

Fine-Needle Aspiration Biopsy Diagnosis of Gastrointestinal Stromal Tumors Using Morphology, Immunocytochemistry, and Mutational Analysis of *c-kit*

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BACKGROUND. Differentiating gastrointestinal stromal tumors (GISTs) from other intramural mesenchymal tumors of the GI tract on fine-needle aspiration biopsies (FNABs) is difficult. Recent studies have shown that GISTs are immunophenotypically and genetically distinct. GISTs exhibit consistent immunohistochemical expression of CD-117 (KIT) and often express activating mutations of this protooncogene. The aim of the current study was to employ immunocytochemistry and mutational analysis of the *c-kit* gene to aid in the diagnosis of GISTs on FNAB.

METHODS. Five endoscopic ultrasound-guided FNABs of gastrointestinal spindle cell neoplasms performed at the Veterans Affairs Medical Center (VAMC) in Portland, Oregon, from 1998–1999 were reviewed. A panel of immunocytochemical stains was performed on each cellblock including CD-117 (KIT), smooth muscle actin (SMA), desmin, S-100, and CD34. Genomic DNA (gDNA) was extracted, and amplification of exons 9, 11, 13 and 17 of *c-kit* was performed by polymerase chain reaction (PCR) on CD-117 (KIT) and CD34 positive cases. Direct sequencing of amplicons identified the mutations.

RESULTS. Five patients were diagnosed with GISTs based on morphology and immunocytochemical positivity for CD-117 and CD34. PCR analysis of *c-kit* exon 11 revealed three cases with novel-sized PCR bands in addition to the expected wild-type-sized PCR product. Amplicons from these cases contained an in-frame deletion mutation. One of the two cases with wild-type-sized exon 11 amplicons was found to be heterozygous for a point mutation producing an amino acid substitution (W557R). No mutations in exon 9, 11, 13, or 17 of *c-kit* were found in the remaining case.

CONCLUSIONS. Ancillary techniques such as immunocytochemistry and *c-kit* gene mutational analysis may aid in the diagnosis of GISTs on FNABs. *Cancer (Cancer Cytopathol)* 2001;93:269–275. © 2001 American Cancer Society.

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Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumor of the gastrointestinal tract. GISTs are immunophenotypically and genetically distinct from other mesenchymal tumors of the gastrointestinal tract and the consistent immunohistochemical expression of CD-117 (KIT) is perhaps the best defining feature of GISTs.^{1–4} KIT is a 145 kD transmembrane glycoprotein that is the product of the *c-kit* gene. A member of the receptor tyrosine kinase subclass III family, KIT is closely related to the receptors for PDGF, M-CSF and flt3 ligand. KIT is normally expressed by hematopoietic progenitor cells, mast cells and germ cells, and by the pace-

maker cells of the gut — the interstitial cells of Cajal (ICC). In contrast to other gastrointestinal mesenchymal tumors, such as leiomyomas and schwannoma, GISTs are often immunohistochemically positive for CD34, the hematopoietic progenitor cell antigen, and negative for desmin, smooth muscle actin (SMA), and S-100 protein.

The clinical behavior of GISTs is difficult to predict using conventional prognostic factors. Features favoring malignancy include a size > 6 cm, a mitotic index > 5 mitotic figures per 50 high-power fields, site of origin, necrosis, hemorrhage, hypercellularity, and nuclear atypia.^{5,6} Although the patient's age, tumor location, tumor size, and mitotic index have the greatest prognostic significance, these factors cannot accurately predict the outcome of individual patients.⁵

Previous studies have shown that some GISTs have gain-of-function mutations of the juxtamembrane domain of *c-kit*, including deletions or point mutations in exon 11.^{1,7,8} Recently, activating mutations of exon 9 and 13 have been reported in GIST.⁹ Mutations of *c-kit* have not been found in other mesenchymal tumors of the gastrointestinal tract (e.g., leiomyosarcoma). The presence of a mutation in the juxtamembrane domain of *c-kit* has recently been suggested to be an independent prognostic factor in GISTs.^{10,11} Approximately 60% of malignant GISTs have mutations in the exon 11 of *c-kit*; these mutations are rare in benign GISTs. The presence of a mutation in the juxtamembrane domain of *c-kit* may portend a significantly poorer prognosis.¹⁰⁻¹²

The introduction of the endoscopic ultrasound fine-needle aspiration biopsy (EUS-FNAB) has resulted in earlier diagnosis of GISTs and has pushed the diagnosis of GISTs into the realm of cytology. Endoscopic ultrasound in conjunction with fine-needle aspiration can assess some of the conventional prognostic features of GISTs such as tumor size, tumor location, irregular margins, echofeatures, cellularity, nuclear atypia, and necrosis, but an accurate assessment of mitotic activity cannot be determined. Because of the overlapping morphology of spindle-cell lesions, GISTs are cytologically difficult to distinguish from other gastrointestinal mesenchymal neoplasms. Thus, although EUS-FNAB provides some of the critical information to diagnosis patients with GISTs, there are significant limits.

Our aim in the current study was to employ immunocytochemistry and mutational analysis of the *c-kit* gene to aid in the diagnosis of gastrointestinal stromal tumors on FNAB material. Mutational analysis of *c-kit* was performed by polymerase chain reaction (PCR) amplification of genomic DNA (gDNA) from

specimens followed by analysis of amplicon size and direct sequencing.

MATERIALS AND METHODS

Specimens

We identified five patients with spindle cell neoplasms diagnosed as GISTs by EUS-FNAB at the Veterans Affairs Medical Center in Portland, Oregon (PVAMC), from 1998–1999. All of the cases included air-dried Diff-Quik slides (Dade Behring, Inc., Düringen, Switzerland), ethanol-fixed Papanicolaou stained slides, and formalin-fixed paraffin-embedded cell blocks.

EUS-FNAB was performed using a Pentax FG36UX echoendoscope (Pentax Precision Instruments, Orangeburg, NJ) and the Wilson–Cook Echotip 22-gauge needle (Wilson–Cook Medical, Winston-Salem, NC). Informed consent was obtained before each procedure. Under real-time EUS guidance, the needle was inserted through the working channel of the echoendoscope and then advanced through the gastrointestinal mucosa into the center of the lesion. The central stylet was advanced to clear the needle, syringe suction applied, and the sample obtained with a to-and-fro motion of the needle in the tumor under direct EUS observation. Three to six passes were made, and adequacy of the specimen was assessed. There were no complications from any of the procedures.

Immunocytochemistry

The tumors were immunocytochemically analyzed for CD34, CD-117 (KIT), SMA, desmin, and S-100 protein. Immunohistochemistry was performed at the PVAMC for the antibodies at specified dilutions as follows: *c-kit* (1:400, with pretreatment), CD34 (1:10, with pretreatment), desmin (1:300, with pretreatment), SMA (1:400, with pretreatment), muscle-specific actin (1:100, with pretreatment), and S-100 (1:2000, without pretreatment). Pretreatment steps, if indicated, consisted of incubating slides in a Dako Target retrieval solution (Dako, Glostrup, Denmark) within a water-bath steamer for 20 minutes before antibody application.

Sections of fixed tissue were rehydrated and submitted to pretreatment steps if indicated. The slides were set in a Biotek Techmate 1000 (capillary-gap) automated immunostainer (Ventana Medical Systems, Inc., Tucson, AZ). The immunostainer exposed the slides to a blocking solution for 15 minutes (TBS/0.3% BSA/0.05% Tween 20) and then followed with the application of primary antibody for 1-hour incubation at room temperature. After three washing steps with TBS/Tween 20, the second antibody incubation occurred for 25 minutes with a mixture of biotinylated secondary antibodies in dilution buffer: antimouse

IgG at 1:400 (Vector £BA-2000, Vector Laboratories, Burlingame, CA); antimouse IgM at 1:200 (Vector £BA-2020, Vector Laboratories); and antirabbit IgG at 1:400 (Vector £BA-1000, Vector Laboratories). After this incubation, the slides were washed again in a three-step series. A quench solution (80% methanol, 1.0 M sodium azide, and 3% hydrogen peroxide) was applied to the slides, followed by an additional washing step series. The slides were then incubated with an avidin-biotin complex (Vectastain Elite ABC kit, Vector Laboratories) for 30 minutes and then washed. Following the ABC application, the DAB solution (K3466, Dako) was applied for 10 minutes; then the slides were washed in water, counterstained with hematoxylin, dehydrated, and coverslipped.

Dissection of Cells and DNA Extraction

To prepare gDNA from the cell blocks, 20-micron sections were prepared on glass slides and then scrapped into a microcentrifuge tube with a sterile scalpel-blade. In one case (Case 2) where DNA extraction could not be obtained from the cellblock, a representative ethanol-fixed slide was soaked in xylene for 24 hours to remove the coverslip and then the cells were scraped off the slide with a sterile scalpel-blade. gDNA was prepared using the QIAamp DNA Minikit (Qiagen, Chatsworth, CA).

PCR Amplification of Exon 11 of *c-kit*

Amplification of 500 ng of gDNA was performed using the primer pairs listed below.¹¹ The primers are designated using the sequence notation system of Andre et al.¹³ (GenBank U63834, National Institute of Health, Bethesda, MD).

For amplification of exon 9 the following primer pairs were used:

Forward *c-kit* 74056 5'-ATTTATTTTCCTAGAGTAAGC-CAGGG-3'

Reverse *c-kit* 74360 5'-ATCATGACTGATATGGTA-GACAGAGC-3'

For amplification of exon 11 the primers listed below were used (the forward primer was used in combination with one of the two reverse primers):

Forward *c-kit* 75594 5'-CCAGAGTGCTCTAATGACTG-3'

Reverse *c-kit* 75784 5'-ACTCAGCCTGTTTCTGG-GAAACTC-3'

Reverse *c-kit* 75840 5'-CTGTTATGTGTACCCAAA-AAGG-3'

For amplification of exon 13 the following primer pairs were used:

Forward *c-kit* 75961 5'-ATTTTGAAACTGCACAAAT-GGTCC-3'

Reverse *c-kit* 76499 5'-GCAAGAGAGAACAACAG-TCTGGG-3'

For amplification of exon 17 these primer pairs were used as follow:

Forward *c-kit* 81318 5'-TGTATTCACAGAGACTT-GCC-3'

Reverse *c-kit* 81534 5'-GGATTTACATTATGAAAG-TCACAGG-3'

c-kit Mutational Analysis

The products of amplification were screened by gel electrophoresis using a 0.7% agarose plus 0.65% Synergel plus TBE gel (Synergel, Diversified Biotech; Boston, MA). The use of the combination of agarose and Synergel allowed clear distinction of exon 11 amplicons whose size differs by only three base pairs (bp) (data not shown). The amplification products were sequenced in both directions using the ABI Big Dye sequencing *c-kit* and an ABI model 377 sequencer (Applied Biosystems, Foster City, CA). Mutations were verified by sequencing of the amplicons from at least two independent amplifications.

RESULTS

Clinicopathologic Features

Of the five patients diagnosed with spindle cell neoplasm consistent with GIST by EUS-FNAB, two patients were male and three were female with an age range of 59–74 years, mean 62.5 years. All of the tumors were located in the submucosa of the stomach. Patients 2, 3, and 4 did not have surgical resection of their tumors and have remained free of disease progression for 3–16 months. Patients 1 and 5 underwent surgical resection within two months of the EUS-FNAB diagnosis of GIST. Demographic data, clinical, histologic, and *c-kit* mutation results of GISTs analysed in this study are summarized in Table 1.

Cytologic Features and Immunocytochemical Analysis

The GISTs studied had cytologic features of a cellular aspirate consisting of fascicles and sheets of spindle and epithelioid cells and numerous single cells with naked nuclei. The stroma of the cohesive sheets was loosely fibrillary and stained bright magenta on Diff-Quick. High-power examination demonstrated light eosinophilic cytoplasm with indistinct borders surrounding a tapered to oval nucleus. The nuclear to cytoplasmic ratio was low. Nuclear pleomorphism was identified in all cases. The nucleus characteristically had smooth nuclear membranes, diffuse fine chromatin, and inconspicuous nucleoli. Rare intranuclear cytoplasmic inclusions were identified. Despite the marked cellularity, the backgrounds in all of the smears were relatively clean and free of excessive

TABLE 1
Clinical Data and *c-kit* Mutations of GISTs

Case	Age	Gender	Site	Cell type	EUS description	Clinical follow-up	Exon 11 mutant band	Sequencing of <i>c-kit</i> exon 11t	Sequencing of <i>c-kit</i> exons 9 and 13
1	70	F	Stomach	Spindle	> 3 cm hyperechoic heterogenous, cystic mass	Resected. 0 mitosis/50 HPF, no necrosis or nuclear pleomorphism	+	Deletion	Exon 9 WT Exon 13 WT
2	59	M	Stomach	Spindle	< 2 cm hypoechoic homogenous mass	Not resected. Repeat EUS in 1 year; NEP @ 16 months	-	Point mutation	Exon 9 WT Exon 13 WT
3	61	F	Stomach	Epithelioid	< 3 cm hypoechoic, homogenous mass	Not resected. Repeat EUS in 1 year; NEP @ 3 months	-	Wild type	Exon 9 WT Exon 13 WT Exon 17 WT
4	62	F	Stomach	Epithelioid	1.4 cm hypoechoic heterogenous mass	Not resected. Repeat EUS in 1 year; NEP @ 9 months	+	Deletion	ND
5	74	M	Stomach	Epithelioid	4.1 cm ulcerated, hypoechoic heterogenous mass	Resected. 31 mitosis/50 HPF microscopic necrosis and nuclear pleomorphism	+	Deletion	Exon 9 WT

HPF: high-power field; NEP: no evidence of progression; ND: not done.

blood and necrotic debris. Mitotic figures were observed exclusively in Case 5. Epithelioid cytologic features with large round cells with distinct cell borders were seen in three cases. All cases were immunohistochemically positive for CD-117 and CD34 and negative for SMA, muscle-specific actin, desmin, and S-100 (Fig. 1).

***c-kit* Mutational Analysis**

Analysis of the PCR amplified products of *c-kit* exon 11 revealed a novel smaller band in addition to the wild-type band in Cases 1, 4, and 5 (Table 1). Sequence analysis of Cases 1 and 5 revealed in-frame deletion of 3 bp resulting in deletion of a single codon. Sequence analysis of Case 4 revealed an in-frame deletion of 6 bp resulting in deletion of two codons. Amplification of gDNA from Cases 2 and 3 produced only a single wild-type band. The amplicon from Case 2 was heterozygous for a point mutation resulting in a missense mutation (W557R). The amplicon from Case 3 was sequenced and found to be homozygous wild type. The predicted amino-acid sequences of the identified mutant *c-kit* alleles are shown in Figure 2. PCR amplification of gDNA from certain cases was also performed using primers flanking *c-kit* exons 9, 13, and 17. A single wild-type-sized amplicon was produced from each reaction. The sequence of all exon 9, 13, and 17 amplicons was homozygous wild-type with respect to *c-kit* (Table 1).

DISCUSSION

Historically, mesenchymal tumors specific to the gastrointestinal tract have been classified as smooth muscle or stromal tumors.^{14,15} GIST has been used either as an umbrella term to include all gastrointestinal mesenchymal tumors or to refer to mesenchymal tumors that did not have clear-cut neural or smooth-muscle origin. Recently, these histologically ambiguous tumors have been shown to have a unique immunohistochemical profile, which includes consistent expression of CD-117 (KIT). In fact, expression of KIT has become the most specific diagnostic criteria for GISTs.¹⁻⁴

Most of the published cytologic studies of smooth muscle or stromal tumors of the gastrointestinal tract have combined GISTs into a group of neoplasms encompassing leiomyoma, schwannoma, leiomyosarcoma, and an epithelioid variant of leiomyosarcoma.¹⁶⁻¹⁸ In a study of four cases, Dodd et al.¹⁶ summarized the cytologic findings of GISTs as a cellular spindle cell neoplasm that consisted of numerous small bland spindle cells distributed individually and as cohesive fragments. In their series, cytologic evidence of malignancy could not be identified; however, subsequent histologic evaluation showed a range of findings from benign to frankly sarcomatous stromal tumors. Tao and Davidson¹⁷ reviewed 16 FNABs of smooth tumors from the gastrointestinal tract and found that cytologic features that favored malignancy included loose groupings, single cells, multinucleated cells, stripped

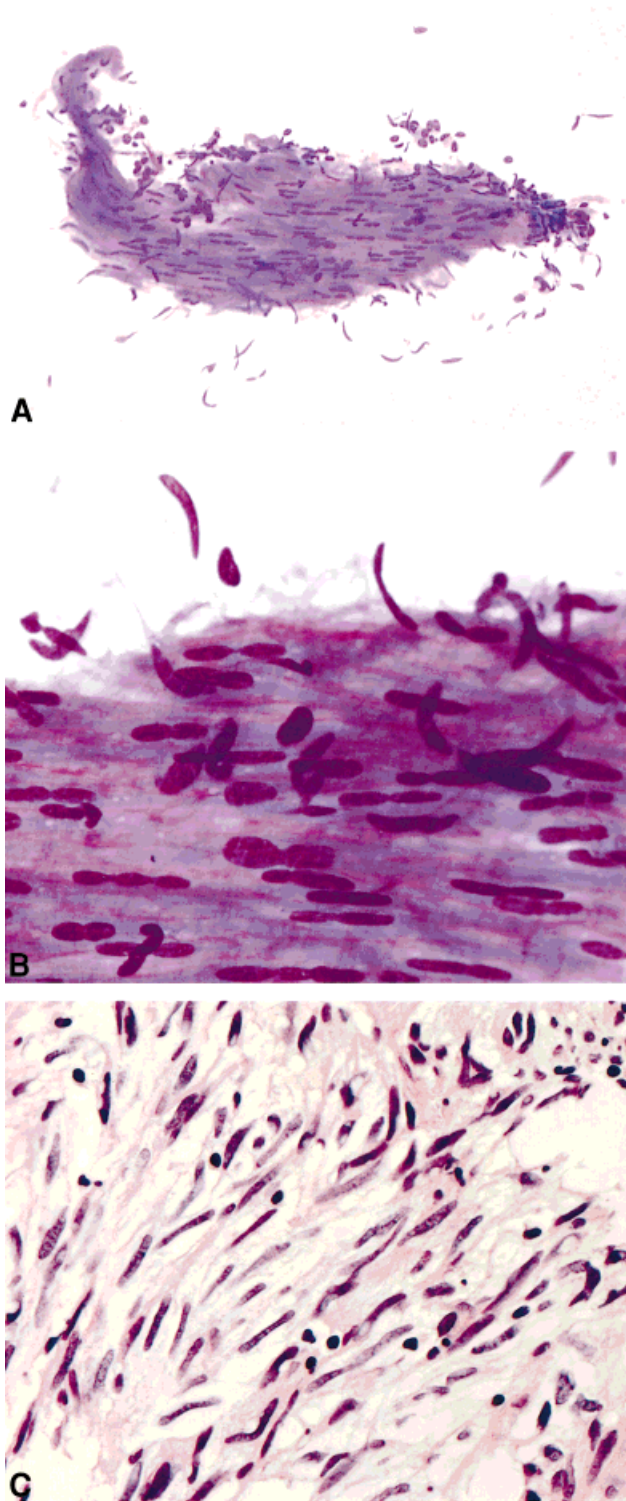


FIGURE 1. This is a GIST, spindle cell type, arising in a 70-year-old man with a 3-centimeter stomach wall mass. A) A loosely cohesive group of spindle cells is shown in a fascicular arrangement (Diff-Quick, $\times 100$); B) Individual cells with ovoid to elongated nuclei and fibrillary cytoplasmic processes are evident (Diff-Quick, $\times 400$); C) This cell block demonstrates loosely packed spindle cells (hematoxylin and eosin, $\times 400$).

nuclei, and blood vessels. A case study by King et al.¹⁸ addresses the difficulty of confirming the diagnosis of GIST on cytology alone and suggested analysis by transmission electron microscopy (TEM). Those authors of the current study found that, ultrastructurally, the cells contained long cytoplasmic processes with multiple shorter, finger-like projections arising from the terminal portion of the processes. The lack of other cellular differentiation was also a consistent TEM finding.

Li et al.^{19,20} recently published two studies on GISTs and found that there was a lack of correspondence between the cytologic and histologic features of malignancy. In these studies, the classic cytologic features of malignancy, such as mitosis, nuclear pleomorphism, increased nuclear to cytoplasmic (N/C) ratio and irregular nuclear membranes were variably present in malignant GISTs. In our study, all of the smears were cellular with bland spindle cells arranged in whorls and bundles. Numerous single cells and cells with stripped nuclei were identified in the background. Although mitotic activity was identified in one case that was subsequently diagnosed as a malignant GIST on resection, it would have been impossible to definitively diagnose this tumor as malignant based solely on cytology. Thus, the specific diagnosis of GISTs and the distinction between malignancy and benignity by cytology alone may be difficult given that features of malignancy are inconsistently present and that there is significant morphologic overlap with other spindle cell neoplasms of the gastrointestinal tract.

In 1998, Hirota et al.¹ analyzed a series of 49 GISTs and noted that most GISTs express the protooncogene, KIT, which codes for a tyrosine kinase receptor involved in cell development. A number of cells, including hematopoietic stem cells, melanocytes, erythrocytes, and the interstitial cells of Cajal (ICC), which are pacemaker cells located in the myenteric plexus, express KIT. Those authors and others have theorized that the origin of the GISTs may be related to the ICC, as both express Kit and CD34 and because both are found within the smooth muscle wall of the gastrointestinal tract.^{2,21} GISTs can be differentiated from other spindle cell neoplasms of the gastrointestinal tract by employing a battery of immunohistochemical stains. CD-117 (KIT) positivity is seen in benign, malignant, and all histologic variants of GISTs. CD34, a marker found in early hematopoietic stem cells and ICC, is identified in 70–80% of GISTs.^{21,22} Focal SMA positivity is described in 20–40% of cases, whereas GISTs are characteristically negative for S-100 and desmin.

Several investigators have identified a gain-of-

Wild-Type	KPMYEVQWKV VEEINGNNYV YIDPTQLPYD HKW
Case 1	KPMYEVQWKV VEEINGNNYV YIDPTQLPY■ HKW
Case 2	KPMYEVQ R KV VEEINGNNYV YIDPTQLPYD HKW
Case 4	KP■■EVQWKV VEEINGNNYV YIDPTQLPYD HKW
Case 5	KPMYEVQWK■ VEEINGNNYV YIDPTQLPYD HKW

FIGURE 2. A predicted amino acid sequence of mutant *c-kit* alleles is shown. The wild-type KIT amino acid sequence (residues 550–582) is shown in the top line. A solid box indicates the location of the deleted residues in Cases 1, 4, and 5. An open box containing the mutated residue indicates the location of the missense mutation in Case 2.

function mutation in the juxtamembrane domain (exon 11) of *c-kit* in GISTs. These gain-of-function mutations allow for constitutive activation of the tyrosine kinase receptor without binding of the cognate ligand, stem cell factor. These mutations are identified in approximately 60% of GISTs and include deletions, insertions, and point mutations in exon 11. A number of clinical series have reported that these mutations are found predominately in histologically and clinically malignant GISTs.^{10,11,12} In one series of over 100 resected GISTs, the presence of an exon 11 mutation correlated with the likelihood of disease recurrence and predicted for poorer survival (86% vs. 49% 5-year survival).¹² In another series using fine-needle aspiration material, 8 of 16 cases demonstrated in-frame deletions of exon 11, all of which were subsequently found to be histologically malignant or borderline.²⁰ The spectrum of *c-kit* mutations in GIST has been expanded recently with the finding of gain-of-function mutations of exon 9 and 13 in a minority of GIST tumors.⁹

In our series, we identified *c-kit* exon 11 mutations in 4 of 5 tumors examined (Cases 1, 2, 4, and 5; see Table 1, Figure 1). Each of these mutations has been previously reported to activate mutation in one or more cases of GIST and to disrupt the conserved juxtamembrane domain known to be important in control of KIT kinase activation.^{8,11,23,24} Thus, 80% of the cases in our series have a mutation of exon 11. Although this frequency is higher than many earlier series, it is similar to several recent reports of exon 11 mutations in 57–71% of GISTs.^{1,7,9–11}

The results of our mutational analyses differ from previous series in several important ways. First, our series consisted entirely of patients with early-stage tumors (maximal size was 4.1 cm, two cases had tumors < 2 cm in diameter), and only one of the tumors met pathologic criteria for being malignant (Case 5). Nonetheless, we found evidence for *c-kit* mutations in four of the five cases. These observations suggest that acquisition of an activating mutation of *c-kit* may be

one of the earliest oncogenic events in the development of GISTs. Because the kinase activity of KIT is required for the embryologic development of ICC, activation of KIT may be the key element that drives the proliferation of ICC cells and leads to GIST formation.²⁵ Thus, gain-of-function KIT mutations may serve a gatekeeper role in the development of GISTs analogous to the role of *bcr-abl* in chronic myelogenous leukemia and the role of adenomatous polyposis coli (*APC*) gene in colorectal cancer.^{27–31} Second, most previous series were retrospective series with long clinical follow-up allowing detection of malignant behavior (metastases or local recurrence). In these series, the presence of an exon 11 *c-kit* mutation was strongly correlated with a malignant rather than benign clinical course. In contrast, our study is somewhat prospective in that only two of the five patients have undergone primary resection. In two cases with *c-kit* mutations (Cases 2 and 4), the patients have not undergone surgical resection and there has been a 9–16 month follow-up period without evidence of progression. Although follow-up is obviously limited, our data suggest that the presence of an exon 11 *c-kit* gain-of-function mutation in a GIST does not always predict an immediate malignant behavior. Further studies are needed to define other prognostic markers (such as loss of heterozygosity at 1p36) that will allow clinical prediction of the malignant potential of GISTs.³²

Differentiating GISTs from other intramural mesenchymal tumors on FNAB is difficult because of the overlapping morphology of spindle cell lesions. However, the use of ancillary techniques such as immunohistochemistry and *c-kit* gene mutational analysis can aid in the diagnosis of GISTs on fine-needle aspiration material. The use of FNAB to diagnose primary or metastatic GISTs will be particularly important as better treatments are developed. Conventional medical treatment of unresectable metastatic or malignant GISTs produces response rates of < 10%.³³ However, a novel tyrosine kinase inhibitor (STI571) has been re-

cently shown to inhibit the kinase activity of both wild-type and exon 11 mutant *c-kit*.³⁴ This compound is currently undergoing testing for the treatment of unresectable recurrent or metastatic GIST in multicenter Phase II and III trials.

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