

# Association of *KIT* Exon 9 Mutations with Nongastric Primary Site and Aggressive Behavior: *KIT* Mutation Analysis and Clinical Correlates of 120 Gastrointestinal Stromal Tumors<sup>1</sup>

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## ABSTRACT

**Purpose:** Activating mutations of the *KIT* juxtamembrane region are the most common genetic events in gastrointestinal stromal tumors (GISTs) and have been noted as independent prognostic factors. The impact of *KIT* mutation in other regions, such as the extracellular or kinase domains, is not well-defined and fewer than 30 cases have been published to date.

**Experimental Design:** One hundred twenty GISTs, confirmed by *KIT* immunoreactivity, were evaluated for the presence of *KIT* exon 9, 11, 13, and 17 mutations. The relation between the presence/type of *KIT* mutation and clinicopathological factors was analyzed using Fisher's exact test and log-rank test.

**Results:** Forty-four % of the tumors were located in the stomach, 47% in the small bowel, 6% in the rectum, and 3% in the retroperitoneum. Overall, *KIT* mutations were detected in 78% of patients as follows: 67% in exon 11, 11% in exon 9, and none in exon 13 or 17. The types of *KIT* exon 11 mutations were heterogeneous and clustered in the classic "hot spot" at the 5' end of exon 11. Seven % of cases showed internal tandem duplications (ITD) at the 3' end of exon 11, in a region that we designate as a second hot spot for *KIT* mutations. Interestingly, these cases were associated with: female predominance, stomach location, occurrence in older patients, and favorable outcome. There were significant associations between exon 9 mutations and large tumor size

( $P < 0.001$ ) and extragastric location ( $P = 0.02$ ). Ten of these 13 patients with more than 1-year follow-up have developed recurrent disease.

**Conclusions:** Most *KIT*-expressing GISTs show *KIT* mutations that are preferentially located within the classic hot spot of exon 11. In addition, we found an association between a second hot spot at the 3' end of exon 11, characterized by ITDs, and a subgroup of clinically indolent gastric GISTs in older females. *KIT* exon 9 mutations seem to define a distinct subset of GISTs, located predominantly in the small bowel and associated with an unfavorable clinical course.

## INTRODUCTION

GISTs<sup>3</sup> are the most common mesenchymal neoplasms of the intestinal tract. The terminology and histogenesis of GIST have been long controversial. Originally regarded as smooth muscle neoplasms, a subset of these tumors designated as plexosarcomas or GANT (gastrointestinal autonomic nerve tumors) were shown later on to have a peculiar neuronal differentiation (1). With the advent of immunohistochemistry, *KIT* negative tumors, such as schwannomas, leiomyomas, and leiomyosarcomas, were excluded from the noncommittal term of "gastrointestinal stromal tumors." Presently, based on immunophenotypic and ultrastructural similarities (2), it is widely accepted that the precursor cell in GISTs is the ICC, an intestinal pacemaker cell.

Most, if not all, GISTs express the *KIT* receptor, a type III receptor tyrosine kinase, which is known to have diverse roles in several major cell systems during embryogenesis and in the postnatal organism: namely in hematopoiesis, in the pigmentary system, in gametogenesis, and in intestinal pacemaker cells (3–5). *KIT* loss-of-function mutations have severe defects in the development and maintenance of these systems (3, 6, 7). *KIT* receptor signaling is initiated by the binding of *KIT* ligand, receptor dimerization, and concomitant activation of the *KIT* kinase (4). A significant number of GISTs have been shown to harbor gain-of-function mutations in the *KIT* proto-oncogene, resulting in ligand-independent *KIT* receptor function (8).

Predicting the clinical behavior of GISTs has always been problematic for both pathologists and clinicians and was applied with variable success using multiparameter approaches. Features associated with an unfavorable prognosis include large

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<sup>3</sup>The abbreviations used are: GIST, gastrointestinal stromal tumor; ICC, interstitial cell(s) of Cajal; MF/50HPF, mitotic figure(s) per 50 high-power field; ITD, internal tandem duplication; AWD, alive with disease; DOD, dead of disease; JM, juxtamembrane; AML, acute myeloid leukemia.

Table 1 *KIT* exon 9, 11, 13, and 17 primer sequence used, with the corresponding annealing temperatures ( $T_A$ ) and expected PCR size products

c- <i>KIT</i> exon no.	Primers	Primer sequences 5'→3'	$T_A$ (°C)	Product sizes
9	hEx9-F	TTCCTAGAGTAAGCCAGGG	53	298 bp
	hEx9-R	AATCATGACTGATATGGT		
11	hEx11-F	CAGGTAACCATTATTTGT	53	326 bp
	hEx11-R	TCATTGTTTCAGGTGGAAC		
13	hEx13-F	ATCAGTTTGCCAGTTGTGCT	53	250 bp
	hEx13-R	TTTATAATCTAGCATTGCC		
17	hEx17-F	GTTTTCACTCTTTACAAGT	53	277 bp
	hEx17-R	TTACATTATGAAAGTCACAGGAAAC		

size, increased mitotic activity, and tumor location (9, 10). Initial molecular analyses suggested that *KIT* mutations occur preferentially in biologically more aggressive GISTs (11, 12). The majority of *KIT* mutations that occur in GISTs are located in the JM portion (exon 11) of *KIT* gene. Mutations have been rarely described in the extracellular domain or kinase domain (13–15). More recently, Heinrich *et al.* (16) described activating mutations in *PDGFRA* in one-third of *KIT* wild-type GISTs.

The objectives of the present study were to evaluate the impact of different *KIT*-activating mutations on other clinicopathological factors, in a well-characterized group of GIST patients, treated and prospectively followed at a single institution.

## MATERIALS AND METHODS

### Histopathology and Immunohistochemical Review.

Tumor specimens included in this study were retrieved among patients who received diagnoses of primary or recurrent GIST and who were admitted and treated at Memorial Sloan-Kettering Cancer Center between July 1982 and March 2002. Patients were entered into a clinical sarcoma database and followed prospectively. Histological slides from the primary tumors were reviewed in all of the cases (by C. R. A., and J. M. W.), to confirm the diagnosis and to assess the morphological subtype (spindle *versus* epithelioid), number of MF/50HPF, and presence of peritoneal seeding or liver metastasis. An immunohistochemical reaction for KIT was performed in every case. Cases in which paraffin tissue was not available or which were negative for KIT were excluded from the study. KIT (CD117) immunohistochemistry was performed with a polyclonal rabbit antibody (DAKO Corp., Carpinteria, CA), at a 1:500 dilution in citrate buffer. Endogenous mast cells or ICC from myenteric plexus were used as internal-positive controls.

**Molecular Analysis.** One hundred twenty cases were identified in which the diagnosis of GIST was confirmed after pathological review and immunoreactivity for KIT, and in which adequate frozen tissue was available for molecular analysis. Tissue collection and molecular analyses were approved by the Institutional Review Board. DNA was isolated from snap-frozen tumor tissue samples stored at  $-70^{\circ}\text{C}$ , using a standard phenol-chloroform organic extraction protocol. One  $\mu\text{g}$  of genomic DNA was subjected to PCR using Platinum TaqDNA Polymerase High Fidelity (Life Technologies, Inc., Gaithersburg, MD). All of the cases were first subjected to a PCR reaction using primers for *KIT* exon 11. The cases that showed a mutation on the first PCR were not studied further. The cases

without exon 11 mutations were subjected to PCR using *KIT* exon 9 primers. From the second PCR, the cases without exon 9 mutations were further studied using *KIT* exon 13 and 17 primers. All of the primer sequences (17), annealing temperatures, and expected product sizes are shown in Table 1. The PCR conditions were as follows: (a)  $94^{\circ}\text{C}$  for 4 min; (b)  $94^{\circ}\text{C}$  for 30 s,  $53^{\circ}\text{C}$  for 30 s,  $72^{\circ}\text{C}$  for 30 s, (35 cycles); and (c)  $72^{\circ}\text{C}$  for 3 min. The PCR products were identified by agarose gel electrophoresis using a 2% MetaPhor agarose gel (BioWhittaker Applications, Rockland, ME). The expected sizes of the PCR products ranged from 250 to 326 bp in length. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA) before sequencing. All of the *KIT* sequencing reactions were performed from forward and reverse directions using a 300-nm PCR primer. All of the exon 11 insertions were confirmed by subcloning of the PCR products using a T/A Cloning kit (Promega, Madison, WI) and subsequent sequencing. Every ABI sequence was compared with a National Center for Biotechnology Information (NCBI) Human *KIT* gene nucleotide sequence and blasted using a NCBI Standard Nucleotide Blast Search to determine the location and type of mutation within a particular exon. We also performed a DNA dilution series to test the sensitivity of determining *KIT* mutation by direct sequencing, using DNA extracted from a blood sample collected from one of the familial GIST patients (associated with a germline *KIT* mutation). *KIT* mutations were detected up to a 65% dilution.

**Clinical Follow-Up and Statistical Analysis.** Overall survival, local recurrence-free survival, and metastasis-free survival were used as end points of the study. The stage at diagnosis was evaluated as follows: localized to the gastrointestinal organ, intra-abdominal spread, or presence of distant (liver) metastasis. Local recurrence was defined as presence of peritoneal disease. The site of distant metastatic disease was restricted to liver.

The clinicopathological and molecular findings of the tumors were analyzed for possible associations with each other and also for their relation to overall survival, time-to-local recurrence, and distant metastasis. The following clinicopathological variables were studied: age, sex, tumor size, location, stage at diagnosis, mitotic index, and morphological type (epithelioid *versus* spindle cell). The association of these variables with the presence or type of *KIT* mutation (exon 11 or not, exon 9 or not, any mutation or not) was tested using Fisher's exact test. The log-rank test was used to assess the relationship of

individual clinicopathological and molecular variables to time-to-local recurrence, time to metastasis, and overall survival.

## RESULTS

**Patient Demographics.** There were 56 females and 64 males, with a mean age of 57.8 years (range, 12–87 years) and peak incidence in the 6th–8th decades of life. There were two pediatric patients, two girls 12 and 15 years of age. Eighteen (15%) patients were younger than age 40, and 62 (52%) patients were older than 60. Clinical history revealed a second malignancy in 14 (12%) patients, the majority of which (12 patients, 10%) were epithelial malignancies, such as breast carcinoma (4 patients); colorectal adenocarcinoma and urothelial carcinoma in 2 each; and prostate, lung, esophageal, and head and neck carcinoma in one patient each. One patient had a history of B-cell small lymphocytic malignant lymphoma of the stomach and 1 year later was diagnosed with a GIST in the same location. One patient had a soft tissue sarcoma (high-grade extremity malignant fibrous histiocytoma) as a second malignancy. In three patients, GIST was found incidentally while being explored/managed for esophageal carcinoma, rectal adenocarcinoma, and uterine leiomyomas.

All except one patient presented with a solitary tumor. The only patient with multiple GISTs was a 45-year-old female who presented with metachronous primary small tumors (<5 cm) involving the stomach, small bowel (jejunum, terminal ileum), and cecum. Histologically, all of the tumors (stomach, small bowel, large bowel) were composed of intersecting short fascicles of uniform spindle cells, showing minimal nuclear pleomorphism or variation in nuclear size or shape. No tumor necrosis was present and mitotic activity was very low (<1 MF/50HPF). Immunohistochemically, the tumor cells were diffusely and strongly positive for KIT. The intestinal wall adjacent to and away from the tumors showed a diffuse thickening of the Auerbach myenteric plexus, with an increased number of KIT-positive cells, consistent with ICC hyperplasia. The patient reported multiple other family members diagnosed with stromal tumors of the gastrointestinal tract (not available for our pathological review) and cutaneous hyperpigmentation, particularly of the hands and perineum. This patient was suspected for familial GIST, and molecular analysis from both tumor and normal tissue indicated the presence of a *KIT* germline mutation.

**Tumor Characteristics.** The tumor locations were evenly distributed between stomach (53 cases; 44%) and small bowel (56 cases; 47%). Eleven tumors (9%) were located either in the rectum (7 cases) or retroperitoneum (4 cases). There were 28 tumors with a diameter <5 cm; 44 tumors with diameter  $\geq$ 5 cm but <10 cm; and 48 tumors  $\geq$  10 cm. The mean tumor size was 7.8 cm. The sizes of the incidentally found GISTs ranged from 1.5–2.5 cm; two were located in the stomach and one in the small bowel.

Nineteen (16%) tumors showed predominantly an epithelioid morphology (>95% of the tumor), whereas the remaining 101 (84%) of the tumors had a spindled histology. The distribution of tumors characterized by an epithelioid phenotype was evenly distributed among stomach (9 cases) and small bowel (8 cases). Two cases were located in the rectum. There was a 2:1 female:male ratio in this subset and six patients were younger

than 40 years age. The two pediatric GIST cases both showed a pure epithelioid morphology; and the tumors were located in the stomach.

Histological features of spindle GISTs included long fascicles of bland spindled cells with pale to eosinophilic fibrillary cytoplasm. The nuclei were uniform in appearance and in many cases were indented at one pole by cytoplasmic vacuoles. Prominent nuclear palisading, resembling Verocay-bodies often seen in schwannoma, was also seen in a number of cases. Nuclear pleomorphism was consistently absent. The predominantly epithelioid GISTs were composed of sheets of round to oval-shaped cells, with abundant glassy, eosinophilic cytoplasm, and well-defined cell membranes. In the majority of these cases, the tumor cells had a distinctive plasmacytoid appearance, with bi- or multinucleation and scattered bizarre nuclei. Focal cytoplasmic vacuolization was seen in a few cases. In contrast to the spindle GISTs, where the extracellular matrix was either loose or focally myxoid, in the epithelioid subtype of GISTs, stromal hyalinization was noted, at least focally in most cases. Eighty-five (71%) tumors had a low mitotic count of <10 MF/HPF, whereas 35 (29%) tumors had  $\geq$ 10 MF/50HPF.

In the overwhelming majority of cases, the KIT immunostaining was strong and diffuse. No differences in staining pattern were noted between GISTs with epithelioid or spindle-cell morphology.

**Molecular Results.** Overall, mutations were detected in 94 (78%) of the patients: 81 (67%) of the cases showed *KIT* mutations in exon 11, 13 (11%) in exon 9, and none in exon 13 or 17. The types of exon 11 mutations were heterogeneous, but the majority (90%) clustered at the 5' end, a previously described classic "hot spot" (Fig. 1). There were 38 (31%) simple deletions, 22 (18%) point mutations, and 13 (11%) point mutations followed or preceded by a deletion. The most common exon 11 *KIT* mutation was a WK (Trp-Lys) deletion at Codon 557 in 15 (12%) of the cases, followed by a V559D substitution in 9 (7%) of the cases (see Fig. 1).

Eight cases (7%) showed an insertion of 6–20 amino acids, representing ITDs at the 3' end of exon 11 in a portion that we designate as a second or ITD hot spot (Fig. 2). All eight of the exon 11 ITD tumors were located in the stomach, occurred in patients older than 60 years of age, had spindle cell morphology, and were associated with a low mitotic count. Seven of these eight patients were female, and five tumors were larger than 10 cm.

Exon 9 mutations were identified in 13 cases and consisted of identical tandem duplications of six nucleotides, encoding Ala-Tyr, as described previously (13, 14). The majority of tumors with exon 9 mutations were located in the small bowel (77%), large in size (77%,  $\geq$ 10 cm), from patients <60 years of age (70%), females (62%), and had a spindle cell morphology (85%). Only 1 of the 13 cases showing exon 9 mutations was located in the stomach and 2 were located in the rectum.

Almost two-thirds (63%) of the 19 GISTs with epithelioid phenotype lacked *KIT* mutations. Only 7 of 19 (37%) of the tumors in this subset showed mutations, 5 in exon 11 and 2 in exon 9. None of the nine gastric epithelioid GISTs had exon 11 mutations, and only one of them showed an exon 9 mutation. The majority of the small bowel epithelioid GISTs showed mutations, five of eight in exon 11 and one of eight in exon 9.

550	560	570	580	590	# of cases
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG					26 WT
QKPMYEVQWKV	[E]	EEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEVQWKV	V	VEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEVQ	[V]	VEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			15
QKPMYEVQWK	[E]	EINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
Q	[V]	VQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QK	[Q]	QWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEV	[E]	EEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEVQ	[E]	EINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			2
QKPMYEVQW	[I]	INGNNYVYIDPTQLPYDHKWEFPRNRLSFG			3
QK	[W]	WKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKP	[V]	VVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPM	[V]	VVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEVQW	[G]	GNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEVQWKV	[V]	VVEEINGN[ ]TQLPYDHKWEFPRNRLSFG			1
Q	[V]	VVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEVQWKV	[E]	EE[ ]PTQLPYDHKWEFPRNRLSFG			1
QKPMYEVQWKV	[E]	EE[ ]TQLPYDHKWEFPRNRLSFG			1
QKPMYEVQWKV	[E]	EEI[ ]PYDHKWEFPRNRLSFG			1
QKPMY	[D]	DTQLPYDHKWEFPRNRLSFG			1
QKPMY	[T]	TQLPYDHKWEFPRNRLSFG			1
QKPMYEV	[Q]	QLPYDHKWEFPRNRLSFG			1
QK	[L]	LYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMY	[E]	EWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEVQW	[L]	LVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKP	[K]	KVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEV	[H]	HVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEV	[V]	VVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPM	[L]	LVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMY	[D]	DVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEVQ	[T]	TNGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEVQW	[R]	RNGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEVQW	[R]	RNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
Q	[L]	LVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEVQ	[S]	SVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEVQ	[G]	GKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEVQ	[R]	RKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEVQWK	[A]	AVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEVQWK	[D]	DVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			9
QKPMYEVQWK	[G]	GVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			3
QKPMYEVQWKV	[D]	DVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			3
QKPMYEVQWKV	[G]	GVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEVQWKV	[P]	PVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEVQ	[F]	FQVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1
QKPMYEVQW	[Q]	QVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG			1

[ ] deletion    [x] deletion with amino acid change    [x] point mutation    [xx] and insertion point mutation

Fig. 1 A summary of deletions, point mutations, and simple insertions found in *KIT* exon 11 in the 120 cases studied. On the right side, the number of cases per type of mutation is recorded. The first row depicts the wild-type (WT) sequence of *KIT* and the number of WT GISTs found in this study.

The two epithelioid GISTs arising in an extra gastrointestinal location were of wild type. None of the epithelioid GIST with exon 11 mutations had tandem-duplications at the 3' end. All three incidental GISTs showed mutations within exon 11, deletions in two cases and point mutation in one case. The two GIST cases from pediatric patients were both wild type.

Direct sequencing of the PCR products of normal blood samples and tumor tissues from the familial GIST case showed an identical substitution mutation at position 557 in exon 11 (TGG→CGG), W557R. Furthermore, DNA from blood samples of family members contained the same identical exon 11 *KIT* mutation as the present case.

In 11 of the 12 cases in which 2 or more tumor samples (from the primary, local recurrence, or metastasis) were available for molecular analysis from the same patient, an identical *KIT* genotype was identified, including exon 11 deletions (*n* = 4), point mutation (*n* = 2), deletion and point mutation (*n* = 1), exon 9 tandem duplications (*n* = 2), and wild type (*n* = 2). Discrepant results were found in only one case, one sample (primary tumor) showing no mutations for any of the exons studied, whereas a different sample (collected from a subsequent intra-abdominal recurrence, 3 years later) from the same patient had a tandem duplication in exon 9. This discrepant result is most likely attributable to contamination with necrotic debris or



**Fig. 2** A comparative summary of the ITDs at the 3' end of *KIT* exon 11, found in our cases and also from the review of the literature. The first eight cases are from the present study, whereas the remaining cases are reported cases of human GIST (*KIT*), canine mast cell tumors (*KIT*), and human AML (*Flt3*). MCT, mast cell tumor.

550	560	570	580	590	
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG					Reference
<u>human</u>					
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG					
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYD <u>HKWEFPRNRLSFG</u>					
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYD <u>HKWEFPRNRLSFG</u>					
QKPMYEVQWKVVEEINGNNYVYIDPTQLPY <u>DHKWEFPRNRLSFG</u>					
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYD <u>HKWEFPRNRLSFG</u>					
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYD <u>HKWEFPRNRLSFG</u>					
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYD <u>HKWEFPRNRLSFG</u>					
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYD <u>HKWEFPRNRLSFG</u>					#23
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYD <u>HKWEFPRNRLSFG</u>					#26
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYD <u>HKWEFPRNRLSFG</u>					#21
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYD <u>HKWEFPRNRLSFG</u>					#21
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYD <u>HKWEFPRNRLSFG</u>					#21
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYD <u>HKWEFPRNRLSFG</u>					#27
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYD <u>HKWEFPRNRLSFG</u>					#27
<u>Canine MCT and MCT cell lines</u>					#28,29
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYD <u>HKWEFPRNRLSFG</u>					
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYD <u>HKWEFPRNRLSFG</u>					
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYD <u>HKWEFPRNRLSFG</u>					
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYD <u>HKWEFPRNRLSFG</u>					
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYD <u>HKWEFPRNRLSFG</u>					
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYD <u>HKWEFPRNRLSFG</u>					+ 6bpIn11STOP
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYD <u>HKWEFPRNRLSFG</u>					+ 363bpIn11+40bpEx12
QKPMYEVQWKVVEEINGNNYVYIDPTQLPYD <u>HKWEFPRNRLSFG</u>					
<u>Human AML_mFlt3</u>					#30
QFRYESQLQMIQVTGDNEYFYVDFRDYEYDLKWEFPRNLEF					
P L					
QFRYESQLQMIQVTGDNEYFYVDFRDYEYDLKWEFPRNLEF					
QFRYESQLQMIQVTGDNEYFYVDFRDYEYDLKWEFPRNLEF					
QFRYESQLQMIQVTGDNEYFYVDFREYEYDLKWEFPRNLEF					
QFRYESQLQMIQVTGDNEYFYVDFR <u>YDFR</u> DYEYDLKWEFPRNLEF					
E DREHH					

normal tissue in the original sample from the primary tumor found to have a wild-type phenotype, because histologically >50% of the tumor was described as being necrotic.

**Clinical Follow-Up.** Clinical follow-up was available in all of the patients, and the median follow-up for survivors was 34 months (range, 0.2–235 months). The median overall survival was 140 months [95% CI, 119– (could not be calculated)], whereas the median time to local recurrence was 87 months (95% CI, 72–113 months) and to liver metastases was 96 months (95% CI, 62–124 months). The majority of patients presented at diagnosis with localized disease. Only 6 (5%) of the patients showed liver metastasis at diagnosis and 21 (17%) of these had intraperitoneal spread at the time of primary tumor resection. This incidence might also be a reflection of our referral pattern as a Cancer Center, which may include patients with more malignant tumors. One-third of patients developed either intra-abdominal spread of disease (41 patients) or liver metastasis (39 patients), and 15% developed both at the time of last follow-up. None of the patients with incidentally found GIST developed recurrences, but the follow up was very short, being less than 1 year.

Of the 28 patients with tumor size <5 cm, 7 (25%) developed recurrent disease: liver metastases in 5 cases, local recurrences in 3 (1 patient having both), and although none died of disease, 6 were AWD at the time of last follow-up. The tumor location of these small-sized GISTs, associated with an adverse outcome, were five in the small bowel and two in the rectum, but none were located in the stomach. In this subset, all of the

tumors that developed liver metastases had a spindled cell morphology. In three of four cases with increased mitotic activity ( $\geq 10$  MF/50HPF), the small-sized GISTs developed recurrent disease.

Large tumor size ( $\geq 10$  cm) and high mitotic activity ( $\geq 10$  MF/50HPF) were significantly associated with both shorter survival ( $P = 0.004$ ,  $P < 0.0001$ , respectively) and shorter time-to-local recurrence ( $P = 0.03$ ,  $P < 0.0001$ , respectively). Furthermore, patients with lower mitotic counts had a longer time-to-distant metastasis than patients with higher counts ( $P < 0.0001$ ). There was also a significant association between the presence of intra-abdominal spread at the time of diagnosis and a shorter time-to-local recurrence ( $P = 0.005$ ) and a shorter survival ( $P = 0.03$ ).

The ITD of *KIT* exon 11 correlated with older female patients ( $P = 0.007$ ) and gastric location ( $P = 0.006$ ). None of these patients developed intra-abdominal spread or distant metastases, and all showed no evidence of disease (NED) at last follow-up. However, the follow-up was longer than 3 years in only four patients and was shorter than 1 year in the remaining 4.

All 10 of the patients with exon 9 mutations, with follow-up information for more than 1 year, developed intra-abdominal disease and/or distant metastases, and are either DOD or AWD. Significant associations between exon 9 mutations and large tumor size ( $P < 0.001$ ), nongastric site ( $P = 0.02$ ), and spindle cell morphology ( $P < 0.001$ ) were found.

A significant association was found between the GISTs with epithelioid morphology and lack of mutation ( $P < 0.0001$ ).

This subset of GISTs was associated with intraabdominal seeding in 11 of 19 (58%) and liver metastasis in 12 of 19 (63%); and 13 of 19 (68%) of the cases were either AWD or DOD at the last follow-up. Follow-up of the two pediatric GIST patients showed that both developed local recurrence and distant metastases but were still AWD after 5.6 and 17 years, respectively.

## DISCUSSION

The *KIT* gene is the cellular homologue of the oncogene *v-kit* of the Hardy-Zuckerman 4 feline sarcoma virus (18, 19). It encodes the receptor tyrosine kinase KIT. KIT is composed of an intracellular tyrosine kinase, a JM region, and an extracellular domain with a ligand binding site. Normally, KIT ligand binding to the KIT receptor mediates receptor dimerization, activation of kinase activity and autophosphorylation (4). The activated receptor phosphorylates various substrates, associates with cytoplasmic signaling molecules and thus triggers distinct signaling cascades. KIT receptor activation mediates survival and proliferation of several cell types as well as other cellular responses. In GIST and hematological malignancies, oncogenic activation of *KIT* leads to ligand-independent activation of the KIT receptor and associated signal transduction cascades, resulting in constitutive signaling for cell proliferation and survival (8, 20). The percentage of GISTs that have been reported *KIT* mutation positive varies from 65% (11) to 92% (21). The majority of the mutations have been found in the JM domain, in the hot-spot region of exon 11, involving codons 550–560 (22). In the two largest series, exon 11 mutations were found in 71 (57%) of the 124 cases and 103 (52%) of the 200 cases (11, 14). The mutations vary from single bp substitutions to complex deletions/insertions but are invariably in-frame. Other investigators have found exon 11 mutations in 20–50% of patients (12, 22, 23). However, the JM region mutation rate reached 71% in a recent report (21) and was 67% in the present study. Methodological differences among these retrospective studies, such as the histopathological diagnostic criteria or the type of tumor tissue available for DNA extraction (archival material *versus* frozen tissue) might be responsible for the variable incidence rate of *KIT* mutations. Another potential factor is the different techniques applied in different studies to detect *KIT* mutations. A certain number of mutations, such as point mutations, would be missed by simple PCR product-length analysis (24), whereas using a denaturing high-pressure liquid chromatography might detect a higher number of *KIT* mutations (25). Furthermore, a systematic sequencing of the entire cDNA *KIT* coding sequence (21) was shown to detect a significantly higher rate of *KIT* mutations than did the evaluation of only a restricted segment of the mutational hot-spot JM region (12, 22, 23).

We have identified a second hot spot for *KIT* mutations located at the 3' end of exon 11, including codons 576–590, which are composed of ITDs. Eight cases in the present series, representing 10% of exon 11 mutated GISTs, showed this type of in-frame duplication, which seemed to define a homogeneous subgroup of indolent gastric GISTs in older females. Seven similar cases were previously described, but a distinct clinical phenotype was not recognized (21, 23, 26, 27). Interestingly, a similar *KIT* mutation has been described in a subset of canine spontaneous mast cell tumors (MCT; Refs. 28, 29). In these

tumors, the ITDs varied from 45 to 70 bps in length and were always present in the 3' region of exon 11 (Fig. 2). This region is adjacent to the 5' end of *v-kit* and thus comprises the junction between the viral gag and feline KIT sequences in the HZ4-FeSV (18, 19). Analysis of a canine mastocytoma cell line showed that the ITD in *KIT* was associated with autophosphorylation of *KIT* in the absence of ligand binding (28). Furthermore, strikingly similar duplications within the 3' end of exon 11 of the closely related type III receptor tyrosine kinase-encoding *Flt3* are detected in ~20% of patients with AML (Fig. 2). The ITD mutations of *Flt3* have oncogenic potential, resulting in constitutive autophosphorylation and strong factor-independent activation of STAT5 (30, 31).

Simple insertions preceded by a substitution are also very rare but are located in the classic hot spot of exon 11. We identified only four such cases, including one in the present series, in which one amino acid insertion [Pro (P)] at position 558–559, is preceded by one amino acid substitution: either Q(K558Q, K558\_V559insP) in our study (see Fig. 1) or a N(K558N, K558\_V559insP) in the three cases previously reported in the literature (11, 15, 27).

Less frequently, *KIT* mutations can occur either in the extracellular domain (exons 9) or kinase domain (exon 13; 13). The two largest series to date (13, 14), focusing on non-JM domain *KIT* mutations, reveal a less than 10% incidence of GISTs with *KIT* mutations in these two domains. There have also been rare reports (21) of GISTs with mutations in the second part of the kinase domain (phosphotransferase domain) in exon 17, which is typically altered in mastocytosis and in mast cell and germ cell tumors (32, 33).

*KIT* exon 9 mutations seem to define a distinct subset of GIST, because all of the cases reported to date show an identical duplication of six nucleotides encoding Ala-Tyr at the COOH terminus of the extracellular domain. Exon 9 mutations have been documented in less than 30 cases (13, 14, 25, 27, 34, 35). Lasota *et al.* (14) were the first to point out that exon-9-mutated GISTs might be associated with an intestinal location and with a more aggressive clinical behavior. Our results confirm these findings because the majority of exon-9-mutated GISTs occurred in the small bowel and were also associated with an unfavorable clinical course. In contrast to these results, others have not found an association of exon-9-mutated GISTs with either a nongastric location (34) or an unfavorable outcome (35). The results of Lasota *et al.* (22), as well as our results, show that the analysis of *KIT* mutation patterns in consecutive tumor samples from the same patient remain identical in different recurrences. In contrast, Andersson *et al.* (15) found multiple mutations within the same tumor and also loss or addition of mutations during the course of disease, whereas Sakurai *et al.* (34) identified different mutations in different samples from the same patient.

The incidence of exon 11 *KIT* mutations does not appear to be related to the tumor site. Previous studies have reported similar frequencies of exon 11 mutations in the two most common sites, stomach and small bowel, and also in other less common locations, such as esophagus (36) and ano-rectum (26).

In a recent study of 13 small-sized (<1 cm) GISTs, which were incidentally found either at autopsy or laparotomy for unrelated disease, Corless *et al.* (25) found that the majority

(85%) of their cases showed a *KIT* mutation with predilection for exon 11. Our results support their findings, because all three of the incidentally diagnosed GISTs in this study showed an exon 11 mutation, suggesting that the activating *KIT* mutations are not size-dependent and most likely occur early in the GIST development. Moreover, the majority of these small, incidental GISTs also appear morphologically “benign,” with low mitotic count and lack of necrosis, supporting more recent reports (21) that the presence of *KIT* mutations does not correlate with tumor grade. The presence of activating *KIT* mutations in morphologically benign GISTs suggest that these mutations play a fundamental role in early GIST development, but it is possible that other, as-yet-undefined, molecular or cytogenetic transforming mechanisms are implicated in initiating malignant progression (37). Although, initial studies suggested that exon 11 *KIT* mutations are more common in “malignant” than in benign GISTs (11, 22), others have failed to find a significant association between *KIT* mutation status and histological grade (21). The results of the present study also fail to identify any correlation between the presence of exon 11 *KIT* mutation and clinicopathological factors that are typically associated with an aggressive behavior, such as size, mitotic activity, or stage.

In a recent study, Wardelmann *et al.* (27) suggested a relationship between the presence of *KIT* mutation and histological phenotype. Their results showed that all GISTs with *KIT* mutations displayed a spindle cell phenotype, whereas mutations were absent in all seven tumors with an epithelioid component. Although, in keeping with their results, a statistically significant association was found in our study between the presence of *KIT* mutation and histological appearance, 6 of the total 19 epithelioid GIST had *KIT* mutations. On closer analysis, all of the epithelioid GISTs in the present study that carried an exon 11 *KIT* mutation were located in the small bowel. None of our nine gastric epithelioid GISTs showed exon 11 *KIT* mutations, and only one showed a tandem-duplication in exon 9. Furthermore, the majority of cases in the Wardelman *et al.* study were also located in the stomach (six of seven), and only one tumor was located in the small bowel. Moreover, in a recent comparative *KIT* mutational status with cytogenetic abnormalities, Debiec-Rychter *et al.* (38) found that all of their four gastric epithelioid GISTs were wild type but carried the same 14q and 22q losses as any other subtype of GISTs (39–41). Therefore, it seems that the majority of gastric epithelioid GIST are wild type for *KIT* mutations and that another mechanism of *KIT* activation is responsible for their tumorigenesis. From the clinical point of view, despite some reports suggesting a favorable/benign outcome of a subset of these tumors (42), more than one-half of our epithelioid gastric GISTs developed liver metastases, and these patients were either AWD or DOD. Also another interesting link appears to be related to the two pediatric patients in this series, both with wild-type gastric epithelioid GISTs. Additional studies in pediatric GISTs are needed to confirm this unusual pattern.

The presence of *KIT* mutations in GISTs has been related to survival (12). The first large study suggesting that *KIT* exon 11 mutations represent an adverse factor in clinical outcome included a retrospective analysis of 124 patients with primary GIST (11). Their overall *KIT* mutation rate was only 57%. After a median follow-up of 3.3 years, the 5-year disease-specific

survival was 86% in 53 patients without a detectable *KIT* mutation, compared with 49% in 71 patients with mutations ( $P = 0.0001$ ). On multivariate analysis, the presence of a *KIT* mutation was an independent predictor of survival. In more recent reports, extending the mutation analysis to exons 9 and 13 in addition to the JM domain of *KIT* (13, 14, 21), the overall mutation rate was found to be much higher, ranging from 88 to 92%. In the present study, the overall *KIT* mutation rate was 78%. More recently, Singer *et al.* (43) reported in a smaller series of 48 GISTs including a 92% *KIT* mutation rate, that missense exon 11 mutations had a statistically significant higher recurrence-free survival as compared with exon 11 deletion/insertion.

Most *KIT* mutations in sporadic GISTs are somatic, although several families with a germline mutation either in the JM or tyrosine kinase domain have been detected (44). Only one patient in the present study entered into this category. She presented with multiple GISTs in a background of myenteric Auerbach hyperplasia and was confirmed to carry a previously described germline point mutation in exon 11, W557R (45). Her family history included other members with GISTs and also cutaneous pigmentation. Familial GISTs share certain clinicopathological and genetic features that distinguish them from sporadic GISTs. This subset of GISTs are usually multiple, small in size, and occur in a background of diffuse hyperplasia/dysplasia of the Auerbach’s plexus, both close to and remote from the tumor tissue (46). In addition, a significant number of familial GIST patients also have systemic cutaneous hyperpigmentation, particularly around the mouth, on the neck, hands, perineum, and axillae (44, 47). In some cases, abnormalities of mast cells have been reported, such as urticaria pigmentosa as well as mastocytosis (48, 49). All except one (50) familial GISTs reported to date are characterized by a germline mutation in the JM domain (exon 11) of *KIT* gene.

The presence of second malignancies in GIST patients is not very well documented in the literature. Corless *et al.* (25) found that almost one-third of incidentally diagnosed GISTs were secondary to other malignancies, predominantly carcinomas of the gynecological or gastrointestinal tract. We found that 12% of our patients had a second malignant neoplasm in their clinical history, with predilection epithelial malignancies.

In conclusion, the majority of *KIT*-positive GISTs show *KIT* mutations (78% in the present study), located with predilection in the JM region (exon 11). In keeping with previous reports, our results confirm the exon 11 mutations heterogeneity within the classic hot spot, such as deletions and/or point mutations. Gastric epithelioid GIST lacked exon 11 mutations. In addition, we identify a second or ITD hot spot at the 3’ end of exon 11, characterized by ITDs, which define a homogeneous subgroup of indolent gastric GISTs in older females. *KIT* exon 9 mutations seem to define a distinct subset of GISTs, because all of the cases reported to date show a duplication of six nucleotides encoding Ala-Tyr, occur predominantly in the small bowel, and are associated with an unfavorable clinical course.

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