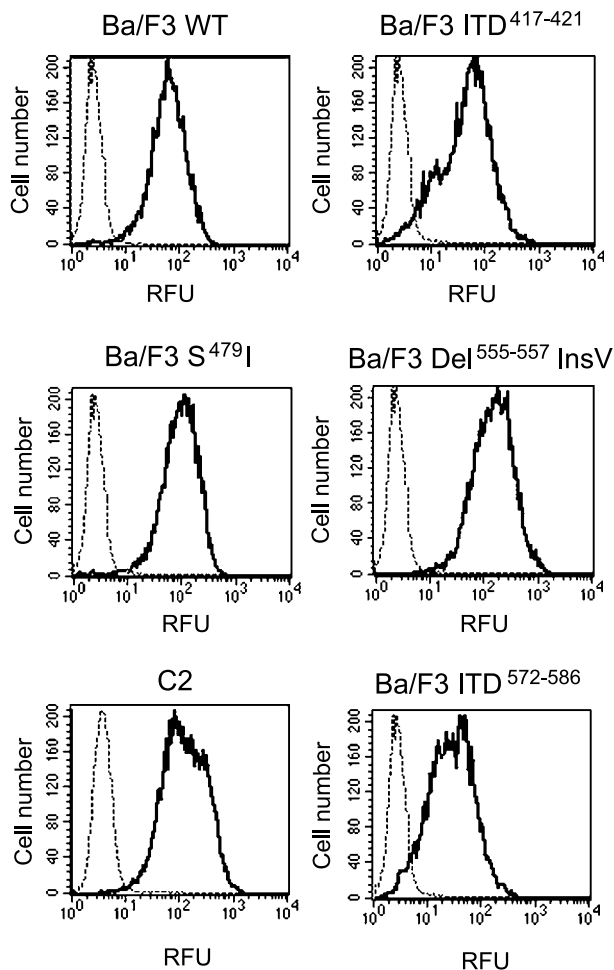


FIGURE 2. Analysis of cell surface expression of KIT by FACS. Ba/F3 cells were transfected with WT or mutant forms of canine KIT and selected with G418. Surface expression of KIT in transfected Ba/F3 cells and in canine C2 mastocytoma cells (positive control) was assessed by FACS using ACK45 anti-KIT monoclonal antibody (solid lines). Untransfected Ba/F3 cells (dotted lines) were used as a negative control. RFU, relative fluorescence units.

vating the KIT tyrosine kinase. To confirm the causative role of KIT tyrosine kinase activation in growth factor-independent cell proliferation and to validate the Ba/F3 system for the functional characterization of canine KIT, we next examined the effect of AG1296, an inhibitor of several receptor tyrosine kinases including KIT (12), on KIT tyrosine phosphorylation and KIT-dependent proliferation in Ba/F3 cells.

Immunoprecipitation-Western blotting experiments showed that AG1296 dose dependently inhibits SCF-stimulated tyrosine phosphorylation of WT KIT expressed in Ba/F3 cells. AG1296 also dose dependently inhibited the ligand-independent phosphorylation of all four tested canine KIT mutants in Ba/F3 cells, although compared with the other mutants, the S⁴⁷⁹I mutant was relatively insensitive (Fig. 4A). AG1296 also dose dependently inhibited the SCF-stimulated proliferation of Ba/F3 cells expressing WT canine KIT as well as the growth factor-independent proliferation of cells expressing the four canine KIT mutants. In all cases, 50% inhibition

was reached at approximately 0.3 to 3 $\mu\text{mol/L}$ of AG1296 (Fig. 4B). IL-3-stimulated proliferation of Ba/F3 cells was much less insensitive to AG1296, with 50% inhibition reached at $\sim 30 \mu\text{mol/L}$.

Of the four mutants, S⁴⁷⁹I mutant was the least sensitive to the inhibitor. To determine whether this insensitivity was shared by other exon 9 mutants, we also examined the N⁵⁰⁸I mutant. We found that this mutation constitutively activated KIT tyrosine phosphorylation, but, unlike S⁴⁷⁹I, it was sensitive to AG1296 (Supplementary Fig. S1).

Finally, mutations in exon 17, such as D⁸¹⁶V, are known to activate KIT but to be insensitive to most tyrosine kinase inhibitors (17). Similarly, we found that the Del⁸²⁶⁻⁸²⁸ InsDT mutation activated KIT but was insensitive to inhibition by AG1296 (Supplementary Fig. S1).

Discussion

In the current study, we examined the types and frequency of KIT mutations in canine mast cell tumors from 191 dogs. We also assessed the effects of four of the most common mutations on KIT function to gain insight into the pathogenesis of canine mast cell tumors and other mast cell diseases. This was the largest and most comprehensive analysis of the role of KIT mutations in canine mast cell tumors to date.

Mutations in KIT were found in just over one quarter (26.2%) of the dogs. A majority of the mutations (64%) were in exon 11, but we also found a significant number (34%) in exons 8 and 9. Only a single dog had a mutation in exon 17 (Del⁸²⁶⁻⁸²⁸ InsDT). Interestingly, like the human D⁸¹⁶V mutant, this canine mutant seems to be insensitive to tyrosine kinase inhibitors. Mutations in exon 11 are associated with higher grades of canine mast cell tumors (18) and an increased incidence of recurrent disease and death due to mast cell tumors (19). Mutations in this exon are also found in a majority of human gastrointestinal stromal tumors and are associated with a poor prognosis (20). Of the mutations in exon 11, most (25 of 32) were ITDs. This high frequency of ITDs in exon 11 agrees with previous findings in canine mast cell tumors (5, 6, 8, 21), although the overall distribution of mutations contrasts with that in human mastocytosis, where mutations in exon 17 (i.e., codon 816) are found in the majority of cases and mutations in exon 11 are rare (1).⁸

Further analysis of the specific sites of the mutations within exon 11 reveals two hotspots, one between residues 555 and 559 and a second between residues 571 and 590 (Fig. 1). Previous studies of canine mast cell tumors revealed the clustering of mutations in these regions (6, 18). One of the two hotspots in exon 11 is around a key tryptophan at residue 556 (corresponding to residue 557 in human KIT), which is inserted into a pocket in the protein kinase domain when the receptor is inactive (22). The other hotspot consists of a variety of ITDs between residues 571 and 590. Interestingly, the hotspots surround but do not include two tyrosines (residues 567 and 569, corresponding to residues 568 and 570 in human) that are

⁸ C. Bodemer et al. Pediatric mastocytosis is a clonal disease associated D⁸¹⁶V and other activating c-Kit mutations, submitted for publication.

principal sites of autophosphorylation following receptor dimerization, supporting the idea that the phosphorylation of these two residues and/or sequences surrounding these two residues are essential for kinase activation (22).

Exon 11 encodes the intracellular juxtamembrane region, which, in the inactive state, maintains the two lobes of the kinase domain in a closed conformation (1). Thus, mutations in exon 11, including Del⁵⁵⁵⁻⁵⁵⁷ InsV and ITD⁵⁷²⁻⁵⁸⁶, probably activate KIT tyrosine phosphorylation by disrupting the structure of the juxtamembrane region, releasing it from the kinase domain.

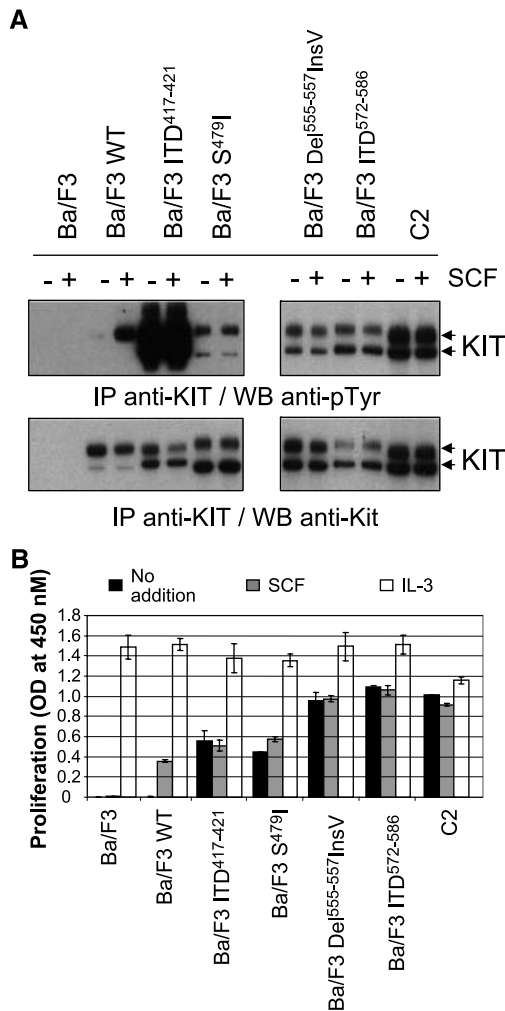


FIGURE 3. Ligand-independent activity of mutant canine KIT proteins in mouse Ba/F3 cells. **A.** Tyrosine phosphorylation of KIT in Ba/F3 and C2 cells. Untransfected Ba/F3 cells, Ba/F3 cells expressing WT or mutant canine KIT, and C2 canine mastocytoma cells were serum starved for 3 h and treated for 5 min with (+) or without (-) 250 ng/mL recombinant murine SCF. Tyrosine phosphorylation of KIT was analyzed by immunoprecipitation (IP) of KIT followed by Western blotting (WB) with a rabbit anti-KIT antibody or an anti-phosphotyrosine (pTyr) antibody. **B.** Proliferation of Ba/F3 cells expressing WT or mutant canine KIT. Ba/F3 cells expressing WT or mutant canine KIT and C2 canine mastocytoma cells were plated in 96-well plates and grown for 48 h with RPMI 10 with or without 0.1% conditioned medium from X63-IL-3 cells (IL-3) or 250 ng/mL murine SCF. Cell growth was assessed by measuring mitochondrial conversion of WST-1 into blue formazan dye with a spectrophotometer. Experiments were done in triplicate. Columns, mean; bars, SD. OD, absorbance.

Although less common than mutations in exon 11, a substantial portion (17 of 50) of the mutations were in exons 8 (Q⁴³⁰R and ITD⁴¹⁷⁻⁴²¹) and 9 (S⁴⁷⁹I and N⁵⁰⁸I). We also recently found that mutations in exons 8 and 9 are very common in childhood mastocytosis.⁸ The ITD at residue 417-421 includes a residue (419 in human) that is also mutated in human patients with mastocytosis and myeloproliferative disorder-acute myeloid leukemia (23-25). Our recent study of childhood mastocytosis also identified a hotspot for mutations consisting of codons 417 to 419.⁸ In addition, the N⁵⁰⁸I substitution (equivalent to residue 505 in human), which also activated KIT, is near a residue (509 in human) that is mutated from lysine to isoleucine in familial mastocytosis (26). It also lies within a hotspot for mutations (codons 501-509) in human gastrointestinal stromal tumor (1) and childhood mastocytosis.⁸ These residues lie COOH terminus to and within the fifth immunoglobulin-like domain. A recent crystallographic study showed that the fifth immunoglobulin-like domain forms homotypic contacts in the ligand-dependent dimer of KIT, and it was suggested that oncogenic mutations in this region act by enhancing the binding affinity of these interactions, leading to activation of the intracellular kinase domain (27).

Our characterization of canine *KIT* mutants was greatly simplified by the use of mouse Ba/F3 cells; previous studies of canine *KIT* mutants have required the generation of equivalent mutations in the human or mouse cDNA (6, 11). These studies in Ba/F3 cells showed not only that all of the tested mutants activate KIT tyrosine phosphorylation but also that they induced growth factor-independent cell proliferation.

In these studies, we used C2 canine mastocytoma cells as a positive control for KIT expression and detection. These cells express *KIT* with a 48-bp tandem duplication in the juxtamembrane domain (6). We show here that, like the tested mutant proteins, KIT in the C2 cells is constitutively tyrosine phosphorylated in the absence of SCF. Consistent with this, Gleixner et al. (11) recently reported that inhibitors of KIT can block the proliferation of C2 cells in culture.

We further showed that AG1296, an inhibitor of KIT and other receptor tyrosine kinases, inhibits not only the ligand-independent tyrosine phosphorylation of the four tested mutants but also their ability to cause growth factor-independent proliferation. This agrees with a previous report by Ueda et al. (12) that AG1296 inhibits the constitutive tyrosine phosphorylation and induction of growth factor-independent proliferation by murine KIT with an activating mutation in the juxtamembrane domain. Although AG1296 potently inhibited the induction of Ba/F3 cell proliferation by the canine KIT mutants, it was a weak inhibitor of IL-3-stimulated cell proliferation. Unexpectedly, tyrosine phosphorylation and induction of cell proliferation by the S⁴⁷⁹I mutant was relatively insensitive to AG1296. This insensitivity does not seem to be a general feature of exon 9 mutations because the N⁵⁰⁸I mutant was sensitive to AG1296. We found the same results using the tyrosine kinase inhibitor PP2 (data not shown), supporting the idea that the inhibition of both cell proliferation and KIT tyrosine phosphorylation is due to the inhibition of tyrosine kinase activity. The fact that both AG1296 and PP2 inhibited

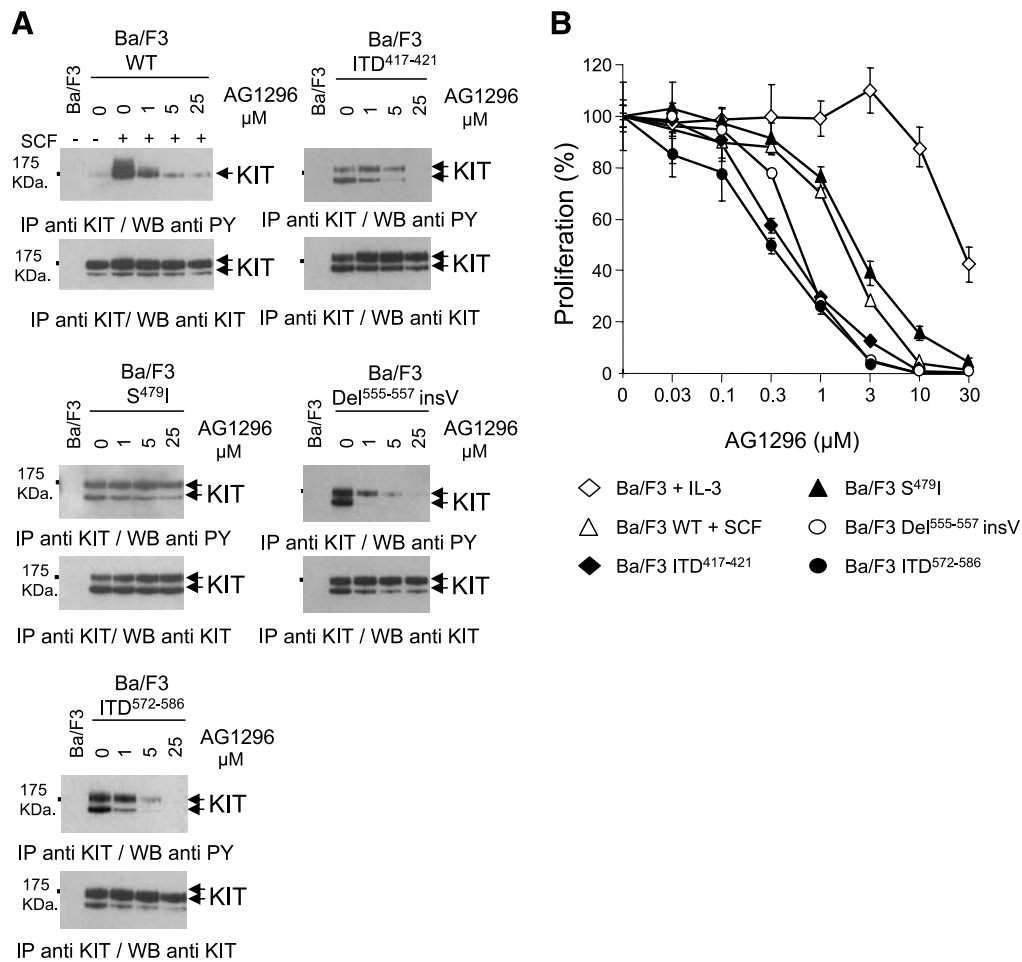


FIGURE 4. Effect of the tyrosine kinase inhibitor AG1296 on ligand-independent activity of mutant canine KIT. **A.** Effect of AG1296 on tyrosine phosphorylation of KIT in Ba/F3 cells. Untransfected Ba/F3 cells and Ba/F3 cells expressing WT or mutant canine KIT were serum starved for 3 h and treated for 5 min with (+) or without (-) 250 ng/mL recombinant murine SCF in the presence of 0 to 25 μ mol/L of AG1296. Tyrosine phosphorylation of KIT was analyzed by immunoprecipitation of KIT followed by Western blotting with a rabbit anti-KIT antibody or an anti-phosphotyrosine antibody. **B.** Proliferation of Ba/F3 cells expressing WT or mutant canine KIT. Ba/F3 cells expressing WT or mutant canine KIT were plated in 96-well plates and grown for 48 h with RPMI 10 with or without 0.1% conditioned medium from X63-IL-3 cells (IL-3) or 250 ng/mL murine SCF and in the presence of 0 to 30 μ mol/L of AG1296. Cell growth was assessed by measuring mitochondrial conversion of WST-1 into blue formazan dye with a spectrophotometer. Experiments were done at least twice. Points, mean; bars, SD. Values are presented relative to cell proliferation in the absence of AG1296.

mutant KIT-induced cell proliferation at lower concentrations than IL-3-induced cell proliferation suggests that the effects of these agents are KIT specific and not due to disruption of pathways generally involved in cell proliferation or survival.

Overall, the rank order of inhibition for the four tested mutants was the same in the Western blots and the cell proliferation. However, there was a difference in the dose-response curves between the cell proliferation and tyrosine phosphorylation experiments: in all cases, AG1296 was roughly 10-fold more potent at inhibiting cell proliferation than KIT tyrosine phosphorylation. Similar results were found with PP2, another tyrosine kinase inhibitor (data not shown). Such differences are often observed with inhibitors of protein kinases (e.g., see refs. 28, 29) and may have multiple causes. First, nonlinearities in the Western blots may have caused the IC₅₀ for tyrosine phosphorylation to appear at a higher concentration. Second, the downstream pathways leading to cell proliferation may have been more sensitive to perturbations

in the kinase activity than autophosphorylation. Third, preexisting phosphotyrosine on KIT (i.e., occurring before the addition of inhibitor) may be lost slowly and cause the autophosphorylation to appear to be less sensitive to an inhibitor than cell proliferation. This would not be apparent with WT KIT, which is only phosphorylated in the presence of SCF. Fourth, the inhibitors could affect multiple enzymes or pathways. Indeed, AG1296 and PP2 are known to inhibit several tyrosine kinases (30-32), and it is possible that the phosphorylation of downstream effectors or the activity of tyrosine kinases downstream of KIT is more sensitive to AG1296 and PP2 than KIT itself. Because both AG1296 and PP2 were potent inhibitors of mutant KIT-induced cell proliferation in all cases, it seems that, at a minimum, tyrosine kinase activity is necessary for their transforming activity. Furthermore, the fact that the rank order of potency in the Western blots is the same as in the cell proliferation assay argues that KIT tyrosine phosphorylation and the induction of cell proliferation are

directly linked. Overall, the differences in the dose-response curves are most likely due to the limitations of the assays, and these results suggest that constitutive activation of the tyrosine kinase domain drives the induction of cell proliferation by canine KIT mutants.

In sum, our results suggest that activating mutations in *KIT* are common and play a causative role in canine mast cell tumors. This agrees with a previous study showing that the tyrosine kinase inhibitor SU11654 (Sugen) causes significant shrinkage or stabilization of canine mast cell tumors (9). Another study by Kobie et al. (10) recently reported that the tyrosine kinase inhibitor STI571 (Glivec) induces the regression of xenografted canine mast cell tumors in severe combined immunodeficient mice. We also show here for the first time that mutations in exons 8 and 9 are common, whereas mutations in exon 17 are rare in canine mast cell tumors. This contrasts with the reported distribution of *KIT* mutations in both adult and childhood forms of mastocytosis (1).⁸

The results presented here should help clarify how transforming mutations in *KIT* lead to disease. In particular, they point out the importance of the fifth immunoglobulin-like domain in receptor activation. This information should aid in the development of treatments for mast cell tumors as well as human KIT-mediated diseases. Finally, we showed that Ba/F3 cells can be used to directly express and characterize canine KIT, avoiding the need to generate equivalent mutations in mouse or human cDNAs.

Materials and Methods

Animals

The analysis of *KIT* mutations in canine mast cell tumors was done as part of a phase III study on the treatment of canine mast cell tumors with the tyrosine kinase inhibitor AB1010. Female and male dogs, regardless of breed, were recruited with owner consent from 25 veterinary clinics in France and the

United States. Dogs had to have at least one histologically diagnosed measurable grade 2 (intermediate) or grade 3 (poorly differentiated) mast cell tumor (33) that was recurrent after a failure of surgery (as standard care) and/or was nonresectable.

Cloning and Sequencing of Exons 8 to 13 and 17 to 19 of *KIT* from Canine Mast Cell Tumors

Cutaneous biopsies (~5 mm) were taken from an enlarged mass (previous tumor, cutaneous metastasis, or internal organ) previously confirmed to be a mast cell tumor. Biopsies were submerged immediately in RNAlater (Qiagen) to a volume of ~1 mL and stored at room temperature. Total RNA was isolated using an RNeasy Mini kit (Qiagen) as recommended by the manufacturer. The extracted RNA (200 ng) was reverse transcribed in a 25- μ L reaction containing random hexamers (Stratagene) and the StrataScript First-Strand Synthesis System (Stratagene). A 2.5- μ L sample of the resulting cDNA was then amplified by PCR using the primers covering exons 8 to 13 and 17 to 19 (Table 3). PCR was carried out for 40 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s. Amplimers were purified with the GeneClean III kit (Qbiogene) and directly sequenced with the BigDye Terminator v.1.1 kit (Applied Biosystems) and sequencing primers (Table 3) on an ABI Prism 3130 sequencer (Applied Biosystems). Observed mutations were systematically checked in a new reverse transcription reaction. This method was able to detect the mutated allele when present in 5% to 10% of the cells.

Cloning and Expression of Full-Length WT and Mutant Canine KIT

RNA from normal and tumor biopsies was extracted and reverse transcribed as described above (see "Cloning and Sequencing of Exons 8 to 13 and 17 to 19 of *KIT* from Canine Mast Cell Tumors") except using oligo(dT) to prime cDNA and ThermoScript reverse transcriptase (Invitrogen). Each full-length cDNA was then amplified by PCR using canine

Table 3. Cloning and Sequencing Primers Used in This Study

Exons Targeted	Primer	Sequence (5'→3')	Nucleotides Targeted
Primers for cloning exons 8-13 and 17-19			
8-11	1159S	GGGAACGAAGGAGGCACTTACACA	1159-1182
	1843AS	CATCCGACTTAATCAGGCCATA	1821-1843
11-13	1651S	ATGTATGAAGTACAGTGAAGG	1651-1672
	2131AS	AAGTGCCACTTCTCCGTGATC	2083-2103
17-19	2321S	CTAGAGGACTTGCTGAGCTT	2293-2312
	2721AS	ATCCAGCAGCTTTCATGA	2702-2721
Primers for sequencing exons 8-13 and 17-19			
8-9	1159S	GGGAACGAAGGAGGCACTTACACA	1159-1182
	1494AS	GTTGTAAGCCCTACTCGAC	1471-1494
9-11	1336S	GTCCAGGAGCTGAGCAGAG	1137-1159
	1843AS	CATCCGACTTAATCAGGCCATA	1821-1843
11-13	1651S	ATGTATGAAGTACAGTGAAGG	1651-1672
	2131AS	AAGTGCCACTTCTCCGTGATC	2083-2103
17-19	2321S	CTAGAGGACTTGCTGAGCTT	2293-2312
	2721AS	ATCCAGCAGCTTTCATGA	2702-2721
Primers for full-length cloning using the Gateway system			
1-21	GWATG sense	ggggacaagtgtgtacaaaaagcAGGCTATCGCAGCCACCGGATGAG	-17 to +5
	GWR	ggggaccactttgtacaagaagctgggtGATCGCTCTTGTTGGGGAGAC	2948-2968

NOTE: Nucleotides targeting the canine *KIT* sequence (Genbank accession number NM001003181) are shown in uppercase letters. Nucleotides added to the PCR primers for cloning using the Gateway system are shown in lowercase letters. Underlined letters indicate the predicted start (ATG) codon.

KIT-specific primers containing attB sites at their 5' ends (Table 3), which allows the rapid and efficient cloning of PCR products into the attP-containing donor vector (pDON R201) using the Gateway system (Invitrogen). Each PCR product-containing vector was then sequenced to verify both the *KIT* sequence integrity and the presence of the introduced *KIT* mutations. Next, a LR recombination reaction was done between donor vectors and the LXSNGW attR-containing destination vector. Ba/F3 cells (5×10^6 in 300 μ L RPMI 10) were mixed with 100 μ L of PBS containing 15 μ g *KIT*-encoding LXSNGW plasmid and subjected to electroporation using a Gene Pulser (Bio-Rad Laboratories) at 250 V and 960 μ F for 30 ms. The transfected cells were selected in RPMI 10 containing 1 mg/mL G418 for 14 d.

Cells and Growth Conditions

Untransfected Ba/F3 cells or Ba/F3 cells expressing WT canine *KIT* were grown at 37°C in RPMI 10 (RPMI 1640 with L-glutamine, with 100 units/mL penicillin, 100 μ g/mL streptomycin, and 10% heat-inactivated FCS) supplemented with 0.1% conditioned medium from X63-IL-3 cells (34). Ba/F3 cells expressing mutants of *KIT* were grown in RPMI 10 without added cytokines. The C2 canine mastocytoma cell line, which expresses *KIT* with a 48-bp tandem duplication in the juxtamembrane domain (6), was provided by Douglas H. Thamm (Department of Clinical Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, WI) and grown at 37°C in RPMI 10.

Fluorescence-Activated Cell Sorting

Surface expression of *KIT* was verified by FACS using 10 μ g/mL of ACK45 anti-*KIT* monoclonal antibody (BD Biosciences). Events were collected using a FACScan (Becton Dickinson) and analyzed using FlowJo software (Treestar).

Analysis of *KIT* Tyrosine Phosphorylation

Ba/F3 cells (5×10^6) expressing WT or mutant *KIT* were serum starved for 3 h in RPMI. In some experiments, cells were treated for 1.5 h with RPMI containing 0 to 25 μ mol/L of AG1296 (Calbiochem). Cells were then treated for 5 min at 37°C with or without 250 ng/mL recombinant murine SCF, which was purified from the conditioned medium of SCF-producing CHO cells (gift of S. Lyman, Immunex). The cells were then placed on ice and washed with ice-cold PBS and lysed in 500 μ L of ice-cold HNTG buffer [50 mmol/L HEPES (pH 7), 50 mmol/L NaF, 1 mmol/L EGTA, 150 mmol/L NaCl, 1% Triton X-100, 10% glycerol, 1.5 mmol/L MgCl₂] containing a mixture of protease inhibitors (Roche Applied Science) and 100 μ mol/L Na₃VO₄. Cell lysates were mixed overnight at 4°C with 2 μ g of purified rat anti-mouse CD117 (*KIT*) monoclonal antibody ACK45 (BD Biosciences) and 50 μ L of a 20% slurry of protein G-Sepharose (Amersham Biosciences Europe GmbH) in PBS. The beads were washed in cold HTNG and resuspended in 20 μ L of 2 \times electrophoresis sample buffer. The immunoprecipitated proteins were then separated by SDS-PAGE on an 8% acrylamide gel. Proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore SAS). Membranes were blocked in TBS contain-

ing 0.05% Tween 20 and 5% bovine serum albumin for 30 to 45 min at room temperature. Western blotting for *KIT* was done using 1:1,000 dilution of a polyclonal rabbit anti-*KIT* antibody (Cell Signaling Technology), followed by 1:10,000 horseradish peroxidase-conjugated anti-rabbit antibody, and, for phosphotyrosine, using 1:1,000 anti-phosphotyrosine antibody 4G10 (Cell Signaling Technology), followed by 1:20,000 horseradish peroxidase-conjugated anti-mouse antibody. Immunoreactive bands were detected using enhanced chemiluminescent reagents (Pierce).

Assay of Cell Proliferation

A total of 10^4 cells per well were seeded into 96-well plates in 100 μ L of RPMI 1640 with 10% fetal bovine serum with or without 0.1% conditioned medium from X63-IL-3 cells or 250 ng/mL murine SCF at 37°C. The murine SCF, which activates dog *KIT*, was purified from the conditioned medium of SCF-producing CHO cells (gift of S. Lyman). In some experiments, the medium was supplemented with 0 to 30 μ mol/L of the tyrosine kinase inhibitor AG1296 (Calbiochem). Cells were grown for 48 h at 37°C and then incubated with 10 μ L/well of WST-1 reagent (Roche Applied Science) for 3 h at 37°C. The amount of formazan dye formed was measured using a scanning multiwell spectrophotometer (MultiSkan MS, Thermo-LabSystems). The absorbance of the samples was measured at 450 nm. A background control without cells was used as a blank for the spectrophotometer. Experiments were done in triplicate, and the means and SDs were calculated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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