Mutational Analysis of Gastrointestinal Stromal Tumors (GISTs): Procedural Approach for Diagnostic Purposes

RAFFAELE PALMIROTTA[1], MARIA LAURA DE MARCHIS[1], GIORGIA LUDOVICI[1], BARBARA LEONE[1], RENATO COVELLO[2], SALVATORE CONTI[3], LEOPOLDO COSTARELLI[4], DAVID DELLA-MORTE[1,5], PATRIZIA FERRONI[1], MARIO ROSELLI[6] and FIORELLA GUADAGNI[1]

[1]*Department of Advanced Biotechnologies and Bioimaging, Institute of Care and Scientific Research (IRCCS) San Raffaele Pisana, Rome, Italy;*
[2]*Department of Pathology, "Regina Elena" National Cancer Institute Rome, Italy;*
[3]*Genechron Laboratory - C.R. ENEA Casaccia, Rome, Italy;*
[4]*Department of Pathology, San Giovanni Addolorata Hospital, Rome, Italy.*
[5]*Department of Neurology, Miller School of Medicine, University of Miami, Miami, FL, U.S.A.;*
[6]*Medical Oncology Unit, Tor Vergata Clinical Center, University of Rome, Rome, Italy*

Abstract. *Background: Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors in the digestive tract characterized, in the majority of cases, by activating mutations in the KIT (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog) or PDGFRA (platelet-derived growth factor receptor, alpha polypeptide) genes. Mutations affecting these tyrosine kinase receptors are also responsible for the mechanisms of primary and secondary drug resistance during the treatment with tyrosine kinase inhibitors. We performed mutational analysis to evaluate the pharmacotherapy susceptibility of GISTs, adopting a comprehensive procedural approach, in order to optimize the identification of mutations that may result in cellular resistance to conventional therapy. Materials and Methods: DNA from paraffin-embedded tumor sections from 40 GISTs were analyzed using microdissection, direct sequencing analysis and allelic separation by cloning. Results: KIT mutations were found in 55.0% of the tumor samples. PDGFRA mutations were present in 5.0% of cases. Allelic cloning assay allowed for better definition of the extent of the mutations and clarification of the exact nucleotidic position of complex mutations. Conclusion: Our experience suggests that sequential microdissection, direct sequencing and allelic separation by PCR cloning of large variants may improve the approach to mutational analysis and interpretation of sequence data of KIT and PDGFRA in patients with GIST.*

Correspondence to: Raffaele Palmirotta, Department of Advanced Biotechnologies and Bioimaging, IRCCS San Raffaele Pisana, via della Pisana 235, 00163 Rome, Italy. Tel: +39 0666130413, Fax: +39 0666130407, e-mail: raffaele.palmirotta@sanraffaele.it

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Gastrointestinal stromal tumors (GISTs) represent fewer than 1% of all malignancies, but are the most common mesenchymal tumors of the gastrointestinal tract, with an incidence of approximately 15 cases per million of population, per year and a prevalence of approximately 129 cases per million (1, 2).

Treatment of GISTs has been revolutionized by the discovery of molecular mechanisms responsible for the onset of this disease. It is estimated that the majority of patients with GIST carry somatic mutations in the *KIT* gene (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog), whereas the less frequently hold mutation in the platelet derived growth factor receptor alpha (*PDGFRA*) gene, both encoding structurally similar tyrosine kinase receptors (3-5). *KIT* and *PDGFRA* mutations are mutually exclusive and drive the tumorigenesis of GISTs, as they underlie structural changes of the relative receptors, resulting in a constitutive persistent autoactivation (5).

The identification of these molecular mechanisms fueled the development and clinical use of selective tyrosine kinase inhibitors (TKIs). Imatinib, a TKI successfully adopted in chronic myeloid leukemia (6), was approved in 2002 for the treatment of unresectable and metastatic GISTs (7), and several studies have also confirmed its efficacy as adjuvant therapy of these tumors (5).

Affinity of imatinib depends, in part, on the type of mutation, and on which codon is affected, thus emphasizing the pivotal role of mutational testing for prognosis and treatment of individual patient with GIST (5, 8, 9).

Table I. *Polimerase Chain Reaction primers, product size and reaction conditions for amplification and direct sequencing for assay of v-kit Hardy-Zuckerman-4 feline sarcoma viral oncogene homolog (KIT), platelet derived growth factor receptor alpha (PDGRFA), v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS), v-raf murine sarcoma viral oncogene homolog B1 (BRAF), tumor protein p53 (TP53) and neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS) genes.*

Gene	Exon	Primers	PCR product size (bp)	PCR annealing temp (C°)
KIT	9	F5'-TTCCTAGAGTAAGCCAGGG-3'	297	53
		R5'-TCATGACTGATATGGTAGACAG-3'		
	11	F5'-TCCAGAGTGCTCTAATGACTG-3'	276	58
		R5'-AGGAAGCCACTGGAGTTCC-3'		
	13	F5'-ATCAGTTTGCCAGTTGTGCT-3'	168	58
		R5'-GCTTTACCTCCAATGGTGC-3'		
	17	F5'-TGTGAACATCATTCAAGGCGTAC-3'	214	60
		R5'-CAGGACTGTCAAGCAGAGAATGG-3'		
PDGFRA	12	F5'-TCCAGTCACTGTGCTGCTTC-3'	274	60
		R5'-TTGTAAAGTTGTGTGCAAGGG-3'		
	18	F5'-TACAGATGGCTTGATCCTGAGTC-3'	223	60
		R5'-TTAGAGAGTAAAGTGTGGGAGGATG-3'		
KRAS	1 (1st PCR)	F5'-GTACTGGTGGAGTATTTGATAGTG-3	278	55
		R5'-GGTCAGAGAAACCTTTATCTGTATC-3'		
	1 (Nested PCR)	F5'-TTTTTATTATAAGGCCTGCT-3'	174	54
		R5'-GTCCTGCACCAGTAATATGC-3'		
	2 (1st PCR)	F5'-AGGTGCACTGTAATAATCCAG-3'	309	52
		R5'-ATTATATGCATGGCATTAGC-3'		
	2 (Nested PCR)	F5'-ATCCAGACTGTGTTTCTCCC-3'	256	55
		R5'-AACTATAATTACTCCTTAATGTCAGC-3'		
BRAF	15	F5'-TCATAATGCTTGTTGCTCTGATAGGA-3'	193	55
		R5'-GGCCAAAAATTTAATCAGTGGA-3'		
TP53	5	F5'-GACTTTCAACTCTGTCTCCTTCC-3'	255	59
		R5'-AACCAGCCCTGTCGTCTC-3'		
	6	F5'-AGGCCTCTGATTCCTCAC-3'	198	55
		R5'-CACTGACAACCACCCTTAAC-3'		
	7	F5'-AGGCGCACTGGCCTCATC-3'	179	65
		R5'-AGTGTGCAGGGTGGCAAGTG-3'		
	8	F5'-CCTTACTGCCTCTTGCTTCT-3'	224	56
		R5'-TAACTGCACCCTTGGTCTC-3'		
	9	F5'-CTCAGATTCACTTTTATCACC-3'	152	52
		R5'-AACTTTCCACTTGATAAGAGG-3'		
NRAS	1	F5'-GGTTTCCAACAGGTTCTTGC-3'	153	55
		R5'-CACTGGGCCTCACCTCTATG-3'		
	2	F5'-CACACCCCCAGGATTCTTAC-3'	150	55
		R5'-TGGCAAATACACAGAGGAAGC-3'		

Moreover, with longer duration of TKI-based treatment, the risk of developing resistance to therapy increases, because of the potential acquisition of secondary mutations in the kinase domain (10). This evidence suggests that *KIT* and *PDGFRA* mutational status has a prognostic significance for patients outcome and may help in the management of patients with GISTs (11).

Despite the prognostic value of *KIT* and *PDGFRA* mutations in GISTs, the majority of published guidelines do not focus their attention on the standardization of methods and procedures useful for the molecular detection of these sequence variants (1, 12-15). In order to offer therapeutic indications for the clinical oncologist, in our laboratory, we perform mutational analysis as a diagnostic procedure to evaluate the GISTs pharmacotherapy susceptibility. The knowledge and expertise gained over the years has led us to the definition of operational procedures in order to optimize the diagnostic approach for the identification of mutations relevant to personalized therapy (5).

Here, we present our experience on mutational screening analysis using microdissection, polymerase chain reaction (PCR), direct sequencing and allelic cloning of formalin-fixed paraffin-embedded samples from 40 patients with GIST. Moreover, the applicability of these methods for routine testing is briefly reviewed in the context of the available international literature.

Table II. *Internet databases used for comparing sequence variants obtained by mutational analysis of KIT and PDGRFA genes. OMIM: Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/omim/).*

Gene name (OMIM code)	Databases
KIT (*164920)	http://www.genomed.org/LOVD/CM/home.php?select_db=KIT http://www.hgmd.cf.ac.uk/ac/gene.php?gene=KIT http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=gene&ln=KIT
PDGFRA (*173490)	http://www.genomed.org/LOVD/CM/home.php?select_db=PDGFRA http://www.hgmd.cf.ac.uk/ac/gene.php?gene=PDGFRA http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=bygene&ln=PDGFRA

Materials and Methods

Forty-two paraffin-embedded cancer sections obtained from 40 consecutive patients with GIST (18 women, 22 men; mean age 62 years at the time of diagnosis, ranging from 25 to 88 years) were delivered to our Laboratory for molecular analysis of *KIT* and *PDGFRA* genes. All patients were referred to our Institution following an oncologist's request. Including two patients with two concomitant cancer samples (one case with two stomach samples and one case with stomach and jejunum samples), the biopsy sites were stomach in 27 cases (64.3%), small intestine in ten cases (23.8%), large intestine in two cases (4.8%) and extraintestinal in three cases (7.1%). All biological data were treated for purposes of scientific research and dissemination of the results occurred only anonymously in an aggregated or summarized form. Written informed consent was obtained from each participant and the study was performed under the appropriate Institutional ethics approvals and in accordance with the principles embodied in the Declaration of Helsinki.

Each paraffin-embedded section was collected on microscope slides and first examined under a microscope to ensure that it contained sufficient tumor material and to eliminate possible contaminating normal tissues. Tumor and tumor-free areas were identified within 15 μm-thick deparaffinized sections lightly counterstained with hematoxylin and microdissected by gentle scraping with sterile scalpels into 1.5 ml polypropylene vials, using a hematoxylin and eosin-stained step section from the same block.

DNA extraction from the microdissected area was performed as previously reported (16, 17). Briefly, formalin-fixed paraffin-embedded microdissected area were dipped into xylene to remove paraffin, rehydrated in a series of ethanol and incubated in 100 μl of digestion buffer, containing 1 M Tris-HCl (pH 8.0), 0.5 M EDTA, 0.02% Tween 20, and 100 mg/ml proteinase K. After an incubation of 3 h at 55˚C, proteinase K was inactivated at 95˚C for 10 min, and samples were centrifuged at 16000 ×*g*. The recovered supernatant was first purified by adding a saturated sodium chloride solution, centrifuged for 30 min at 16000 ×*g* and then precipitated by adding two volumes of 100% ethanol. The DNA pellet was finally dried and the pellet was redissolved in 50 ml of DNase-free water. *KIT* and *PDGFRA* primers and PCR conditions are described in Table I.

To separate alleles and characterize complex mutations, the amplified product was cloned into Pcr4-TOPO Vector using TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and the haplotypes of each heterozygote genotype were purified and sequenced (17).

Recent studies reported that a very small portion of GISTs wild type for both *KIT* and *PDGFRA* genes may have somatic mutations in the v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) (18-22), v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) (20, 21), neuroblastoma RAS viral (v-ras) oncogene homolog (*NRAS*) (18, 20) or tumor protein p53 (*TP53*) (23, 24) genes, predicting primary resistance to Imatinib in GISTs (21). Thus, all samples were subjected to mutational analysis of *TP53* (exons 5, 6, 7, 8 and 9), *BRAF* (exon 15), *KRAS* (exons 1 and 2) and NRAS (exon 1 and 2), which was performed by PCR and direct sequencing, as described in Table I. Exons 1 and 2 of *KRAS* were individually amplified using a nested amplification protocol as previously described (16, 17).

DNA extraction was performed in a dedicated area different from that used for set-up of the PCR reactions. Direct sequencing reactions were performed using a Big Dye Terminator (Applied Biosystems, Foster City, CA, USA), and run on an ABI 3130 Genetic Analyzer (Applied Biosystems). In order to exclude pre-analytical and analytical errors, all sequencing analyses were carried out on both strands and were repeated on PCR products obtained from new nucleic acid extractions. In all cases mutation nomenclature follows the recommendations of the Human Genome Variation Society (HGVS) (http://www.hgvs.org). The reference sequences used in this study are: *KIT* (*NM_000222.2*), *PDGFRA* (*NM_006206*), *TP53* (*NM_000546*), *NRAS* (*NM_002524*), *KRAS* (*NM_004985* and *M54968*) and *BRAF* (*NM_004333*) mRNA sequences from GeneBank (http://www.ncbi.nlm.nih.gov). To compare all detected sequence variants we previously identified the most widely used mutations Internet-databases listed in Table II.

Results

Results on DNA sequencing of *KIT* and *PDGFRA* genes obtained on all 42 tumor samples from 40 patients with GIST are listed in Table III.

Overall, 24 patients (24/40, 60.0%) showed evidence of pathogenetic variants, four (4/40, 10.0%) presented simple polymorphisms, whereas 12 (12/40, 30.0%) did not show any sequence variant of either gene (Table III). There were no statistical differences between sexes. Regarding the type, frequency and localization of pathogenetic variants, *KIT* mutations were the most frequent mutations, followed by *PDGFRA* mutations.

Mutations of *KIT* were detected in 22 patients (22/40, 55.0%), five of whom showed a double pathogenic variant and one had two different mutations on two different

Table III. *Tumor study group. Distribution of gastrointestinal stromal tumors by site and features. WT: Wild-type; sp: spindle; ep: epithelioid; m: mixed; *homozygous.*

Tumor N°	Tumor Site	Histology	Gender	Age, years	Gene involved	Exon	Mutations/sequence variants
1	Stomach	sp	F	88	*KIT*	11	c.1679-1681 3 bp del (TTG) (cod Val560 del)
						17	c.2408G>A (Gly803Asp)
2	Ileum	sp	F	43	-	-	WT
3	Duodenum	sp	M	66	*PDGFRA*	12	c.1759G>A (Glu587Lys)
4	Stomach	sp	M	53	*KIT*	11	c.1756 ins 33 bp (cod 574-585 dup + 3 bp del)
						17	c.2394C>T (Ile798Ile)
						17	c.2454G>A (Lys818Lys)
					PDGFRA	18	2496G>A(Val832Val)
5	Colon	sp	M	67	*KIT*	11	c.1740 (cod 580) 12 bp ins (cod 576-580 dup)
						17	c.2466T>A(Asn822Lys)
6 A	Stomach	sp	M	72	*KIT*	11	c. 1661 (cod554) 12 bp del (cod 554-cod 558)
6 B	Stomach	sp	M		*KIT*	11	c. 1744T>G (Trp582Gly)
7	Stomach	sp	M	70	*KIT*	11	c.1677T>C (Val559Ala)
					PDGFRA	18	c.2472C>T (Val824Val)
8	Stomach	sp	M	84	*KIT*	11	c.1751(cod584) 22 bp ins (cod 577-583 dup)
					PDGFRA	18	c.2472C>T (Val824Val)
9	Stomach	sp	M	71	*PDGFRA*	18	c.2525A>T(Asp842Val)
10	Stomach	sp	M	79			WT
11	Duodenum	sp	M	44	*KIT*	11	c.1703 A>G (Tyr568Cys)
						11	c.1707 24bpdel (cod 570-cod 576)
12	Stomach	sp	F	64	*KIT*	11	c.1659 15bp del (cod 554-cod558)
					KIT	17	c.2471T>C (Val824Ala)
13	Duodenum	sp	M	49	*KIT*	11	c.1727 T>C (Leu576Pro) *
14 A	Stomach	ep	M	67	*KIT*	11	c.1756 ins 42bp (cod 572-585 dup)
14 B	Jejunum	sp	M		*KIT*	11	WT
15	Stomach	sp	F	62	-	-	WT
16	Stomach	sp	M	25	-	-	WT
17	Stomach	sp	M	28	*KIT*	11	c.1727T>C (Leu576Pro)
18	Intestinal	sp	M	34	*KIT*	9	c.1383A>G (Thr461Thr)
19	Stomach	sp	M	70	*KIT*	11	c. 1727T>C (Leu576Pro)
20	Stomach	sp	F	76	*KIT*	11	c.1679T>A (Val560Asp)
					PDGFRA	18	c.2472C>T (Val824Val)
21	Liver	sp	F	79	*KIT*	11	c.1669_1674 del (Trp557-Lys558 del)
					PDGFRA	18	c.2472C>T (Val824Val)
22	Stomach	sp	F	61			WT
23	Stomach	sp	M	65	*KIT*	11	c.1661_1675 15 bp del (cod 555-560 del)
24	Sigma	sp	M	82	*PDGFRA*	18	c.2472C>T (Val824Val)
25	Stomach	sp	F	75	*KIT*	11	c.1741(cod 581) dup 24 bp (cod573-cod580)
27	Stomach	sp	F		-	-	WT
28	Duodenum	sp	M	46	-	-	WT
29	Adrenal	sp	M	70	-	-	WT
30	Duodenum	sp	F	55	*KIT*	13	c.1924A>G (Lys642Glu)
31	Pancreas	sp	M	68	-	-	WT
32	Stomach	ep	F	73	*KIT*	11	28bp del (25 bp exon 11, 3 bp intron 10)
					PDGFRA	18	c.2472C>T (Val824Val)
33	Stomach	m	F	45	*KIT*	11	c.1652 del 12 pb (cod 551-cod 555)
34	Stomach	sp	F		-	-	WT
35	Stomach	sp	F	38	*PDGFRA*	18	c.2472C>T (Val824Val)
36	Intestinal	sp	M	83	*KIT*	9	c.1504-1509 dup (cod 502-503 dup)
37	Stomach	sp	F	64	-	-	WT
38	Stomach	sp	M	45	-	-	WT
40	Intestinal	sp	F	72	*KIT*	11	c.1648 24 bp del (Lys550-Lys557)
						13	c.1889 C>T (His630Leu)
42	Stomach	ep	F	62	*PDGFRA*	18	c.2493T>C (Ile831Ile)
43	Stomach	ep	F	62	*KIT*	11	c.1654-1659del (Met552-Tyr553 del)

biopsies. In a total of 28 mutations, the most frequently found were identified in exon 11 (22 cases, 78.6%), followed by exon 17 (3 case, 10.7%), exon 13 (2 cases, 7.1%) and exon 9 (1 case, 3.6%).

The 22 mutations of exon 11 included nucleotide substitutions in 7/22 cases, duplications in 5/22 cases and deletions in 10/22 cases. In 9/22 cases (40.9%), mutations of exon 11 were clustered in the hot-spot region between codons 550 and 560.

Among the large deletions of exon 11, one case showed the concomitant presence of a 24-bp deletion from codon 570 to 576 and a single nucleotide substitution at codon 568. In one case we identified a deletion of 24 bp starting from the first codon of exon 11, while a similar deletion spanning from intron 10 to exon 11 and affecting the exon 11 splice-acceptor site was found in another case, in agreement with previously reported observations (25, 26). In one case we found a homozygous mutation affecting exon 11 of *KIT* gene (Table III).

Regarding the *PDGFRA* mutational analysis, two cases (2/40, 5.0%) carried mutations of the gene, one of which was the most frequent, located in exon 18 (Asp842Val). Mutations of *KIT* and *PDGFRA* genes were mutually exclusive in all cases. Finally, we found six silent polymorphisms for *KIT* and for *PDGFRA* gene in six individual patients.

Of interest, the mutation sites of *KIT* and *PDGFRA* correlated with specific anatomic tumor sites, as has been previously suggested (27- 29). Specifically, 13 out of 22 tumors showing mutations in exon 11 of *KIT* were located in the stomach, all four duplications in exon 11 were associated with gastric localization, while in the two cases with mutations in exon 9 of *KIT* tumors were found in the small intestine. A case of synchronous gastric and jejunal location was previously described (30).

Recent data indicate the onset of activating mutation of *BRAF* or *KRAS* genes as a possible novel mechanism of primary resistance to imatinib in GISTs, which could explain the small percentage of patients who, although carriers of sensitive *KIT* mutations, do not respond to this treatment (21). In our cases, all tested tumors were wild-type for *KRAS* and *BRAF* genes, in agreement with the low percentages found in previous studies (18-22). Similarly, the mutational analysis of *TP53* and *NRAS* genes, considered responsible for several rare forms of imatinib-resistant GIST by some authors, gave negative results (18-22).

Allelic cloning assay, performed on all large variants, allowed us to better define the extent of the mutations and to clarify the exact nucleotidic position of complex mutations (Figure 1). In a specific case, the use of this technique led us to the identification of a complex variant consisting of a 33-bp duplication combined with a 3-bp deletion (Figure 2).

Discussion

New insights into the molecular mechanisms responsible for GISTs have shown that molecular subclassification of GISTs provides valuable information for patient management, prognosis, treatment response, and resistance to therapy (1, 5, 11, 31).

In order to offer therapeutic indications for clinical oncologists, in our laboratory we perform a mutational diagnostic procedure to evaluate the susceptibility of GISTs to TKI therapy. Currently, to our knowledge there are no guidelines for routine molecular testing and only three recent studies addressed the issue of GIST genotyping quality assessment scheme (32- 34).

In the first of these studies, Merkelbach-Bruse *et al*. compared the mutational analysis results of *KIT* and *PDGFRA* genes from six different German molecular laboratories (32). As a result of this inter-laboratory trial the authors concluded that there is a large number of possible pitfalls during the different technical procedures. These issues included different steps of molecular analysis, including the quality of extracted DNA, the excess of target DNA, the number of PCR cycles and the design of the primers. Special attention was paid to the interpretation of electropherograms and the subsequent indication of mutations at the DNA and protein level by a description consistent with the guidelines for sequence variation of the Human Genome Variation Society (HGVS; http://www.hgvs.org/mutnomen) (35). These results prompted the authors to provide a valuable and useful proposal for the mutation testing standard operating procedures (http://www.biomedcentral.com/1471-2350/11/ 106/additional).

The quality control program for mutation detection in *KIT* and *PDGFRA* genes described by Hostein and colleagues was conducted in eleven European laboratories (33). The results demonstrated a global error rate of 4.5% (eight cases on 200 reports). In this case it was also possible to observe an issue concerning sequence data interpretation, which resulted in incorrect nucleotide or protein sequence formula.

The most recent study performed on quality issues is the United Kingdom National External Quality Assessment Scheme (UK NEQAS) for Molecular Genetics GIST that highlighted some concerns related to *KIT* and *PDGFRA* mutation analyses and indicated the interpretation of complex mutations as being one of the main problems (34).

In synthesis, all three studies emphasize the importance of different steps included in the mutational analysis: the quality of DNA extracted, the exact amount of target DNA, the number of PCR cycles, the design of the primers and, in particular, the interpretation of electropherograms and the use of HGVS nomenclature to avoid the variability in nomenclature of complex mutations.

The experience gained over the years in our laboratory has led us to the definition of operational procedures in order to optimize

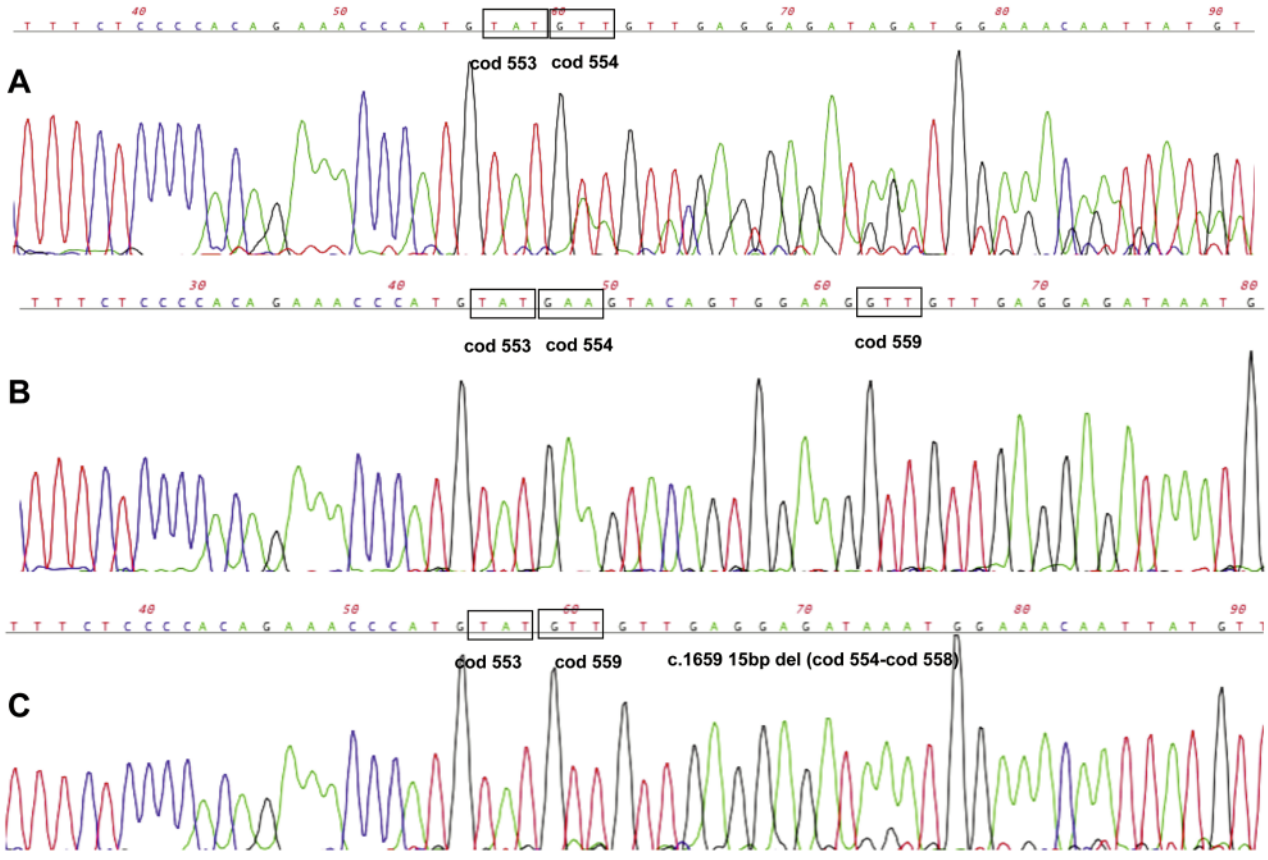

Figure 1. *Direct sequencing of the v-kit Hardy-Zuckerman-4 feline sarcoma viral oncogene homolog (KIT) gene exon 11 (patient 12, see Table III) showing a 15 bp deletion from codon 554 to codon 558 (A). The cloning sequencing of PCR products shows an allele with a wild-type exon 11 (B) and an allele with 15 bp deleted in exon 11 (C).*

the diagnostic approach for the identification of mutations relevant to personalized therapy (5). In previous pharmacogenetic studies we have already emphasized that microdissection of the analyzed tissue represents an essential step in the molecular analysis, since it prevents an erroneous mixture of healthy and transformed tissues being analyzed (16, 17). Indeed, under these circumstances, the PCR reaction might result in an excessive presence of 'background noise' or stochastic occurrence of allelic preferential amplifications. Based on this experience, we performed a preliminary morphological evaluation of biopsy samples before nucleic acid extraction, outlining under microscopy the boundaries between frankly neoplastic tissue and normal tissue. Then for each sample, we proceeded to the extraction, amplification and sequence analysis of different portions of the same microdissected biological sample. An example of this procedure is shown in Figure 3.

Furthermore, particular attention was paid to the design of the primers, a feature which is essential both for the PCR reaction and direct sequencing analysis. All oligonucleotides are designed in such a manner as to include coding regions and the flanking intron-exon junctions, in order to avoid PCR products more than 300 bp large and to minimize primer-primer interactions (Table I).

The analysis of electropherograms, in some cases, is very complex due to the intrinsic nature of the particular frameshift mutation and the difficulty of distinguishing the presence of a variant from the 'background noise' which is normally found in an electropherogram. In our case all electropherograms are evaluated by two independent experts on the basis of a wild-type sequence reference also paying attention to potential homozygous mutations. All identified variants are then compared with those listed in the Internet-databases (Table II) and described following the recommendations of the HGVS.

Mutations in homozygosity can go undetected in an preliminary analysis of the electropherograms. It is therefore recommended to use an alignment program such as BLAST (basic local alignment search tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi) to detect these kind of variants.

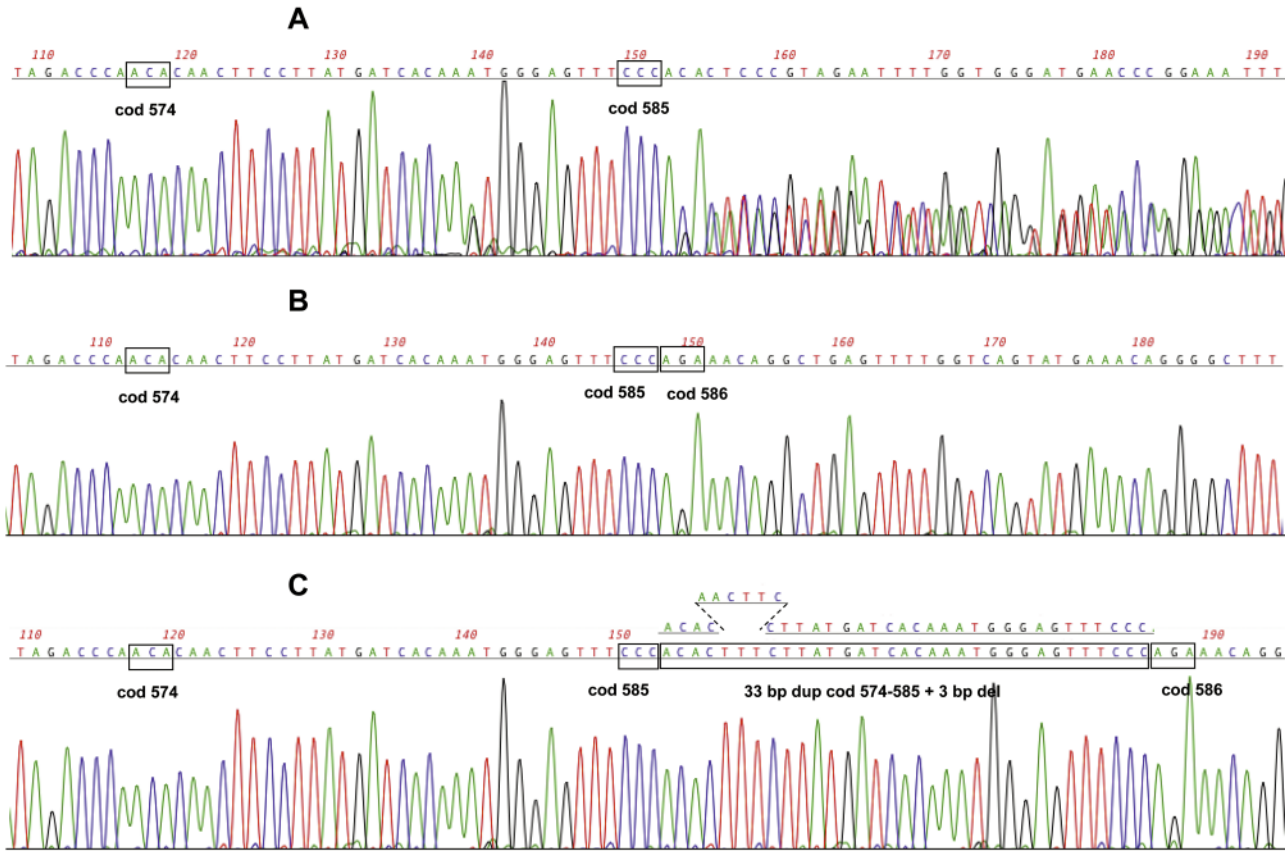

Figure 2. *Direct sequencing of the v-kit Hardy-Zuckerman-4 feline sarcoma viral oncogene homolog (KIT) gene exon 11 showing an extended mutation (patient 4, see Table III) determining a frameshift starting from codon 586 (A). The cloning sequencing of the PCR product reveals a wild-type allele (B) and an allele with a complex mutation consisting of a 33-bp duplication (codons 574-585) combined with a 3-bp deletion (C).*

In addition to these recommendations, in order to optimize the detection and the accurate reporting of mutations, allelic separation by cloning is a step that may increase the identification of complex mutations of *KIT* and *PDGFRA* genes. Our example depicted in Figure 2 is paradigmatic of the difficult interpretation of an electropherogram in which it is possible to recognize a probable second mutation localized at the same locus of a primary pathogenic variant. For this reason, whenever possible, we proceed with amplification and sequence analysis of different clones obtained from the same biological sample carrying a complex mutation.

In conclusion, standardization of genetic analyses in pharmacogenetic studies is necessary to improve the ability to identify already known and new genetic mutations (36) that may result in cellular resistance to conventional therapy. We suggest that some analytical phases, fundamental in clinical decision making, should be better-standardized in scientific studies. In this respect, tissue microdissection, direct gene sequencing, and allelic separation by cloning might represent important steps capable of increasing the power of pharmacogenetic studies in patients with GIST.

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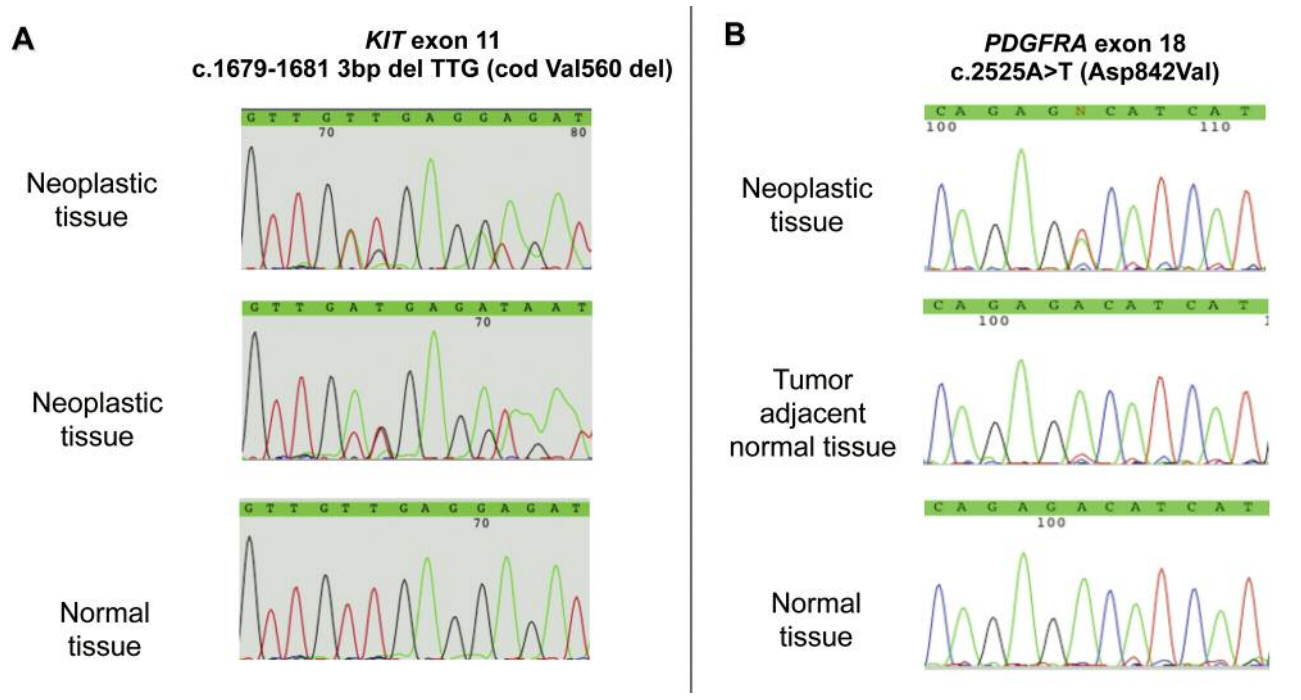

Figure 3. *Sequence analysis of v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT) exon 11 (A) and platelet derived growth factor receptor alpha (PDGRFA) exon 18 (B) on DNA extracted from different microdissected areas of paraffin-embedded tumour sections. The intensity of the peaks corresponding to the mutated allele is markedly dissimilar in each microdissected area.*

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