

# Short Communication

## KIT Extracellular and Kinase Domain Mutations in Gastrointestinal Stromal Tumors

Marcia L. Lux,\* Brian P. Rubin,\* Tara L. Biase,\*  
Chang-Jie Chen,\* Timothy Maclure,\*  
George Demetri,† Sheng Xiao,\* Samuel Singer,†‡§  
Christopher D.M. Fletcher,\*† and  
Jonathan A. Fletcher\*†

From the Departments of Pathology\* and Surgery,‡ Brigham and Women's Hospital; the Department of Pediatric Oncology,† Dana-Farber Cancer Institute and Children's Hospital; and the Departments of Pathology,\* Surgery,§ and Pediatrics,† Harvard Medical School, Boston, Massachusetts

**Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal neoplasms arising in the gastrointestinal tract. GISTs express the KIT receptor tyrosine kinase, and many cases have activating mutations in the KIT juxtamembrane region. We now report an analysis of *KIT* cDNA and genomic sequences in eight GISTs that lack juxtamembrane region mutations. Six cases contained heterozygous exon 9 mutations in which six nucleotides, encoding Ala-Tyr, were duplicated. The other two cases contained homozygous exon 13 missense mutations, resulting in substitution of Glu for Lys<sup>642</sup>, that were associated with constitutive KIT tyrosine phosphorylation. Sequence analysis of DNAs from nonneoplastic companion tissues revealed that both the exon 9 and exon 13 mutations were somatic. These are the first descriptions, in any tumor, of mutations in *KIT* exons encoding the C-terminal end of the extracellular domain and the first part of the split kinase domain. These findings indicate that KIT may be activated by mutations in at least three domains—extracellular, juxtamembrane, and kinase—in GISTs. (*Am J Pathol* 2000, 156:791–795)**

The KIT protein is a type III receptor tyrosine kinase (RTK).<sup>1</sup> Interactions between KIT and its ligand, SCF, are important in the development and maintenance of hematopoietic cells, melanocytes, germ cells, and the interstitial cells of Cajal.<sup>2–5</sup> Although expression is restricted to a relatively small number of cell types, KIT nonetheless plays a major oncogenic role. Activating *KIT* mutations

have been identified in human mast cell tumors,<sup>6–9</sup> myelofibrosis,<sup>10</sup> chronic myelogenous leukemia,<sup>10</sup> germ cell tumors,<sup>11</sup> and gastrointestinal stromal tumors (GISTs).<sup>12,13</sup> To date, KIT oncogenic mutations have been identified in exon 2 (myelofibrosis and chronic myelogenous leukemia), the exon 11 juxtamembrane domain (mast cell tumors and GISTs), and the exon 17 phosphotransferase domain (mast cell tumors and germ cell tumors). In the present study we evaluated eight GISTs that expressed KIT (as determined immunohistochemically) but lacked exon 11 mutations. We reasoned that some of these cases might have novel *KIT* mutations and that detection of such mutations might lead to a fuller understanding of KIT activating mechanisms.

### Materials and Methods

#### Patient Samples

The study group consisted of eight primary intraabdominal neoplasms (one gastric, five small bowel, one mesenteric, one abdominal wall) biopsied at Brigham and Women's Hospital and the Dana-Farber Cancer Institute. All cases were evaluated histologically by two of the authors (BPR and CDMF) and were diffusely and strongly immunoreactive with KIT antibody (A-4052, rabbit polyclonal antiserum, 1:100; DAKO Corporation, Carpinteria, CA), according to analysis by an avidin-biotin-peroxidase complex method after microwave antigen retrieval. All cases lacked mutations of *KIT* exons 10 and 11, a region containing the entire coding sequence for the KIT juxtamembrane domain, as determined by sequencing of genomic DNAs isolated from frozen tumor materials.

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Drs. Lux and Rubin contributed equally to this work.

Address reprint requests to Dr. Brian P. Rubin or Dr. Jonathan A. Fletcher, Department of Pathology, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115. E-mail: bprubin@bics.bwh.harvard.edu or jfletcher@rics.bwh.harvard.edu.

### RT-PCR Analysis of *KIT* Transcripts

RNAs were isolated from frozen tissue, using TRIzol (GIBCO BRL Life Technologies, Gaithersburg, MD), and cDNAs were synthesized using AMV reverse transcriptase and random 9-mer primers (TaKaRa Shuzo Co., Seoul, Korea), according to the manufacturer's protocols. Polymerase chain reaction (PCR) amplifications were performed using Taq DNA polymerase and oligonucleotide primer sequences as described by Furitsu et al.<sup>6</sup> DNAs were amplified in 20- $\mu$ l PCR reactions of 0.5 minute at 94°C, 0.5 minute at 60°C, and 1 minute at 72°C for 35 cycles. Amplified products were purified with the QIAquick gel extraction kit (Qiagen, Valencia, CA) and directly sequenced in the forward and reverse directions with ABI BigDye terminators (Applied Biosystems, Foster City, CA). Sequences were analyzed on an ABI Prism 377 sequencer (Applied Biosystems), and alignments and mutation scanning were performed using Sequence Navigator (Applied Biosystems) and BLAST (National Center for Biotechnology Information) software.

### Genomic DNA Sequencing

DNAs were isolated from frozen GIST specimens by the use of NaOH boiling preps. DNAs were isolated from paraffin sections of nonneoplastic companion tissues (evaluated to exclude the possibility of constitutional polymorphisms), using standard proteinase K digestion methods. Intronic primers were chosen using the Whitehead Genome Center Primer3 software, and all intronic primers were numbered according to *KIT* genomic sequence Genbank number U63834. The PCR cycling conditions were identical to those in the cDNA amplifications.

Exon 9: F/KIT/74056, 5'-ATTTATTTTCCTAGAG  
TAAGCCAGGG-3'  
R/KIT/74360, 5'-ATCATGACTGATATG  
GTAGACAGAGC-3'  
Exons 12-13: F/KIT/75961, 5'-ATTTTGAACTGCAC  
AAATGGTCCTT-3'  
R/KIT/76499, 5'-GCAAGAGAGAACAA  
CAGTCTGGGTAA-3'

The PCR products were purified, as described above, and cycle sequencing was performed using the following intronic primer pairs:

Exon 9: Same as for PCR  
Exons 12-13: F/KIT/76032, 5'-CACCATCACCCTT  
ACTTGTGTCT-3'  
R/KIT/76403, 5'-GACAGACAATAAAAG  
GCAGCTTGGAC-3'

### Fluorescence in Situ Hybridization

Total yeast DNA was isolated from CEPH yeast artificial chromosome clone, 840\_E\_11, containing the human *KIT* locus at chromosome band 4q12. Two hundred nanograms of 840\_E\_11 DNA was biotin labeled by random-octamer priming (BioPrime Kit; Gibco, Rockville, MD), and 500 ng of the labeled YAC was cohybridized with a

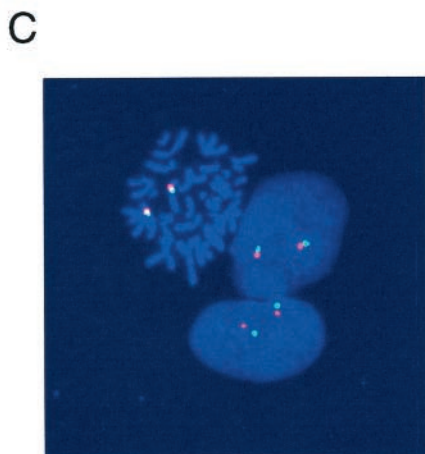
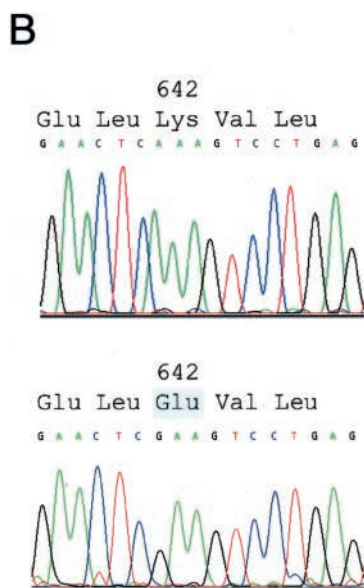
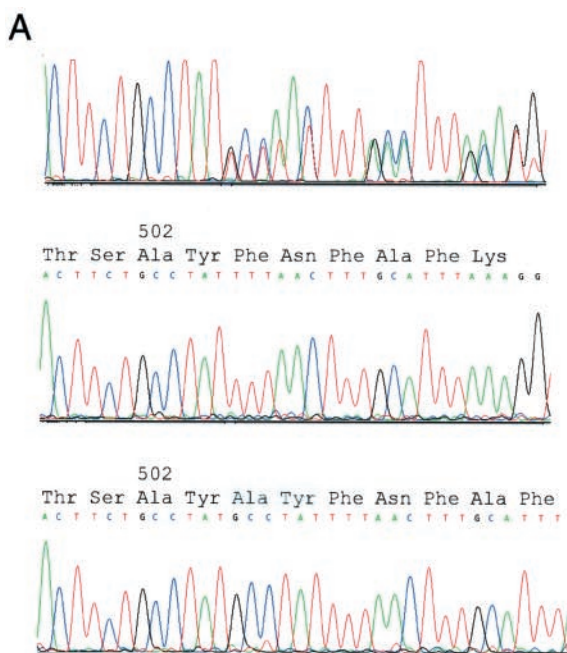
digoxigenin-labeled chromosome 4 pericentromeric probe (*D4Z1*) against cytogenetic preparations of GIST cells. YAC and centromere probe detection was carried out with streptavidin-FITC (Zymed Laboratories, South San Francisco, CA) and rhodamine anti-digoxigenin (Zymed Laboratories), respectively.

### Evaluation of *KIT* Tyrosine Phosphorylation

GIST and malignant peripheral nerve sheath tumor (MPNST) cells were lysed in ice-cold 1% NP-40, 50 mmol/L Tris (pH 8.0), 100 mmol/L sodium fluoride, 30 mmol/L sodium pyrophosphate, 2 mmol/L sodium molybdate, 5 mmol/L EDTA, 2 mmol/L sodium vanadate, 5  $\mu$ g/ml aprotinin, 5  $\mu$ g/ml leupeptin, and 50  $\mu$ g/ml phenylmethylsulfonyl fluoride (lysis buffer). Lysates were incubated with anti-*KIT* (C-19; Santa Cruz) for 2 hours, followed by 20  $\mu$ l of protein A-Sepharose (Zymed Laboratories) for 1 hour at 4°C. Immunoprecipitates were washed three times in lysis buffer, then eluted at 100°C into 40  $\mu$ l of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and resolved by SDS-PAGE under reducing conditions (4-12% gradient gels). Immunoblotting was accomplished by electrophoretic transfer to polyvinyl pyrrolidone membranes (Millipore), blocking in phosphate-buffered saline containing 0.2% Tween-20 and 5% dry milk for 1 hour, then sequential incubation with murine anti-phosphotyrosine (PY99; Santa Cruz) and horseradish peroxidase anti-mouse Ig (Amersham, Piscataway, NJ). Detection was made by chemoluminescence (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce, Rockford, IL).

### Results and Discussion

Initial mutation screening was carried out by sequence analysis of the entire *KIT* coding sequence, and potential mutations were then confirmed by genomic sequencing. Each of the eight GISTs contained a single mutation. Six cases had heterozygous exon 9 mutations (1530ins6) in which six nucleotides were duplicated (Figure 1A). In one of these cases, the 1530ins6 mutation was demonstrated both in the primary tumor and in a subsequently biopsied metastasis. cDNA and genomic sequencing indicated a 50:50 ratio, for wild-type:mutant transcripts and alleles, respectively, in the 1530ins6 mutation cases (Figure 1A). These findings suggest that transcriptional regulation of the mutant 1530ins6 allele is unperturbed, compared to that of the nonmutant allele. In contrast, the remaining two GISTs contained homozygous exon 13 missense mutations, 1945A>G. Wildtype *KIT* genomic and cDNA sequences were undetectable in both of these cases (Figure 1B), and loss of *KIT* allelic heterozygosity was evidenced by loss of constitutional polymorphisms in exon 10, intron 16, and intron 17 (case 7) and in exon 10 and intron 17 (case 8). Dual-color fluorescence *in situ* hybridization, using a *KIT*-containing YAC clone, showed no evidence of *KIT* deletion, or other rearrangement, in either copy of chromosome 4 from the GISTs with exon 13 mutations (Figure 1C). Together, these data demonstrate



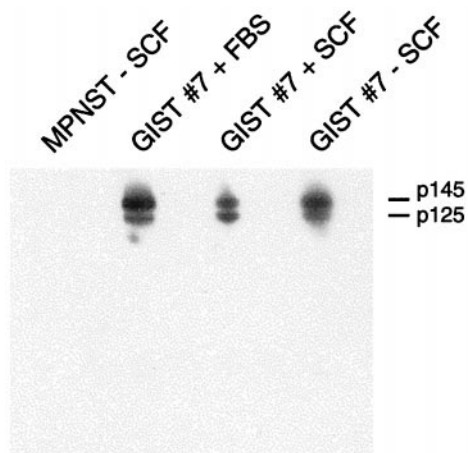
loss of the wild-type *KIT* allele and are consistent with duplication of the 1945A>G mutant allele.

The exon 9 1530ins6 mutations are predicted to encode *KIT* proteins with duplication of amino acid residues Ala<sup>502</sup> and Tyr<sup>503</sup>. The exon 13 1945A>G mutations result in substitution of a Glu at Lys<sup>642</sup>. To confirm that the exon 9 and 13 mutations were acquired, we sequenced nonneoplastic cell genomic DNAs from the same patients. The companion nonneoplastic specimens contained only wild-type *KIT* sequences, indicating that the mutations were somatic—and presumably tumor-restricted—rather than being germline mutations or polymorphisms (Figure 1B).

Most GISTs are characterized by diffuse *KIT* protein expression, and many GISTs contain oncogenic *KIT* mutations.<sup>12</sup> To date, GIST *KIT* mutations have been reported only in the exon 11 juxtamembrane domain.<sup>12–17</sup> Oncogenic *KIT* exon 11 mutations are associated with constitutive ligand-independent receptor dimerization and activation of the kinase domain.<sup>9,12</sup> Although *KIT* exon 11 juxtamembrane domain mutations are found in most GISTs, a subset of cases lack such mutations. In our experience (B. P. Rubin and J. A. Fletcher, unpublished data) 32 of 45 GISTs expressed *KIT* (as determined immunohistochemically) and contained exon 11 mutations. The remaining 13 cases expressed *KIT* but lacked exon 11 mutations. We now report that eight of these 13 GISTs contain somatic mutations in *KIT* exon 9 (extracellular domain) or exon 13 (first part of the split tyrosine kinase domain). Clinicopathological correlations, for the entire series, will be reported separately.

Although *KIT* exon 9 mutations have not been described previously, it is likely that the 1530ins6 mutations activate the receptor via ligand-independent oligomerization. Extracellular region mutations have been characterized in several other receptor tyrosine kinase genes, including *FGFR2*, *FGFR3*, and *RET*, and in all cases these mutations are associated with constitutive receptor oligomerization.<sup>18–21</sup> Although the mechanistic implications of the novel exon 13 (1945A>G) mutations are unclear, it is worth noting that Lys<sup>642</sup> is conserved in all members of the type III RTK family, highlighting its likely functional importance. *KIT* is constitutively tyrosine-phosphorylated, in a ligand-independent manner, in a cell line established from one of our exon 13 mutant (1945A>G) GISTs (Figure 2). Therefore, the exon 13 mutation is almost certainly activating. The Lys<sup>642</sup>→Glu (K642E) substitution imparts a negative charge and would likely alter the three-dimen-

**Figure 1.** Genomic evaluations of *KIT* by sequencing (**A** and **B**) and fluorescence *in situ* hybridization (**C**). **A:** Sequence analysis of total genomic DNA demonstrates a heterozygous exon 9 6-bp insertion/duplication in a GIST (**top**). Sequence analysis of cloned PCR products, from the same GIST as shown at top, demonstrates the wild-type (**middle**) and mutant (**bottom**) alleles. **B:** Sequence analysis of total genomic DNA demonstrates a homozygous exon 13 A>G missense mutation. The GIST sequence is at bottom, and the wild-type sequence, obtained from adjacent nonneoplastic tissue, is at top. **C:** Fluorescence *in situ* hybridization demonstrates nondeleted *KIT*, in the same GIST as in **B**, consistent with loss of the chromosome 4 homolog containing the wild-type *KIT* allele and duplication of the chromosome 4 homolog containing the mutant *KIT* allele. FITC signals (green) identify the *KIT* loci at chromosome band 4q12, and rhodamine signals (red) identify the chromosome 4 pericentromeric region.



**Figure 2.** Demonstration of high-level, ligand-independent, KIT tyrosine phosphorylation in a GIST cell line, established from case 7, expressing the KIT K642E oncoprotein. Immunoprecipitations and Western blotting were with KIT and phosphotyrosine antibodies, respectively. K642E mutant KIT is tyrosine phosphorylated in GIST cells cultured in 15% fetal bovine serum (**second lane**), serum-free media with stem cell factor 100 ng/ml (**third lane**), and serum-free media without stem cell factor (**fourth lane**). Low-level KIT activation, in a malignant peripheral nerve sheath tumor cell line (ST88-14),<sup>25,26</sup> is not detected on this 10-second film exposure (**first lane**).

sional structure of the mutant protein. This potential conformational change might either encourage receptor oligomerization or, alternatively, might be associated with constitutive kinase activation in the absence of receptor oligomerization.<sup>22</sup> It is intriguing that wild-type *KIT* sequences were undetectable in either of the two GISTs with exon 13 mutations. This finding is notable, because heterodimerization—i.e., interaction between mutant and nonmutant receptors—is a potential activating mechanism for receptor tyrosine kinase oncoproteins. The homozygous nature of the K642E mutations demonstrates that heterodimerization is not requisite, *in vivo*, for oncogenic activation. It is conceivable that heterodimerization blunts the activating impact of the K642E mutation, thereby providing selective advantage to cells in which the wild-type allele has been deleted. Alternatively, this mutation might be intrinsically less activating than the generally heterozygous mutations found in exons 9 and 11. GIST precursors might require two copies of the mutant allele (loss of the wild-type allele followed by duplication of the mutant) for neoplastic transformation.

In summary, our finding of novel *KIT* exon 9 and 13 mutational hotspots underscores the central role of KIT oncogenic activation in GIST pathogenesis. Functional characterization of the *KIT* exon 9 and 13 mutations should provide new insights into the varied mechanisms of KIT-mediated oncogenesis in GISTs and other tumors. KIT signal transduction pathways regulate proliferation, differentiation, migration, and survival of various cell types, including hematopoietic stem cells, mast cells, melanocytes, germ cells, and the interstitial cells of Cajal.<sup>2-4,6,23,24</sup> Therefore anti-KIT oncological therapies will likely be toxic, particularly to the bone marrow compartment. Future elucidation of KIT oncogenic mechanisms might enable development of highly specific therapies that target particular KIT mutational

pathways and minimize toxicity to KIT-dependent non-neoplastic cell lineages.

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