KIT Gene Deletions at the Intron 10–Exon 11 Boundary in GI Stromal Tumors

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Most gastrointestinal stromal tumors (GISTs) harbor oncogenic mutations in the *KIT* gene, and the majority of these mutations affect the juxtamembrane domain of the kinase encoded by exon 11. Screening GISTs for *KIT* gene mutations is important for translational research studies and for providing prognostic information on the likelihood of tumor response to treatment with the kinase inhibitor imatinib mesylate (Gleevec). In a series of GISTs analyzed in our laboratory by a combination of denaturing HPLC and direct DNA sequencing, we identified 19 cases with *KIT* exon 11 deletions that included from 1 to 14 bp of intron 10 sequence and resulted in loss of the normal splice acceptor site at the beginning of exon 11. Predicted use of the next potential splice-acceptor site was confirmed by cDNA sequencing in 4 cases. Thus, the resulting mutant isoform, deletion KPMYEVQWK 550–558, was the same in all 19 cases. Only two other examples of deletions across the intron 10–exon 11 boundary have been reported, yet among 722 GISTs analyzed in our laboratories these deletions were not uncommon, accounting for 3.9% of exon 11 mutations and 2.6% of all tumors. Loss of *KIT* intron 10 sequences may be under-recognized if the forward primer is too close to exon 11, or if cases are examined exclusively at the cDNA level. Laboratories that offer clinical screening for *KIT* mutations in GI stromal tumors should be aware of this class of mutations. (*J Mol Diagn* 2004, 6:366–370)

Oncogenic activation of the receptor tyrosine kinase KIT plays a central role in the development of most gastrointestinal stromal tumors (GISTs). KIT expression is detectable by immunohistochemistry in approximately 95% of GISTs,^{1,2} and phosphorylation of the kinase is evident by immunoblotting in the great majority of tumors.^{3,4} A vari-

ety of mutations have been identified in *KIT* exons 9, 11, 13, and 17, including deletions, insertions, single nucleotide substitutions, and combinations thereof, that are invariably in-frame.^{2,5–11} The resulting mutant isoforms have constitutive kinase activity *in vitro* and support the development of tumors in nude mice.^{2,3} Several kindreds with germline *KIT* gene mutations have been identified in which affected individuals are at high risk for the development of multiple GISTs, providing additional evidence of an oncogenic role for KIT in these tumors.^{12–17} Interestingly, a minority of GISTs (5 to 7%) appear to develop through a parallel pathway involving activating mutation of the KIT-homologous receptor tyrosine kinase PDGFRA.^{18,19}

The importance of KIT and PDGFRA in the biology of GISTs is underscored by the clinical response of many of these tumors to treatment with imatinib mesylate (Gleevec), an inhibitor that has activity against both of these kinases.^{20–22} Based on correlative studies of a recent phase II trial of imatinib for advanced GIST, there is a relationship between kinase mutation status and response to therapy.²³ Patients whose tumor harbored a *KIT* exon 11 mutation had a higher response rate and better overall survival than those with *KIT* exon 9-mutant tumor and those with tumor lacking *KIT* or *PDGFRA* mutation. Similar observations have emerged from phase I and II trials conducted in Europe.²⁴ Thus, mutation screening of GISTs is not only of research interest, but has prognostic value in evaluating tumors for which imatinib therapy may be considered. In addition, identification of *KIT* and *PDGFRA* mutations can be useful in confirming the diagnosis of GISTs that have unusual immunophenotypic features (eg, KIT-negative tumors).^{25,26}

A number of methods have been applied in assessing GISTs for kinase gene mutations, including direct DNA sequencing, single-strand conformational polymorphism (SSCP) detection, and length analysis of PCR products (LAPP).^{5,6,8,11} We have found that a combination of denaturing HPLC (DHPLC) and DNA sequencing is highly sensitive for both deletion/insertion type mutations and point mutations.^{18,23,27} Among 722 GISTs analyzed in our laboratories by this approach, we identified 19 examples of unusual *KIT* exon 11 deletions that are the subject of this report.

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Materials and Methods

GI Stromal Tumors

Paraffin-embedded samples of formalin-fixed GISTs were obtained from the archives of the Departments of Pathology at OHSU Hospital, the Portland VA Medical Center, Kaiser Northwest Laboratories, and the Brigham & Women's Hospital. In accordance with Institutional Review Board (IRB) requirements at all four institutions, each sample was assigned a unique number and all information was stored in a password-protected database. In addition, samples were included from recent clinical trials in which patients were consented for research use of their tumor tissue. A total of 722 GISTs from these various sources have been analyzed in our laboratories for *KIT* gene mutations. Data from subsets of these cases have been previously reported.^{18,23,27}

Preparation of Genomic DNA

Genomic DNA was extracted as previously described. Briefly, a hematoxylin and eosin (H&E)-stained section of the embedded tumor was reviewed by standard light microscopy to determine which areas were suitable for analysis (>80% tumor cells). These areas were then removed from the paraffin block or scraped from matched, unstained slides using a sterile scalpel blade. Following deparaffinization in xylenes and graded ethanols, DNA was extracted from the rehydrated tissue using the QIAamp mini kit (Qiagen, Valencia, CA).

PCR Amplification and Analysis of *KIT* Exon 11

Amplification of *KIT* gene exon 11 was performed using our previously published protocol.²⁷ The forward primer used was CCAGAGTGCTCTAATGACTG, and the reverse primer used was ACCCAAAAAGGTGACATGGA. Amplicons were screened both for length alterations (deletions and/or insertions) and single nucleotide polymorphisms by DHPLC (WAVE system, Transgenomic, Inc., Omaha, NE), as detailed in a previous report.²⁷ Specimens with aberrant HPLC profiles were sequenced bidirectionally using the ABI Prism Big Dye terminator kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI 310 sequencer (Applied Biosystems).

Preparation and Analysis of cDNA

Formalin-fixed, paraffin-embedded samples of tumor tissue were identified, collected, and deparaffinized as described above. The tissue was rehydrated in 10 mmol/L Tris-HCl, 0.1 mmol/L EDTA, 2% EDTA and digested with 500 μ g/ml proteinase K (Qiagen) at 60°C for at least 4 hours (longer if the tissue was not fully lysed). Total RNA was purified using the StrataPrep Total RNA microprep kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. RNA (1 μ g) was converted to cDNA using 1 unit of SuperScript RNase H reverse transcriptase (Gibco Biologicals, Carlsbad, CA) and 0.2 μ g random primers

(Gibco) in a 30- μ l volume incubated at 37°C for 60 minutes. *KIT* cDNA sequences from exons 10 and 11 were amplified by PCR using a reverse primer (GAGTCGGACAAAGAC-CCTTT) matched with one of two forward primers: AAATC-CATCCCCACACCC (247-bp product) or CCTTTGCTGAT-TGGTTTCGT (201-bp product). The PCR conditions were as follows: 1 minute at 95°C, 45 cycles of 1 minute at 94°C/1 minute at 56°C/1 minute at 72°C, and then 7 minutes at 72°C. Samples yielding proper-sized amplicons, as confirmed by DHPLC, were sequenced as described above. In one of the four cases analyzed, direct DNA sequencing yielded satisfactory results in only one direction. Therefore, the amplicon was TOPO cloned as previously described, and a subclone was sequenced bidirectionally to confirm the initial sequence result.¹⁸

Results and Discussion

Analysis of GIST samples from 722 patients in our laboratories yielded 578 cases (80.0%) in which a mutation in exon 9, 11, 13, or 17 of the *KIT* gene was identified. The great majority of these mutations affected the juxtamembrane region of *KIT* encoded by exon 11 (67.7% of all GISTs). Among these exon 11 mutations were 19 examples in which there was a deletion that spanned the intron 10–exon 11 boundary. As illustrated in Figure 1, these deletions included from 1 to 14 bp of the 3' end of intron 10 and resulted in loss of the splice acceptor site for exon 11. In each case, a new potential splice acceptor AG was created, based on either the last two nucleotides of codon K558 (cases 1 to 14) or juxtaposition of an intron 10 adenosine with the terminal guanine of this codon (cases 15 to 19). The deletion resulting from use of this splice acceptor was predicted as KPMYEVQWK550–558. In 14 of the 19 cases, there was a C immediately 5' to the splice acceptor AG, consistent with the preference for a CAG sequence at the splice acceptor site. Based on DHPLC profiles, 6 of the 19 cases appeared to be hemizygous or homozygous for the detected deletion.

To prove that the new splice acceptor site was, in fact, used we prepared cDNA from the paraffin-embedded tumor of four of the cases (no frozen material was available). Using two different forward primers, amplicons of either 201 or 247 bp were successfully generated from the exon 10–11 boundary. Sequencing of these amplicons confirmed loss of codons 550–558 in all four samples (Figure 2). Interestingly, the cDNA products of all four cases were uniformly mutant even though three of the cases appeared heterozygous at the genomic level. One explanation for this apparent discrepancy is that wild-type genomic DNA contributed by non-tumor cells masked hemi/homozygosity of the tumor for the mutant allele. Alternatively, there may be selective transcription of the mutant allele in tumors that are truly heterozygous. For comparison, cDNA analyses were also performed on four exon 11-mutant tumors that did not include intron 10 sequences; three of these showed heterozygous allelic expression as expected, while the fourth appeared to express only the mutant allele, despite apparent genomic heterozygosity. Thus, this phenomenon is not unique to

	75631	INTRON 10	EXON 11
Case# 1)	aggtgatctct <u>gtttttcc</u> ctttctccccacag		AAACCCATGTATGAAGTACAGTGGAA <u>AG</u> GTTGTTGAGGAG
2)	aggtgatctat <u>ttttttcc</u> ttttctccccacag		AAACCCATGTATGAAGTACAGTGGAA <u>AG</u> GTTGTTGAGGAG
3)	aggtgatctat <u>ttttttcc</u> ttttctccccacag		AAACCCATGTATGAAGTACAGTGGAA <u>AG</u> GTTGTTGAGGAG
4)	aggtgatctat <u>ttttttcc</u> ttttctccccacag		AAACCCATGTATGAAGTACAGTGGAA <u>AG</u> GTTGTTGAGGAG
5)	aggtgatctat <u>ttttttcc</u> ttttctccccacag		AAACCCATGTATGAAGTACAGTGGAA <u>AG</u> GTTGTTGAGGAG
6)	aggtgatctat <u>ttttttcc</u> ttttct <u>tt</u> ccccacag		AAACCCATGTATGAAGTACAGTGGAA <u>AG</u> GTTGTTGAGGAG
7)	aggtgatctat <u>ttttttcc</u> ttttctcc <u>cc</u> acag		AAACCCATGTATGAAGTACAGTGGAA <u>AG</u> GTTGTTGAGGAG
8)	aggtgatctat <u>ttttttcc</u> ttttctc <u>ct</u> ccacag		AAACCCATGTATGAAGTACAGTGGAA <u>AG</u> GTTGTTGAGGAG
9-10)	aggtgatctat <u>ttttttcc</u> ttttctccc <u>cc</u> acag		AAACCCATGTATGAAGTACAGTGGAA <u>AG</u> GTTGTTGAGGAG
11-14)	aggtgatctat <u>ttttttcc</u> ttttctcccc <u>ac</u> ag		AAACCCATGTATGAAGTACAGTGGAA <u>AG</u> GTTGTTGAGGAG
15-18)	aggtgatctat <u>ttttttcc</u> ttttctcccc <u>a</u> ag		AAACCCATGTATGAAGTACAGTGGAA <u>G</u> GTTGTTGAGGAG
19)	aggtgatctat <u>ttttttcc</u> ttttctcccc <u>a</u> g		AAACCCATGTATGAAGTACAGTGGAA <u>G</u> GTTGTTGAGGAG
Wild-type	aggtgatctat <u>ttttttcc</u> ttttctcccc <u>a</u> g		AAACCCATGTATGAAGTACAGTGGAAAGGTTGTTGAGGAG K ⁵⁵⁰ P M Y E V Q W K V V ⁵⁶⁰ E E

Figure 1. Genomic deletions that include the intron 10–exon 11 boundary. Sequence from the end of intron 10, beginning at nucleotide 75631, and the beginning of exon 11 is shown, with the deletions of all 19 cases indicated in **gray**. Predicted splice acceptor sites are **boxed**. Wild-type sequence and amino acid codons are shown at the **bottom** of the figure for comparison. Note that in cases 6 and 8 the deletion was accompanied by a single nucleotide substitution at the 5' end (**underlined**). A separate single nucleotide polymorphism is **underlined** in the intron 10 sequence of case 1.

tumors with mutations that begin in intron 10 and may be worthy of further study.

Deletion of KPMYEVQWK550–558 in *KIT* exon 11 was one of the five mutations identified by Hirota and colleagues² in their original report of oncogenic *KIT* mutations in GI stromal tumors. However, that study was based on cDNA analysis, precluding identification of a possible intronic component of the deletion. Emile and colleagues⁶ reported a GIST with a genomic deletion that extended 7 bp into intron 10 (identical to our case 5), and deduced that use of the next possible splice acceptor site would result in the deletion KPMYEVQWK550–558. Our results, confirmed at the cDNA level, demonstrate that this phenomenon is not uncommon. Indeed, it is possible that the KPMYEVQWK550–558 deletions reported by Taniguchi et al (two cases),¹¹ Miettinen et al,²⁸

and Antonescu et al,⁵ also included intron 10 sequences, but this issue was not addressed in these studies.

It should be noted that deletion KPMYEVQWK550–558 can also result from an in-frame deletion that begins precisely at the first coding nucleotide of exon 11 (codon 550). We have observed five such deletions in our series (data not shown). Of course, at the protein level the final mutant isoform is the same as in tumors that have deleted part of intron 10 and use the alternative splice acceptor. It is not surprising, then, that the clinicopathologic features of the intron 10-deleted tumors are not substantively different from those with more typical exon 11 deletions. All 19 tumors were positive for *KIT* (CD117) by immunohistochemistry and had a spindle or mixed spindle/epithelioid morphology. All of the tumors with an assigned site of origin derived from the stomach. Available infor-

	Exon 10	Exon 11
<u>Wild-type</u>	CTGACCTACAAATATTTACAG L T Y K Y L Q	AAACCCATGTATGAAGTACAGTGGAAAGGTTGTTGAGGAG K ⁵⁵⁰ P M Y E V Q W K V V ⁵⁶⁰ E E
<u>CDNA From Cases #1, 5, 11 & 15</u>	CTGACCTACAAATATTTACAG L T Y K Y L Q	-----GTTGTTGAGGAG - - - - - V V ⁵⁶⁰ E E

Figure 2. cDNA sequences. The cDNA sequences from cases 1, 5, 11, and 15 all showed the identical deletion, as predicted from the genomic analyses.

mation on the age (median 57 years, $n = 10$) and sex (male:female, 1.25:1, $n = 9$) of the patients does not appear to differ from other published series of GISTs (information on cases from ongoing clinical trials is not available). Sixteen of the 19 tumors were malignant, but this very likely reflects the bias of our series for clinical trial patients. On the other hand, three tumors were classified as very low-risk by the criteria of Fletcher et al.,¹ so deletions crossing into intron 10 are not necessarily inherently aggressive. Most significantly, among six patients treated with imatinib for advanced disease, five had a partial remission and one had disease stabilization. These results are entirely consistent with the responses of exon 11-mutant GISTs in general.^{23,24}

To our knowledge, the only other documented example of a genomic deletion across the *KIT* intron 10–exon 11 boundary was described by Lee et al.⁹ Case 4 in their report was interpreted as a deletion/substitution KPMYEV550–555I, but it appears from the published sequence that loss of the splice acceptor site was not appreciated. It is likely that the AG of codon Q556 served as an alternative splice acceptor in that tumor, such that the resulting deletion was KPMYEVQ550–556. Interestingly, a KPMYEV550–555I deletion/substitution was also listed in the original report by Hirota and colleagues.² In that case, the isoleucine substitution was documented at the cDNA level and, therefore, the underlying genomic mutation must have differed from case 4 of Lee et al.⁹ Another example of a KPMYEV550–555I deletion/substitution was reported by Taniguchi et al.,¹¹ but their report did not include the supporting sequence and it is unclear whether intronic sequences were part of that deletion. Our series has yielded two cases with the deletion/substitution KPMYEV550–555I and one case with deletion KPMYEVQ550–556, but none of these included intron 10 sequences.

The juxtamembrane (JM) domain of KIT exerts an autoinhibitory effect on kinase activity through interaction with the N-terminal kinase domain.^{29,30} Length alterations or point substitutions in the JM domain disrupt this interaction and result in kinase activation. As autoinhibition of kinase activity by the JM domain is a property of other members of the receptor tyrosine kinase family, it is possible that length-altering mutations that involve the intron/exon boundary of JM domains will be found in other kinase genes. PDGFRA is of particular interest in this regard. Many of the reported intra-chromosomal deletions that lead to fusion of the *FIP1L1* and *PDGFRA* genes in patients with hypereosinophilic syndrome result in loss of the splice acceptor site for exon 12, which encodes the PDGFRA JM domain.³¹ An alternate AG splice acceptor within the sequence of exon 12 serves to maintain the reading frame in these cases.

In summary, our findings and other published reports indicate that deletions across the intron 10–exon 11 boundary of *KIT* can result in two mutant isoforms, deletion KPMYEVQ550–556 and deletion KPMYEVQWK550–558. Of these two isoforms, the latter is not uncommon, accounting for 3.9% of all exon 11 mutations in our series. The choice of the forward primer for exon 11 amplification is important in the identification of these partially intronic

deletions. Indeed, it remains possible that even larger deletions would be found if other primers were used. It is also conceivable that mutations involving the splice donor site at the exon 10–intron 10 boundary could affect the JM domain of KIT, although we have not yet observed any in separate analyses of exon 10 (data not shown). Laboratories engaged in mutation screening of GISTs for research or clinical purposes should be aware of the existence of deletions involving the intron 10–exon 11 boundary, because identification of exon 11-mutant GISTs has prognostic significance in regard to imatinib response.

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