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KIT exon 11 and *PDGFRA exon 18* gene mutations in gastric GIST: proposal of a short panel for predicting therapeutic response

Denise Barcelos[1], Ricardo Artigiani Neto[1*], Leonardo Cardili[1], Mariana Fernandes[1], Fernando Cintra Lopes Carapeto[2], Andréia Neves Comodo[1], Karina Funabashi[1] and Edna Sadayo Miazato Iwamura[1]

Abstract

Background: GIST is the most common mesenchymal tumor of gastrointestinal tract and is more frequent in stomach. Its main mutations affect *KIT* and *PDGFRA* genes. Full genetic analysis panels are currently used to study mutations in GIST and other tumors. Considering that in gastric GIST KIT gene mutations in exon 11 are sensitive to IM whereas PDGFRA gene mutations in exon 18 (D842V) are resistant to the same drug, the aim of this study is to focus on these two molecular targets as a short alternative panel for predicting therapeutic response in gastric GIST which might optimize resources.

Methods: The genotypes of 38 cases of primary GIST were determined by performing bidirectional DNA sequencing.

Results: Exon 11 of *KIT* gene showed mutations in 65.3% and the exon 18 of *PDGFRA* gene showed 9% of cases. So it was possible to determine a subgroup of tumors which presented mutations in *KIT* exon 11 and *PDGFRA* exon 18.

Conclusion: Considering all of the foregoing analyzed globally, the application of short panel has impact on the cost and time of release of results to the physician, allowing a rapid approach to patients eligible for treatment with the target therapy.

Keywords: Exon 11 *KIT* gene, Exon 18 *PDGFRA* gene, FFPE, Gastric GIST, Therapeutic response

Introduction

Predictive markers of therapeutic response are biomarkers that provide upfront information to physicians as whether or not a patient can be beneficiary from a specific therapy. Such data is essential to clinical oncologists to guide therapy (Duffy et al. 2011).

Due the biological factors as heterogeneity (Almendro et al. 2013), only few patients with a particular type of tumor which show specific mutations can be treated with target therapy. Currently, it is known the response rate from several advanced tumors to target therapy.

Target therapies have efficacy in minority of patients. Therefore, detection of patients likely to respond to a specific treatment would be of great clinical value (Lasota and Miettinen 2006).

Gastrointestinal Stromal Tumor (GIST) is rare and represents less than 1% of all gastrointestinal tumors. It is the most frequent gastrointestinal mesenchymal visceral neoplasm (Miettinen et al. 2002). Approximately 60% of GISTs occur in stomach and have better prognosis than tumors located in other sites as small intestine, duodenum and rectum (Miettinen and Lasota 2011). Studies have shown that GISTs bear mutations in approximately 85% of cases, most of them related to kinase tyrosine *KIT* (70–80%) or *PDGFRA* (5–8%) genes (Cerski et al. 2011). About 15% of GISTs do not present detectable mutations (Rubin et al. 2001) (Fig. 1). These tumors may be sensitive or resistant

* Correspondence: ricardoartigiani@hotmail.com
[1]Department of Pathology, Universidade Federal de São Paulo/UNIFESP, Rua Botucatu, 740, São Paulo CEP 04023-900, Brazil
Full list of author information is available at the end of the article

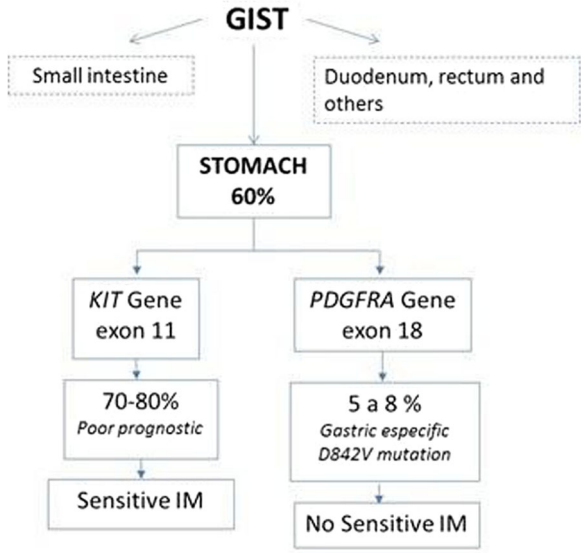

Fig. 1 Scheme summarized regarding the molecular characterization of gastric GIST

to tyrosine kinase inhibitors depending on mutated exon. In fact, there is a link between gene, mutated exon, tumor location and prognostic factors (Dematteo et al. 2002). Most primary tumor mutations of *KIT* gene affect exon 11 (67–75%) and are related to poor prognosis. However, it has been demonstrated that GISTs show responsiveness of approximately 83.5% to tyrosine kinase inhibitor imatinib mesilate (IM) (Heinrich et al. 2008; Demetri et al. 2004)'. Moreover, mutations in *PDGFRA* gene mainly occur in gastric tumors and almost exclusively in exon 18 followed by few mutations in exon 12 and exon 14 (Demetri 2001). The most commonly reported mutation in exon 18 is a substitution of a single nucleotide known as *Asp842Val (D842V)* which is primarily resistant to tyrosine kinase inhibitors (Cassier et al. 2012). Nowadays, several mutation detection methods have been used as conventional direct sequencing (Baskin et al. 2016) and next generation sequence (NGS). Both are sequenced gene panels involving several genes related to various tumors including GIST (Fisher et al. 2016). However, these procedures are very expensive, requiring high-cost equipment and qualified training for technical staff. Formalin-fixed paraffin-embedded samples (FFPE) are very common practices in pathology laboratories worldwide. This type of storage is extremely important for prospective studies and is the largest source of samples for pathology laboratories. Nevertheless, it often difficult the use of DNA for molecular assays (Greer et al. 1994).

Considering that in gastric GIST *KIT gene* mutations in exon 11 are sensitive to IM whereas *PDGFRA gene* mutations in exon 18 (D842V) are resistant to the same drug, the aim of this study is to focus on these two molecular targets as triage tool for detection of a subgroup of patients more likely to be responsive to IM (Fig. 1) *(*Gheorghe et al. 2014*)*.

Materials and methods

We reviewed 38 cases of primary GIST tumors (FFPE), all from gastric sites. The blocks were made available from the Pathology department of the Universidade Federal de São Paulo - UNIFESP/EPM, São Paulo, Brazil. The storage period ranged from 2000 to 2010. All tumors showed morphological characteristics typical of GIST, CD117 positive in immunohistochemistry and they had not received specific treatment before resection. It was analyzed 10-μm cuts from gastric GISTs fixed in formalin and embedded in paraffin. The sections were deparaffinized tissue was manually macrodissected. For DNA extraction, we used a Qiagen QIAamp® DNA Micro commercial kit, according to the manufacturer's instructions. DNA quantification was calculated by Nanodrop 2000c Thermo scientific in A_{260}. The PCR reactions were performed in a thermal cycler (Mastercycler® personal; Eppendorf, Hamburg, Germany). Three different primer combinations were used to study exon 11 of the *KIT* gene. Primers were used at a concentration of 0.4 μM per reaction, generating the following fragments: 174 bp (P1 and P2), 196 bp (P3 and P4) and 215 bp (P5 and P6) (Merkelbach-Bruse et al. 2010) (Fig. 2):

P1:F-5'GATCTATTTTTCCCTTTCTCC3' and P2:R-5' AGCCCCTGTTTCATACTGAC 3′; P3:F-5'GATC TATTTTTCCCTTTCTCC3' and P4:R-5'TACCCAAAA AGGTGACATGG3'; P5:F-5'CCAGAGTGCTCTAATGA CTG3' and P6:R-5'AGCCCCTGTTTCATACTGAC3'. For amplification of the 218 bp fragment of the *PDGFRA* gene, we used the following forward (F) and reverse (R) sequences:

P7:F-5'CAGCTACAGATGGCTTGATC3' and R5'GAA GGAGGATGAGCCTGAC3'. For all reactions, we used Master Mix (Qiagen®), and the concentration of DNA used in each reaction varied between 10 and 30 ng. Amplification of *KIT* gene exon 11 involved one cycle at 94 °C for one min, 94 °C for 40 s, 54 °C for 40 s, one extension period of 72 °C for 40 s and a final extension of 72 °C for 5 min. *PDGFRA* gene exon 18 amplification used the same PCR conditions as above, but at 57 °C. These reactions were adapted from Merkelbach-Bruse et al. (2010). The amplified fragments were visualized in 1.5% agarose gel stained with "Gel Red™ (Biotium®)". Product purification was performed with a QIAquick PCR purification kit (Qiagen, Hilden, Germany). Tape sequencing (forward and reverse) was performed with Big Dye Terminator Cycle Sequencing Ready Reaction Kit v1.0 (Applied Biosystems, Foster City, CA, USA). The primer concentration was 0.4 μM each. We used between 1 and 4 μl of PCR product, depending on the

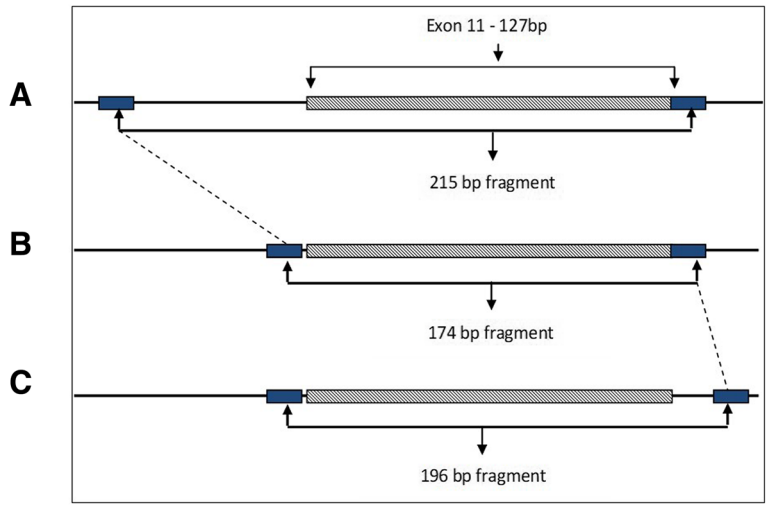

Fig. 2 Schematic figure showing 127 bp of the KIT gene exon 11 and the positions occupied by the primers used in this study. **a** 215 bp fragment; **b** 174 bp fragment and **c** 196 bp fragment. "pm" primer

intensity of the band observed. The PCR program conditions for the sequencing cycle of *KIT* gene exon 11 were: 96 °C for 1 min, 96 °C for 10 s, annealing at 55 °C for 5 s, and extension at 60 °C for 4 min. The conditions for *PDGFRA* gene exon 18 were the same except for the annealing temperature of 60 °C. Sequencing was performed on ABI 3100 Genetic Analyzer equipment (Applied Biosystems, Foster City, CA, USA).

In addition, duplicates were made and the two DNA strands (forward and reverse) were sequenced for validation of the technique.

Results

The results of the evaluation of purity of DNA extracted from the samples were close to 1.8. This indicates that DNA extraction by Qiagen QIAamp® DNA Micro commercial kit was adequate (Funabashi et al. 2012). A relationship between these values and the amplification and sequencing could be not established.

Exon 11 *KIT* gene PCR

Among the primer sets used, the one with the best response to the material was the one that produced 174 bp fragments in 37/38 (97.4%) of cases, followed by the 196 bp fragment in 18/38 (47.3%) and the 215 bp fragment in 20/38 (52.6%).

Thereby, 37 samples were amplified successfully; however, 31.5% could not be sequenced. One of the samples used had two simultaneous mutations so was considered 27 mutations in total. The number and types of mutations found in exon 11 of the *KIT* gene were 5/27 (18.5%) deletions, 9/27 (33.3%) substitutions, 1/27 (3.7%) insertions and 2/27 (7.5%) duplications, with no mutation in 9/27 (33.3%). The type of mutation, genomic location and the position of the codon in the 38 samples used in the study are shown in Table 1.

Exon 18 PCR *PDGFRA* gene

Of all 38 samples, 22/38 (58%) were amplified with the 213 bp primers. Of these, 11/22 (50%) had silent p.V824 V mutations e 2/22 (9.1%) of tumors carried mutations in codon 842. Finally, in 41% (9/22) of sample, there were no detected mutations (Table 1).

Sanger sequencing

The method used was the Sanger sequencing which is known as the gold standard method for GIST genotyping (Martin-Broto et al. 2017). The sensitivity and specificity of the Sanger sequencing method, as well in the complete panel as the short panel is the same, because it is the same method. In general, in terms of accuracy for determination of specific mutations, Sanger Sequencing presents similar accuracy to other methods (Zhang et al. 2015).

According to the criteria listed below, we mention some reasons why using the Short panel (Table 2).

Some authors argue about the weaknesses of the method and its implications on the results of the research (Table 3).

Although NGS methods have many advantages in terms of speed, cost, and parallelism, the accuracy and read length of Sanger sequencing is still superior and has confined the use of NGS mainly to sequencing genomes (Verma et al. 2017). Therefore, it is necessary that the tools that already implanted are not discarded but adapted or improved.

Table 1 Type of mutation, genomic location and the position of the codon in the 38 samples used in the study

Genotypic results for the 38 GIST patients				
Samples	Exon 11 *KIT* mutation		Exon 18 *PDGFRA* mutation	
1	nm	nm	–	–
2	–	–	–	–
3	–	–	–	–
4	p.F584L	c.1753 T > G sub	–	–
5	p.Y570F	c.1708_1710sub	–	–
6	nm	nm	nm	nm
7	nm	nm	nm	nm
8	–	–	nm	nm
9	–	–	nm	nm
10	–	–	nm	nm
11	–	–	nm	nm
12	p.556_567dup42	c.1667_1701dup42	nm	nm
13	p.557_558del	c.1669_1674del6	nm	nm
14	nm	nm	nm	nm
15	p.P573M	c.1717_1719	p.D842V	c.2525A > T
16	–	–	–	–
17	–	–	–	–
18	–	–	–	–
19	p.P585T	1753C > A	–	–
20	p.V559D	1676 T > A	–	–
21	nm	nm	–	–
22	p.579del	c.1735_1737del	nm	nm
23	p.591_592ins16	c.1773_1774ins48	nm	nm
24	nm	nm	–	–
25	–	–	–	–
26	–	–	–	–
27	–	–	–	–
28	p.E561L - Y578C	c.1681G > A - 1733A > G	–	–
29	nm	nm	nm	nm
30	nm	nm	nm	nm
31	nm	nm	nm	nm
32	nm	nm	nm	nm
33	p.Q556_558del 6	c.1667_1672del6	nm	nm
34	p.V559D	c.1676 T > A	p.D842V	c.2525A > T
35	p.W557_558del	c.1669_1674del6	nm	nm
36	P.W557_561del	c.1669_1737del	nm	nm
37	p.566ins7	c.1697	nm	nm
38	p.D572V	c.1715A > T	nm	nm

"-"sample not sequenced, *del*-deletion, *dup*-duplication, *ins*-insertion, *sub*-substitution, *n/m*-no mutation in the exon studied

Discussion

The conventional Sanger sequencing used as a method of detecting mutations in GIST takes time, is laborious and requires specialized technical knowledge for the analysis of mutations (Agaimy et al. 2013; Schöffski et al. 2016).

Traditionally, full-panel sequencing approaches in GISTs involve *KIT* gene and *PDGFRA gene* (Miettinen and Lasota 2006). Multi-exon strategy allows tracking of different mutations, thus providing a broad spectrum of genetic profile of patients. However, such approach is very expensive and show several limitations in FFPE samples (Blanke and Huse 2010; Guerin et al. 2015).

Pre-analytic issues and high-cost equipment are limiting factors for large use of DNA sequencing for medical assistance purposes. Despite these difficulties, data provided by sequencing are essential to identify a subgroup of patients which will benefit from IM therapy.

Considering that the complete panel is extensive, involving mutations in four exons (9, 11, 13 and 17) of the KIT gene and three exons (12, 14 and 18) of the PDGFRA gene, the use of short sequencing panel may be a practical, fast and economical alternative of evaluating tumor sensitiveness to MI.

Furthermore, for each analyzed exon in addition to the duplicates, independent PCR reactions are required for both DNA strands of the sample. Therefore, only one GIST sample requires eight sequencing reactions for the KIT gene and six other sequence reactions if the *PDGFRA* gene exons are also analyzed.

For that reason, it would be important that laboratories with less demand and/or located outside the large urban centers, could benefit from simplified screening protocols that focus on the detection of mutations in specific exons to meet the need for therapeutic response (Zhang et al. 2015).

Currently, an alternative to Sanger sequencing is the next generation sequencing (NGS), this technique a solid potential as molecular test in several types of cancer (Gao et al. 2016). The large sequencing capability of NGS platforms allows detecting all mutation information in multiple samples at the same time. However, the NGS does not use the same work steps of the conventional Sanger method, and in addition, it presents a high cost, besides requiring many times the confirmation of the findings by the Sanger sequencing (Johnston et al. 2012; Pandey et al. 2016; Diekstra et al. 2015), making it difficult to use them in the diagnostic routine. In addition, the mutational spectrum is much broader generating a volume of information unnecessary for the therapeutic routine.

Different authors also discuss the limitations of Sanger's method in relation to other methods and how this implies the results of the analysis. However, only knowing their limitations will it be possible to establish protocols that minimize them so that it can continue to be a benchmark for other methods.

Analyzing other sequencing methods, specifically the analytical validation tests on NGS platforms, is still

Table 2 Analysis criteria that mention why use the short panel

Criteria	Short painel
Steps required	The number of steps for executing the Sanger sequencing method of each analyzed exon is the same. However, the number of exons studied in the short panel is smaller.
Time	The time spent from the extraction of DNA to the analysis of the results is the same per exon studied, however it becomes smaller when we consider that the short panel presents a reduction in the number of exons studied.
Cost	The steps of the Sanger sequencing encompass DNA extraction, PCR, amplicon purification, forward and reverse sequencing PCR, capillary sequencing and analysis. In each of these steps specific kits and reagents are used. The reduction of target exons allows more tests to be performed with the same amount of reagents, which contributes to the reduction of expenses in the diagnostic routine.
Sensibility	It is the same, because the proposal is the use of the same methodology.
Specificity	The specificity of the method is the same. However, we understand that when evaluating only two exons we will have lower specificity of mutation detection and therefore the short panel is a proposal of screening.
Interpretation	The interpretation becomes more concise, objective and fast for technical managers, reducing misinterpretations.
Final consideration	Considering all of the foregoing analyzed globally, the application of short panel has impact on the cost and time of release of results to the physician, allowing a rapid approach to patients eligible for treatment with the target therapy.

extremely challenging for pathology laboratories, for although there are many affordable NGS instruments and easy to access, there are still no standardized guidelines for clinical validation trials NGS (Rathi et al. 2017).

In addition, patterns related to the input of FFPE material were defined, both for use in NGS and for Sanger sequencing. Thus, the reliability of mutation analysis could be improved by manual inspection of sequence data (de Leng et al. 2016).

It is possible that some institutions already select specific exons in the practice of diagnostic routine. However, this study provides a theoretical basis for this practice that would have sensitive implications in the management of GIST patients.

KIT gene: exon 11

Exon 11 of the *KIT* gene has a variable mutation profile that is distributed in different codons throughout the exon, sometimes hindering analysis and interpretation of results. Combined with pre-analytic issues as non-controlled fixation and embedding processes, full sequencing analysis becomes a challenging task. The type frequency and of mutations found in exon 11 of *KIT* gene were deletions (5/27–18.5%), substitutions (9/27–33.3%), insertions (1/27–3.7%) and duplications, (2/27–7.5%). No mutations were found in 9/27 of cases (33.3%).

Duplications have been reported among mutations found in this exon and are mainly associated with

Table 3 Shows some recent articles arguing about advantages and disadvantages of Sanger sequencing in relation to other methods

Title	Age	Authors	Argumentation
Comparison of an in vitro Diagnostic Next-Generation Sequencing Assay with Sanger Sequencing for HIV-1 Genotypic Resistance Testing.	2018	*Tzou PL,* et al. (2018)	Sensitivity of this method is insufficient for identifying low frequency mutations.
A simple and robust real-time qPCR method for the detection of PIK3CA mutations.	2018	*Alvarez-Garcia V;* et al... (2018)	Low sensitivity and the high cost.
Droplet Digital PCR for Mutation Detection in Formalin-Fixed, Paraffin-Embedded Melanoma Tissues: A Comparison with Sanger Sequencing and Pyrosequencing.	2018	*McEvoy AC;* et al. (2018)	Failure to detect mutations in genes and specific samples.
Implementation of next generation sequencing technology for s omatic mutation detection in routine laboratory practice.	2018	*Giardina T;* et al. (2018)	It presents greater difficulties in detecting materials that were not microdissected.
Non-reproducible sequence artifacts in FFPE tissue: an experience report.	2017	*Ofner R,* et al. (2017)	Low reproducibility in FFPE.
Clinical validation of the 50 gene AmpliSeq Cancer Panel V2 for use on a next generation sequencing platform using formalin fixed, paraffin embedded and fine needle aspiration tumour specimens.	2017	*Rathi V;* et al. (2017)	Disadvantages in relation to new technologies such as NGS
Locked nucleic acid probe enhances Sanger sequencing sensitivity and improves diagnostic accuracy of high-resolution melting-based KRAS mutational analysis.	2016	*Ishige T;* et al (2016)	Sensitivity of this method is insufficient for identifying low frequency mutations.
Clinical Applications of Next-Generation Sequencing in Cancer Diagnosis	2016	*Sabour L;* et al. (2017)	Laborious, time consuming and difficulty in distinguishing between normal and altered genotypes.
A combination of immunohistochemistry and molecular approaches improves highly sensitive detection of BRAF mutations in papillary thyroid cancer.	2016	*Martinuzzi C;* et al. (2016)	Low sensitivity

gastric site, female gender and benign behavior (Antonescu et al. 2003). We have found two cases of duplication in our set of cases (i.e., 7.5%), rate similar to values reported in literature (Palmirotta et al. 2013; Minárik et al. 2013).

Deletions are often associated with worse prognosis, mainly if involving Trp557 and/or Lys558. These codons are regarded as the first hot spot of mutations in this exon and are associated to aggressive biological behavior (Wardelmann et al. 2003; Singer et al. 2002). Nevertheless, they present better response to IM. In this study, 16% (4/25) of cases had in-frame deletions. Three of them affected codons 557 and 558, one sample with p.W557_E561del5 and two samples with p.557_558del2. There was also a homozygous in-frame deletion in codon 579 (p.D579del; Fig. 3). The highest frequency of in-frame deletions in the terminal 5′ region of exon 11 involves codons 550–560 (Wardelmann et al. 2003; Wardelmann et al. 2007). The 3′ region of *KIT* gene exon 11 is considered the second most mutated hotspot (Antonescu et al. 2003). Insertions and duplications of large genomic regions most often occur in such region even though the number of nucleotides varies. Moreover, this type of mutation may be difficult to visualize as it does not present changes in the exon body (Lasota et al. 2003; Iesalnieks et al. 2005). We also observed a case like this in our sample: the insertion of 48 bp p.F591_G592ins16 at the 3′ terminal. Although this mutation is not directly involved in the body of exon 11, it could affect the protein structure due to its size and position. We found eight missense substitutions in the analyzed tumors with varied distribution. Substitutions have been found to be the most common mutations in this exon and most often occupy codons 557, 559, 560 and 576. The p.V559D mutation was found in two cases (Silva et al. 2010; Antonescu et al. 2005). When deleted, this codon may be associated with metastatic disease but the p.V559D substitution was not specifically associated with such behavior (Wardelmann et al. 2003). The missense substitution Y570F (c.1708_1710), also found in this study, was previously described in a study analyzing GIST mutations in a Portuguese population, but was not associated to gastric site (Gomes et al. 2008). Codon 572 is often cited in literature, presenting with several types of mutations. While other amino acids have been reported to occupy this codon in GIST and in other tumors (Rossi et al. 2010), the substitution of Aspartic acid (Asp/D) by Valine (Val/V) found in one of our patients had not been previously described (p.D572V). The p.P573M mutation has also not been described previously in gastric GIST tumors, but this codon is involved in several other mutations involving non gastric GIST (Antonescu et al. 2003) and melanoma (Omholt et al. 2011). The missense mutation p.F584 L c.1752 T > G has been described in GIST (Minárik et al. 2012), but it may also be present in other tumors, such as primary adenoid cystic carcinoma of salivary glands (Vila et al. 2009) and breast carcinoma (Hussain et al. 2012).

Finally, the p.P585T mutation was previously described in GIST in a study associating GIST mutations and VEGF expression (de Oliveira et al. 2011; Mazzola et al. 2008). One sample showed two mutations for *KIT* gene exon 11 -- p.E561L and p.Y578C – which had not been described to date. However, other substitutions in codon p.E561 involving different tissues, such as p.E561K in hematopoietic tissues (Hongyo et al. 2005) and GIST (Hanada et al. 2006), p.E561G in melanoma (Kong et al. 2011) and p.E561D in GIST (Nakajima et al. 2009), have already been described.

	codon #	550	551	552	553	554	555	556	557	558	559	560	561	562	563	564	565	566	567	568	569	570	571	572	573	574	575	576	577	578	579	580	581	582	583	584	585	586	587	588	589	590	
	codon sequence	AAA	CCC	ATG	TAT	GAA	GTA	CAG	TGG	AAG	GTT	GTT	GAG	GAG	ATA	AAT	GGA	AAC	AAT	TAT	GTT	TAC	ATA	GAC	CCA	ACA	CAA	CTT	CCT	TAT	GAT	CAC	AAA	TGG	GAG	TTT	CCC	AGA	AAC	AGG	CTG	AGT	TTT	intron
samples	mutation/Amino acid	K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	V	Y	I	D	P	T	Q	L	P	Y	D	H	K	W	E	F	P	R	N	R	L	S	F	
4	p.F584L	K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	V	Y	I	D	P	T	Q	L	P	Y	D	H	K	W	E	*L*	P	R	N	R	L	S	F	
5	p.Y570F	K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	V	*F*	I	D	P	T	Q	L	P	Y	D	H	K	W	E	F	P	R	N	R	L	S	F	
12	p.Q556_567dup42	K	P	M	Y	E	V	*duplication*																																				
13	p.557_558del	K	P	M	Y	E	V	Q	*del*		V	V	E	E	I	N	G	N	N	Y	V	Y	I	D	P	T	Q	L	P	Y	D	H	K	W	E	F	P	R	N	R	L	S	F	
15	p.P573M	K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	V	Y	I	D	*M*	T	Q	L	P	Y	D	H	K	W	E	F	P	R	N	R	L	S	F	
19	p.P585T	K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	V	Y	I	D	P	T	Q	L	P	Y	D	H	K	W	E	F	*T*	R	N	R	L	S	F	
20	p.V559D	K	P	M	Y	E	V	Q	W	K	*D*	V	E	E	I	N	G	N	N	Y	V	Y	I	D	P	T	Q	L	P	Y	D	H	K	W	E	F	P	R	N	R	L	S	F	
22	p.D579del	K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	V	Y	I	D	P	T	Q	L	P	Y	*del*	H	K	W	E	F	P	R	N	R	L	S	F	
23	p.591_592ins16	K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	V	Y	I	D	P	T	Q	L	P	Y	D	H	K	W	E	F	P	R	N	R	L	S	F	*duplic*
28	p.E561L / p.Y578C	K	P	M	Y	E	V	Q	W	K	V	V	*L*	E	I	N	G	N	N	Y	V	Y	I	D	P	T	Q	L	P	*C*	D	H	K	W	E	F	P	R	N	R	L	S	F	
33	p.556_558 del6	K	P	M	Y	E	V	*del*			V	V	E	E	I	N	G	N	N	Y	V	Y	I	D	P	T	Q	L	P	Y	D	H	K	W	E	F	P	R	N	R	L	S	F	
34	p.V559D	K	P	M	Y	E	V	Q	W	K	*D*	V	E	E	I	N	G	N	N	Y	V	Y	I	D	P	T	Q	L	P	Y	D	H	K	W	E	F	P	R	N	R	L	S	F	
35	p.W557_558del	K	P	M	Y	E	V	Q	*del*		V	V	E	E	I	N	G	N	N	Y	V	Y	I	D	P	T	Q	L	P	Y	D	H	K	W	E	F	P	R	N	R	L	S	F	
36	P.W557_561del15	K	P	M	Y	E	V	Q	*del*					E	I	N	G	N	N	Y	V	Y	I	D	P	T	Q	L	P	Y	D	H	K	W	E	F	P	R	N	R	L	S	F	
37	p.566ins	K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	*ins*																										
38	p.D572V	K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	V	Y	I	*V*	P	T	Q	L	P	Y	D	H	K	W	E	F	P	R	N	R	L	S	F	

5 deletion *9* substitution *1* insertion *2* duplication

Fig. 3 The figure shows the 42 codons that compose the KIT gene exon 11, the type of mutation found in the samples (5 - deletions, 9 - substitution, 1 - insertion and 2 - duplications) and the distribution of the mutations in exon, indicating the number of exons and the code of the related aminoacid

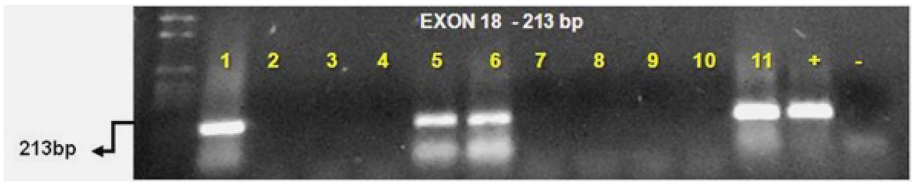

Fig. 4 Agarose (1.5%) gel electrophoresis of 213 bp amplification product exon 18 PDGFRA gene in GIST gastric

PDGFRA gene: exon 18

We obtained high-quality samples by using this primer designed for exon 18 of *PDGFRA* (Fig. 4). However, the size of fragment generated by this set of primers may also have affected the PCR amplification results (13/22, 59%), confirming the need for adjustments to PCR protocol and sequencing for this exon as it was done in *KIT* exon 11. In this study, 2/22 (9.1%) of tumors carried mutations in codon 842 of *PDGFRA exon 18*, which is considered a hotspot for this exon. No mutations were shown in 9/22 (40.9%) of cases.

Two samples (15 and 34) presented *KIT* exon 11 and *PDGFRA* exon 18 gene mutations. The explanation for such findings may be related to intratumoral heterogeneity and although some authors have previously reported that mutations in *KIT* and *PDGFRA* genes are mutually exclusive, morphological heterogeneity is sometimes evident. Therefore, further investigations are required to better elucidate this issue (Kumar et al. 2014; Alic et al. 2014).

However, a few points should be paid attention. First, our study was descriptive and is an initial study only demonstrates the feasibility of the method in FFPE samples. Second, the comparison between original panel and the panel will be proposed in future studies.

FFPE samples and quality

Noteworthy is the fact that our sample included tumors processed in different laboratories, which precluded the necessary quality control procedures. We have found that 18/38 (47.5%) of samples were sequenced for both exons studied. This finding indicates the quality and suitability of performed methods. However, there have been individual responses of different samples to the same protocol. Accordingly, it is difficult to obtain a well succeed sequencing procedure in diagnostic laboratory without making fine adjustments in protocols. The literature points that the average DNA length obtained after extraction is 300-400 bp but this value is lower in FFPE samples (Barcelos et al. 2008; Casale et al. 2010), which explains the increase in amplification from 52.6 to 97.4% obtained in this study. This increased value was only possible when we repositioned primers from 215 bp amplicons to 174 bp amplicons (Merkelbach-Bruse et al. 2010).

Reduction in fragment size does not guarantee PCR reaction efficiency since primers that generated 196 bp fragments presented 47.3% positivity, which was lower than presented by the 215 bp sample. That is true once there are regions in the genome that may be compromised by fixation. In the case of exon 11, mutations could be located in such regions, thus preventing primer annealing. Besides DNA extraction, PCR and sequencing protocols were the same. About 21% (8/38) of samples could not be sequenced for both genes and some samples could be sequenced only for one of them. This may be related to variations in pre-analysis step, such as batch and quality of reagents, since the study period involved years of collection and storage. This phenomenon also occurred with other authors who worked with FFPE samples that turned out to be unusable for molecular analysis (Origone et al. 2013).

Study limitations

Some limiting aspects deserve to be highlighted. The lack of amplification of some FFPE samples represents an important limitation of our study. In addition, studies involving a greater number of samples are necessary since our results are based on a rather reduced casuistry. In the future, an extended investigation produces relevant results to provide the basis for operating protocols.

Conclusion

The use of targeted therapies requires knowledge of specific mutations for the patient to truly benefit from the treatment. Establishing the genotypic profile of GIST can determine response to treatment and modify the entire treatment schedule.

Considering all the foregoing analyzed globally, we conclude that application of short panel has impact on the cost and time of release of results to the physician, allowing a rapid approach to patients eligible for treatment with the target therapy.

Abbreviations

FFPE: Formalin-fixed paraffin-embedded samples; GIST: Gastrointestinal Stromal Tumor; IM: Imatinib mesilate; NGS: Next generation sequence

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Authors' contributions
DB was responsible for the design, study design and implementation of the project and drafted the manuscript. RI and ESMI conceived of the study, and participated in its design and coordination and helped to draft the manuscript. KF, MF, AC held the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. FC and LC participated in the histopathological analysis of biopsies and drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The study was approved by the Ethical Committee of Universidade Federal de São Paulo - Plataforma Brasil, reference number 16183.

Consent for publication
"Not applicable".

Competing interests
The authors declare that they have no competing interests.

Publisher's Note

Author details
[1]Department of Pathology, Universidade Federal de São Paulo/UNIFESP, Rua Botucatu, 740, São Paulo CEP 04023-900, Brazil. [2]University of Texas MD Anderson Cancer Center, Pathology, Houston, TX, USA.

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