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Improved Detection of *KIT* Exon 11 Duplications in Formalin-Fixed, Paraffin-Embedded Gastrointestinal Stromal Tumors

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Gastrointestinal stromal tumors (GISTs) are the most common gastrointestinal mesenchymal tumors driven by KIT or PDGFRA mutations. A majority of these mutations affect *KIT* exon 11 and represent deletions or point mutations, but insertions and duplications have also been reported. The latter have been found exclusively in the 3′ part of *KIT* exon 11. Reported frequency of duplications varies, and a higher frequency has been reported in studies based on frozen tissue. Recently, we have hypothesized that in some cases, the duplications might remain undetected in formalin-fixed, paraffin-embedded GISTs because of the preferential polymerase chain reaction (PCR) amplification of wild-type *KIT* over the mutant allele. In this study, 16 GISTs initially diagnosed as a wild-type *KIT* were evaluated using PCR assay amplifying only the 3′ part of *KIT* exon 11, the region commonly affected by duplications. Denaturing high-pressure liquid chromatography and direct sequencing analyses revealed duplications in 4 (25%) of 16 analyzed cases. Use of the PCR assay amplifying the specific region affected by duplications and yielding 129 bp in wild-type *KIT* can substantially improve the detection of these mutations in formalin-fixed, paraffin-embedded GISTs. *(J Mol Diagn 2007, 9:89–94; DOI: 10.2353/jmoldx.2007.060104)*

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of gastrointestinal tract driven by KIT or platelet-derived growth factor α (PDGFRA) gain-of-function mutations.[1–3] A great majority of these mutations affects KIT juxtamembrane domain encoded by exon 11 and represent deletions and point mutations; however, insertions and duplications have also been reported.[4,5] Although most of the deletions and point mutations cluster in the 5′ part, duplications have been found almost exclusively in the 3′ part of *KIT* exon 11.[6,7] In addition, duplications are predominantly associated with gastric tumor location.[6–9] Reported frequency of duplications varies from 3.6 to 13.6%.[7,10] This variation could be attributed to lower or higher number of gastric GISTs in analyzed tumor cohorts and to methodological differences in mutation detection. Recently, we have hypothesized that lower frequency of duplication in formalin-fixed, paraffin-embedded (FFPE) GISTs could also be related to preferential polymerase chain reaction (PCR) amplification of the wild-type (WT) *KIT* over the mutant allele because of severe DNA degradation in FFPE tissue.[3] In this study, we found support for this hypothesis and show that duplication in *KIT* can be more accurately detected with PCR assay tailored to amplify the 3′ part of *KIT* exon 11 involved in the duplications.

Materials and Methods

Sixteen GISTs analyzed in this study were obtained from the Department of Pathology, University Hospital of Tromsoe. DNA was extracted from FFPE tissues as previously reported.[4] All tumors were considered *KIT*- and *PDGFRA*-WT after screening of *KIT* exon 9, 11, 13, and 17 and *PDGFRA* exon 12, 14, and 18 mutation "hot spots" using previously reported PCR assays.[11]

A region commonly affected by duplications in the 3′ part of KIT exon 11 was identified based on analysis of 50 duplications, including 18 that were previously published,[7] available at our GIST mutation database and 46 cases reported by others in the literature.[5,6,8,12–23] All analyzed duplications, except two (2.1%), have been reported in the *KIT* region c.1729_1794 (g.75722_75787). Primers CK11.1F and CK11.3R closely flanking this region were used for PCR amplification of smaller products, yielding 129 bp in KIT-WT. For comparative purposes, the entire *KIT* exon 11 was subsequently amplified using

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Table 1. PCR Assays Used to Evaluate *KIT* Exon 11

Primer	Nucleotide position*	Primer sequence	PCR	Temperature (°C)	Size (bp)
CK10.7F	g.75575_75595	5′-CCATTTATTTGTTCTCTCTCC-3′	CK10.7F/CK11.3R	50	234
CK11.1F	g.75670_75690	5′-CAGTGGAAGGTTGTTGAGGAG-3′	CK11.1F/CK11.3R	55	129
CK11.3R	g.75789_75808	5′-AGCCCCTGTTTCATACTGAC-3′			
CK11.3.5R	g.75825_75845	5′-AGTCACTGTTATGTGTACCCA-3′	CK10.7F/CK11.3.5R	50	271

F, forward; R, reverse.
*Nucleotide position based on human *KIT* (locus HSU63834) sequence available in GenBank at *http:www.ncbi.nlm.nih.gov*.

two PCR assays based on intronic primers and generating 271- and 234-bp PCR products in selected cases. Primer sequences, PCR conditions, and length of the PCR products are listed in Table 1.

All PCR products were screened for mutations using denaturing high-pressure liquid chromatography (DHPLC) and WAVE System 3500HT (Transgenomic Ltd., Elancourt, France). Partially denaturing conditions for mutation detection were predicted using Navigator Software v.1.6.0. PCR products were analyzed under at least two different temperatures. Simultaneously and independently from DHPLC, PCR products were screened for mutations by direct sequencing as previously reported.[24]

Nomenclature of the mutations identified at the DNA level and deduced mutations at the protein level are based on the recommendations of the Human Genome Mutation Society (*http:www.hgvs.org*). The following *KIT* reference sequences, HSU63834 and XO6182, were obtained from GenBank (*http:www.ncbi.nlm.nih.gov*).

Results

Sixteen *KIT* and *PDGFRA*-WT GISTs from a series of 89 tumors were screened for *KIT* duplication using PCR assay designed to amplify a region in the 3′ part of *KIT* exon 11 commonly affected by duplications. PCR amplification was successful in all cases. DHPLC analyses suggested mutations in four (25%) tumors. Direct sequencing of PCR products identified duplications in all four GISTs indicated by DHPLC. Mutant *KIT* sequences are shown in Figure 1. Identified duplications ranged from 18 to 42 nucleotides and involved region c.1735_1782; one duplication, c.1741_1779dup, has been previously reported in a few cases.[9,25]

There were three male patients and one female patient with ages ranging from 41 to 59 years. Three tumors were gastric GISTs, and one was diagnosed in omentum. Histologically, all four tumors were spindle cell GISTs with very low (1 to 2 mitosis/50 high-power fields) or no mitotic activity. Tumor size varied from 4 to 26 cm. The demographic and clinicopathologic data of the four cases are summarized in Table 2.

To understand the nature of previous false-negative results, we subsequently studied these tumors using two different PCR assays based on intronic primers with target amplicons of 271 and 234 bp of genomic DNA. PCR amplification of 271 bp was achieved in three of four analyzed GISTs. DHPLC analysis of these PCR products indicated mutation only in two cases. Review of previous *KIT* mutation studies based on amplification and direct

Table 2. The Demographic and Clinicopathologic Data of Four GISTs with Duplications

Case	Age	Sex	Surgery (year)	Location	Size (cm)	Mitosis/50HPF	Histology
1	58	F	2001	Stomach	26	0	Spindle
2	41	M	1988	Stomach	4	2	Spindle
3	59	M	1986	Stomach	5	0	Spindle
4	49	M	1994	Omentum	11	1	Spindle

F, female; M, male.

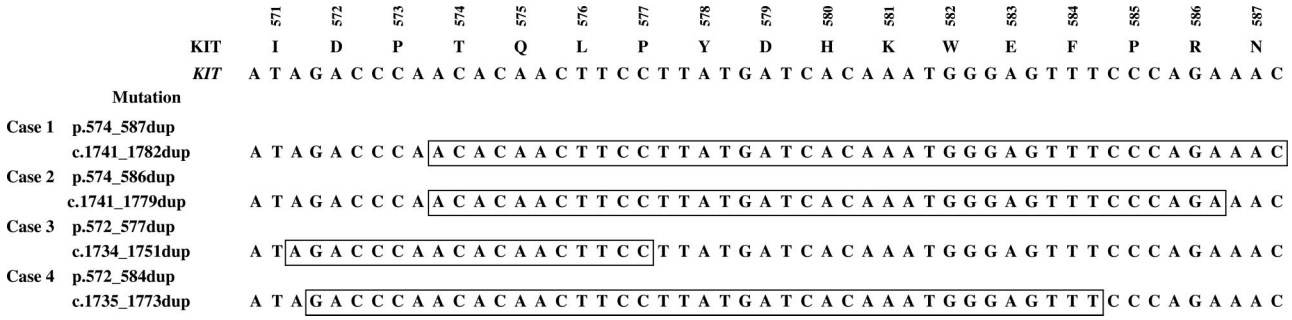

Figure 1. Genomic sequences of mutated *KIT* alleles identified in this study. Part of the genomic and amino acid sequence of WT *KIT* exon 11 is shown above. Duplicated sequences are boxed. Nomenclature of the mutations identified at the DNA level and deduced mutations at the protein level are shown in the second column.

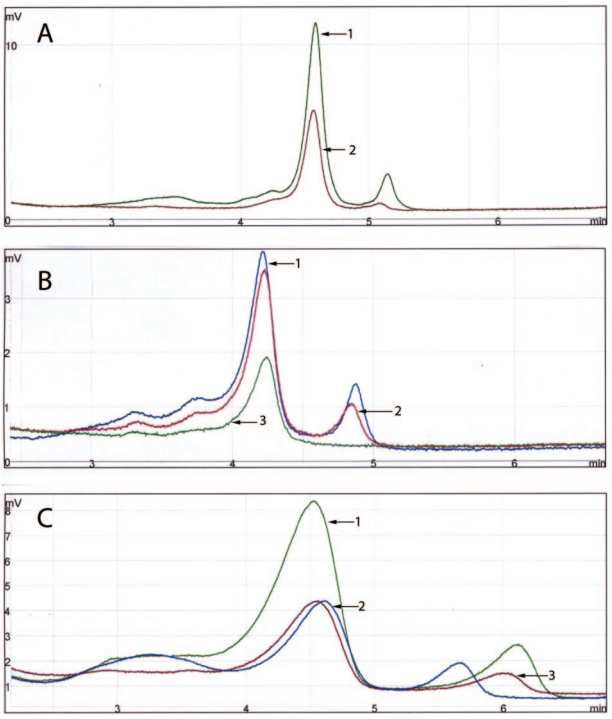

Figure 2. DHPLC analyses of 271-bp (**A**), 234-bp (**B**), and 129-bp (**C**) PCR products amplified from GISTs 1, 2, and 3. Left and right peaks represent products amplified from WT and mutant *KIT* alleles, respectively. **A:** Note the presence of WT and mutant *KIT* peaks in case 1, no indication of mutation (one WT peak) in case 2, and the lack of PCR amplification products in case 3. **B:** Note the presence of WT and mutant *KIT* peaks in case 1 and 2 and no indication of mutation (one WT peak) in case 3. **C:** Note the presence of WT and mutant *KIT* peaks in all three cases.

sequencing of 271-bp PCR products revealed in the cases indicated by DHPLC weak "tail sequence," suggesting possible duplication. These sequences were previously interpreted as a sequencing background.

PCR amplification of 234 bp was possible in all four analyzed cases. Mutations were indicated in three cases and confirmed by direct sequencing. However, accurate identification of mutant allele sequence was possible only in two cases. Although an extended "tail sequence" of longer mutant *KIT* allele was seen in the third case, elevated peaks of mutant *KIT* allele could not be easily distinguished from the elevated background. Figures 2 to 4 show representative data obtained by DHPLC and direct sequencing of PCR products.

Discussion

GISTs are common mesenchymal tumors of gastrointestinal tract, driven by KIT or PDGFRA gain-of-function mutations.[1,2] The frequency of these mutations varies between the studies and may depend to some extent on the constellation of tumor type and location in different series. *KIT* exon 9 mutations have been found almost exclusively in intestinal GISTs,[6,24,25] whereas *PDGFRA* exon 18 mutations are linked to gastric tumors with epithelioid cell morphology.[19,26–28] Although a majority of *KIT* exon 11 mutations have been found in GISTs from different locations, duplications affecting 3′*KIT* exon 11 occur predominantly in gastric tumors; 59 of 67 (88%) GISTs with *KIT* exon 11 duplications reported with clinical data were of gastric origin.[6–10,17–19,22,25] Also in this

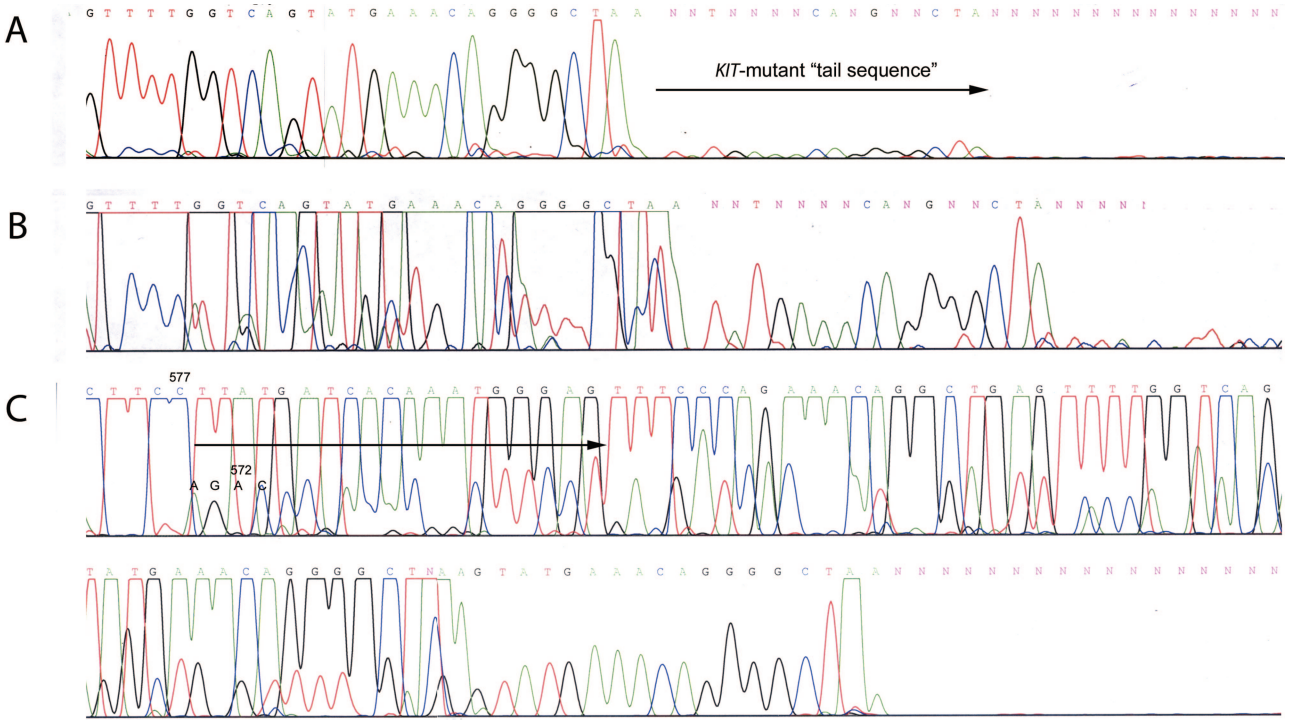

Figure 3. Direct sequencing of 234-bp (**A** and **B**) and 129-bp (**C**) PCR products from GIST 2. Note the hardly visible (**A**) and artificially elevated (**B**) "tail sequence" of mutant *KIT* allele. Elevated peaks of mutant *KIT* allele could not be easily distinguished from the elevated background. **C:** A shift of sequence detected in 129-bp PCR assay indicating duplication is emphasized by an **arrow**. WT and mutant *KIT* allele codons demarcating beginning of duplication are indicated by codon numbers.

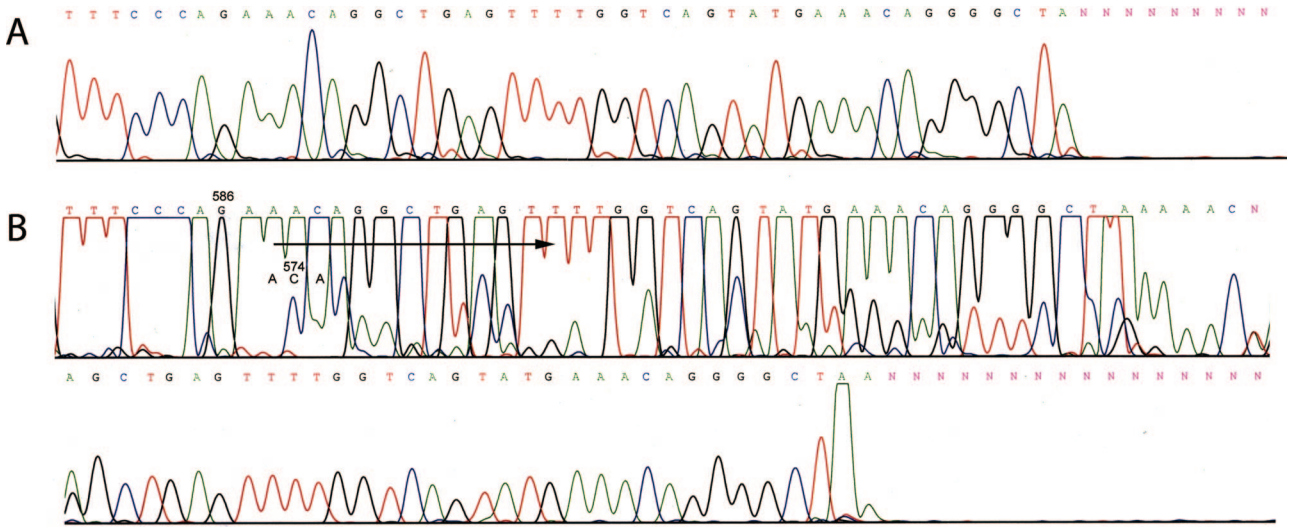

Figure 4. Direct sequencing of 234-bp (**A**) and 129-bp (**B**) PCR products from GIST 3. **A:** Note the lack of mutant *KIT* allele sequence in the products from 234-bp PCR assay. **B:** A shift of sequence detected in 129-bp PCR assay indicating duplication is emphasized by an **arrow**. WT and mutant *KIT* allele codons demarcating beginning of duplication are indicated by codon numbers.

study, three of four analyzed GISTs were gastric, and one was diagnosed in the omentum. Omental GISTs show histological and prognostic features similar to gastric tumors.[29]

Recent studies based on archival FFPE material reported independently by two different groups revealed decreasing mutation detection rate with increasing age of paraffin blocks.[9,30] Although specific factors causing this phenomenon have not been identified yet, there is a possibility that long-term storage of FFPE tissue might increase degradation of nucleic acids. In the current study, two GISTs (cases 2 and 3), which generated most serious diagnostic problems, were prepared by FFPE 18 and 20 years ago, respectively. However, one of the analyzed tumors (case 4) was prepared by FFPE only 5 years ago. Several factors including time between surgery and tissue fixation, prolonged tissue exposure to room temperature, and use of suboptimal formalin and paraffin may increase degradation of nucleic acids and lead to preferential amplification of the *KIT* allele not containing a duplication, thereby leading to false-negative results.

KIT exon 11 duplications reported in GISTs ranged from 3 to 57 nucleotides.[7,8] In this study, three of four analyzed GISTs had relatively large duplications of 39 to 42 nucleotides, whereas one tumor had only 18 nucleotides duplicated. However, this tumor was prepared by FFPE 20 years ago. Size of duplication and degree of degradation (more severe in older paraffin blocks) are two important factors leading to preferential amplification of WT versus mutant *KIT* allele with duplication.

In this study, 4 of 16 GISTs initially diagnosed *KIT*-WT were found to have duplications when evaluated with PCR assay exclusively yielding the amplification of a significantly shorter PCR product from the region commonly affected by duplication. Subsequently, we reevaluated all of these tumors using *KIT* exon 11 PCR assays generating larger products. Although DHPLC analysis indicated mutations in some cases, identification of mutant *KIT* sequences was often impossible because of the low signal, which could not be separated from the elevated background. Potentially, this could be overcome by DHPLC in-line fraction collector to isolate low-signal heteroduplex peaks followed by their direct sequencing to reveal the mutation.[31]

Recent studies have shown that DHPLC-based *KIT* mutation screening is more sensitive than direct sequencing of the PCR products.[32] In this study, one of the duplications was undetected by DHPLC when larger PCR products were analyzed. This observation indicates that both direct sequencing and DHPLC could create false-negative results due to suboptimal amplification of the longer mutant *KIT* allele with duplication when analyzing severely degraded DNA.

Imatinib mesylate, a KIT tyrosine kinase inhibitor also known as Gleevec/Glivec, has been used in the treatment of metastatic GISTs.[33,34] Although the majority of GISTs respond well to imatinib treatment, some cases are variably resistant.[35] Tumors with KIT exon 11 mutations respond to Gleevec treatment better than GISTs with Ala502_Tyr503dup (KIT exon 9 mutation) or those with WT KIT; also, GISTs with PDGFRA Asp842Val are primarily resistant to Gleevec treatment.[14,36,37] Because of these findings, accurate detection of KIT and PDGFRA mutations in GISTs are clinically significant.

In summary, our study confirmed that screening with DHPLC helps to eliminate misinterpretation of the direct sequencing of PCR products but, in some cases, could also create false-negative results due to suboptimal amplification of the longer mutant *KIT* allele with duplication. Both DHPLC- and direct sequencing-based mutation detection of *KIT* exon 11 duplications in GISTs could be improved by using PCR assays targeting smaller amplicons including the region commonly affected by duplication.

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