Prognostic Relevance of *KIT* and *PDGFRA* Mutations in Gastrointestinal Stromal Tumors

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Abstract. *Background: Prediction of biological behavior is crucial for selection of new therapeutic modalities in GIST. Here, we aimed to assess whether KIT and PDGFRA mutations have survival impact in gastrointestinal stromal tumors (GIST). Patients and Methods: Fifty-five Brazilian patients with completely resected GIST were examined for KIT and PDGFRA mutations. The 5-year disease-free survival (DFS) was analyzed. Results: KIT and PDGFRA mutations were identified in 74.5% and 7.3% of patients, respectively. The 5-year DFS rate for all patients was 52.8%. The 5-year DFS rate was lower in patients with tumors having in-frame deletions or concomitant in-frame deletions and insertions affecting codons 557-558 than in patients with tumors having other exon 11 KIT mutations (p=0.023). Conversely, when the patients with concomitant deletion-insertion mutations affecting codons 557-558 were excluded from the analysis, deletions involving codons 557-558 had no influence on 5-year DFS rates. Conclusion: Our findings indicate that a specific KIT mutation may be associated with unfavorable behavior in GIST. This finding may have implications on selecting patients for adjuvant therapy.*

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Key Words: GIST, KIT, PDGFRA, prognosis.

Gastrointestinal stromal tumor (GIST) represents the most frequent mesenchymal tumor of the gastrointestinal tract (1). GIST is highly resistant to chemotherapy, and surgery has been the mainstay treatment of localized or even for locally advanced GIST (2). A large number of newly diagnosed GISTs are already metastatic at the time of surgery. The knowledge of constitutive activation of KIT signaling pathway in GIST carcinogenesis has allowed the introduction of inhibitors of tyrosine kinases in GIST treatment, which have revolutionized the treatment of unresectable and metastatic, or recurrent GIST (3, 4). Imatinib mesylate (STI571; Novartis Pharma AG, Basel, Switzerland), the first tyrosine kinase inhibitor available, is a potent inhibitor of KIT, and other tyrosine kinases such as abelson murine leukemia viral oncogene homolog 1 (ABL), breakpoint cluster region-abelson murine leukemia viral oncogene homolog 1 (BCR-ABL), platelet derived growth factor receptor alpha (PDGFRA) tyrosine kinase, and platelet derived growth factor receptor beta (PDGFRB) tyrosine kinase (5). Imatinib has been shown to induce stable disease, partial response and complete remission, with up to 90% of patients with advanced GIST showing some clinical benefit (6). In this context, the knowledge of tumor biological behavior is crucial for the rational selection of patients to be treated with imatinib-based or new therapeutic modalities.

Prediction of biological behavior of primary tumors remains challenging in GIST. GISTs should never be considered benign. Therefore, primary GIST has been classified according to the risk of malignant behavior (7). Despite this classification, a small subset of tumors of low-risk may behave as malignant tumors and may recur after prolonged follow-up (8). In this regard, the knowledge of genetic abnormalities involved in GIST carcinogenesis and tumor progression has been intensively studied to better evaluate its prognostic and for the rational selection of therapeutic modalities.

Activating mutations on *KIT* (encoding a type 3 transmembrane receptor for mast cell growth factor) are frequent and occur very early during GIST carcinogenesis (9). In addition, activating *PDGFRA* mutations have been identified in a small subset of GISTs lacking *KIT* mutations. Whereas the mutation status has been shown to predict poor response to tyrosine kinase inhibitors, the predictive value of mutation status as in GIST recurrence after resection of primary tumor has not been established yet (10). Therefore, in this study, we aimed to assess whether *KIT* and *PDGFRA* mutations could predict the GIST behavior and have survival impact in a Brazilian cohort.

Patients and Methods

Patients and tumors. Eighty-one patients with completely resected GIST treated between 1993 and 2003 at different Brazilian Institutions were retrospectively included in this study. The Institutional Ethics Committee approved this study. All cases were retrospectively identified and morphologically evaluated at the Department of Pathology at the National Cancer Institute (INCA) and at Consultoria em Patologia by two pathologists (S.R and C.B.). In all cases, the diagnosis was confirmed based on both histopathological features on hematoxylin and eosin-stained slides, and an immunohistochemical panel including KIT immunohistochemical expression (antibody anti-CD117; DAKO Corp, Carpinteria, CA, USA). Tumors were evaluated for size, cell type (spindle, epithelioid or mixed cell type) and mitotic index. Mitotic figures were counted in 50 high-power fields. Based on mitotic activity, tumor size and anatomic site, GISTs were stratified as very low-, low-, intermediate- and high-risk tumors according to criteria previously defined in the literature (11).

Mutation analysis. DNA was isolated from paraffin-embedded tumor tissue as previously described (12). *KIT* mutational status was analyzed by polymerase chain reaction (PCR) amplification and subsequent DNA sequencing of exons 9, 11, 13 and 17. Tumors with wild-type *KIT* were further analyzed for *PDGFRA* mutations in exons 12, 14 and 18. The oligonucleotide sequences and the amplification conditions are detailed in Table I.

PCR products were purified with the GFX TM PCR DNA and Gel Band Purification Kit H (GE Healthcare, Sao Paulo, Brazil) and subsequently sequenced with the DYEnamyc TM ET Terminator Cycle Sequencing Premix Kit H (GE Healthcare) or BigDye Terminator Cycle Sequencing Standard Version 3.1 (Applied Byosistems, Austin, TX, USA). Products were electrophoresed in ABI PRISM TM 377 or 3730 DNA automatic sequencers (Applied Biosystems, Foster City, CA, USA) and analyzed by Sequence Navigator (Applied Biosystems) and Sequencher version 4.1 softwares (Gene Codes Corporation, Ann Arbor, MI, USA) (13).

Immunohistochemical (IHC) analysis. In all cases, the formalin-fixed, paraffin-embedded tissue specimens were cut into 3-4 mm sections followed by section deparaffinization in xylene and subsequently rehydrated in a graded series of ethanol. Heat-induced epitope retrieval was performed by using a steamer and citrate buffer (pH, 6.0; 10 mol/l). The sections were allowed to cool and then immersed in a 3% hydrogen peroxide for 20 min to block endogenous peroxidase. The sections were incubated with primary antibody at 4˚C temperature, overnight. The primary antibody was rabbit polyclonal antibody anti-human KIT (CD117) (1:100 dilution; DAKO Corp, Carpinteria, CA, USA). Sections were then incubated with secondary reagents by using standard avidin-biotin complex (LSAB+; DAKO Corp.). Immunohistochemical staining was assessed in a semiquantitative manner based on the percentage of stained cells. Intensity of KIT staining was subdivided into three categories: weak staining, fewer than 25% of tumor cells were positive; moderate staining, between 25 and 75%; and strong staining, more than 75% of cells stained.

Table I. *List of primers used for PCR and DNA sequencing. PCR amplification consisted of one initial denaturing step of two minutes at 94˚C, followed by 40 cycles of 94˚C for 30 s, 60˚C for 30 s, 72˚C for 60 s, and a final extension step of 10 min at 72˚C.*

Target gene	Primer target	Sequence (5'-3')
KIT	Exon 9F	TTCCTAGAGTAAGCCAGGGC
	Exon 9R	ACAGAGCCTAAACATCCCCT
	Exon 11F	GTGATGATTCTGACCTACAAAT
	Exon 11R	TGTACCCAAAAAGGTGACATG
	Exon 13F	CTGCATGCGCTTGACATCAG
	Exon 13R	CTAGCATTGCCAAAATCATATT
	Exon 17F	GTTTTCTTTTCTCCTCCAACCT
	Exon 17R	CCTTTGCAGGACTGTCAAGC
PDGFRA	Exon 12F	CTCTGGTGCACTGGGACTTT
	Exon 12R	AAAGGGAGTCTTGGGAGGTT
	Exon 14F	TGGTAGCTCAGCTGGACTGAT
	Exon 14R	GGGATGGAGAGTGGAGGATT
	Exon 18F	ACCATGGATCAGCCAGTCTT
	Exon 18R	TGAAGGAGGATGAGCCTGACC

F: Forward; R: reverse.

Statistical analysis. To ensure the quality of the information, data were stored, organized, filtered, encoded and extracted using a Microsoft Access 2000-based methodology developed by the Division of Clinical Research's data management personnel at INCA. Disease-free survival (DFS) was estimated using the Kaplan-Meier method. DFS was calculated from the date of surgery to the date of disease relapse or to the date of last follow-up visit. The 5-year DFS rates were assessed with the respect to the following variables: age; gender; histological subtype, *KIT* and *PDGFRA* mutational status. Differences between survival curves were estimated by the log-rank test. Results were considered significant when the p-value was ≤ 0.05. Data were analyzed using SPSS software, version 13.0 for windows (SPSS Inc. Chicago, IL, USA).

Results

Clinicopathological and demographic data. Over the course of the study, 26 patients initially intended for the study were excluded from further analysis, leaving a cohort of 55 patients for the final study population. The reasons for exclusion were incomplete clinical information and suboptimal quality of the DNA. Our cohort comprised 22 males (40.0%) and 33

Table II. *Distribution of KIT and PDGFRA mutations in GISTs (n=55).*

Tumor no.	Site	Risk behavior	Cell type	Mutation (exon/type)	KIT staining
KIT					
4	Stomach	Very low	Spindle	Ex 11/K558del	>75%
6	Small bowel	Intermediate	Spindle	Ex 11/K558_D572del	>75%
12	Small bowel	Malignant	Spindle	Ex11/ Q556_L576del	25-75%
14	Peritoneum	Intermediate	Mixed	Ex 11/V559D	25-75%
19	Stomach	Low	Epithelioid	Ex 11/W557_K558delinsQ	25-75%
20	Stomach	Very low	Spindle	Ex 11/W557_K558delinsQ	>75%
21	Small bowel	Very low	Spindle	Ex 9/A502_Y503ins	>75%
25	Small bowel	Malignant	Spindle	Ex 9/A502_Y503ins	>75%
28	Small bowel	High	Spindle	Ex 11/W557_K558del	>75%
30	Small bowel	Malignant	Spindle	Ex 11/W557_K558del	>75%
31	Stomach	High	Spindle	Ex 11/W557_K558del	>75%
38	Small bowel	Intermediate	Epithelioid	Ex 11/V555_Q575del	>75%
40	Peritoneum	High	Spindle	Ex 11/W557_K558del	>75%
42	Small bowel	Intermediate	Spindle	Ex 11/N566_P573del	>75%
43	Stomach	High	Spindle	Ex 11/E554_K558del	>75%
44	Stomach	Low	Spindle	Ex 11/V559_G565del	>75%
46	Small bowel	Low	Spindle	Ex 11/Q575_W582delinsR	>75%
47	Stomach	High	Spindle	Ex 11/K558delinsNP	>75%
50	Small bowel	Very low	Spindle	Ex 11/W557_K558del	>75%
51	Stomach	High	Spindle	Ex 11/P551_V555ins	25-75%
55	Colorectum	Very low	Spindle	Ex 11/W557_K558del	>75%
56	Stomach	Intermediate	Spindle	Ex 11/W557G	>75%
59	Small bowel	Very low	Spindle	Ex 11/K550_E554del	>75%
60	Colorectum	Intermediate	Spindle	Ex 11/K550_E554del	>75%
61	Small bowel	Low	Spindle	Ex 11/W557_K558del	>75%
65	Peritoneum	Intermediate	Epithelioid	Ex 11/W557_K558del	>75
70	Colorectum	Very low	Spindle	Ex 11/W557_K558del	<25%
88	Stomach	Low	Epithelioid	Ex 11/K550_E554delV555L	<25%
93	Stomach	High	Spindle	Ex 11/W557_K558del	>75%
97	Small bowel	High	Spindle	Ex 11/D579N	>75%
99	Small bowel	Low	Spindle	Ex 11/W557R	>75%
101	Stomach	Malignant	Spindle	Ex 11/V560D	>75%
104	Small bowel	Intermediate	Epithelioid	Ex 11/E554_N564delinsD	>75%
127	Retroperitoneum	High	Mixed	Ex 11/W557R	>75%
129	Stomach	Intermediate	Spindle	Ex 11/W557_K558del	Na
138	Stomach	Very low	Spindle	Ex 11/N566Y	Na
144	Stomach	High	Mixed	Ex 11/K550_W557del	>75%
147	Stomach	Intermediate	Mixed	Ex 11/N566D	25-75%
148	Stomach	Low	Spindle	Ex 11/E554_V555del	>75%
149	Small bowel	Low	Spindle	Ex 11/E562V	>75%
151	Small bowel	Low	Spindle	Ex 11/W557_K558del	>75%
PDGFRA					
45	Stomach	Very low	Epithelioid	Ex 12/V561A	25-75%
57	Stomach	Low	Epithelioid	Ex 18/D842V	25-75%
87	Stomach	Low	Epithelioid	Ex 12/V561D	25-75%
163	Stomach	Very low	Epithelioid	Ex12/S582_E585del	25-75%

NA: Not available.

females (60.0%) and the mean age was 55.5 (±SD 13.2) years. The most common sites of primary tumors were the stomach (47.3%) and small intestine (36.4%), followed by colorectal (9.1%) and nongastrointestinal (7.3%). In most cases, the morphology comprised spindle cells (70.4%). Twenty-three patients (41.8%) had very low- or low-risk, 12 (21.8%) intermediate-risk and 20 (36.4%) had high-risk or overtly malignant tumors. Overtly malignant GISTs were tumors that had already metastasized at the time of surgery. Six out of 55 patients (21.8%) were classified as having overtly malignant tumors. In these patients, all metastatic lesions were completely excised at surgery. Twelve (21.8%)

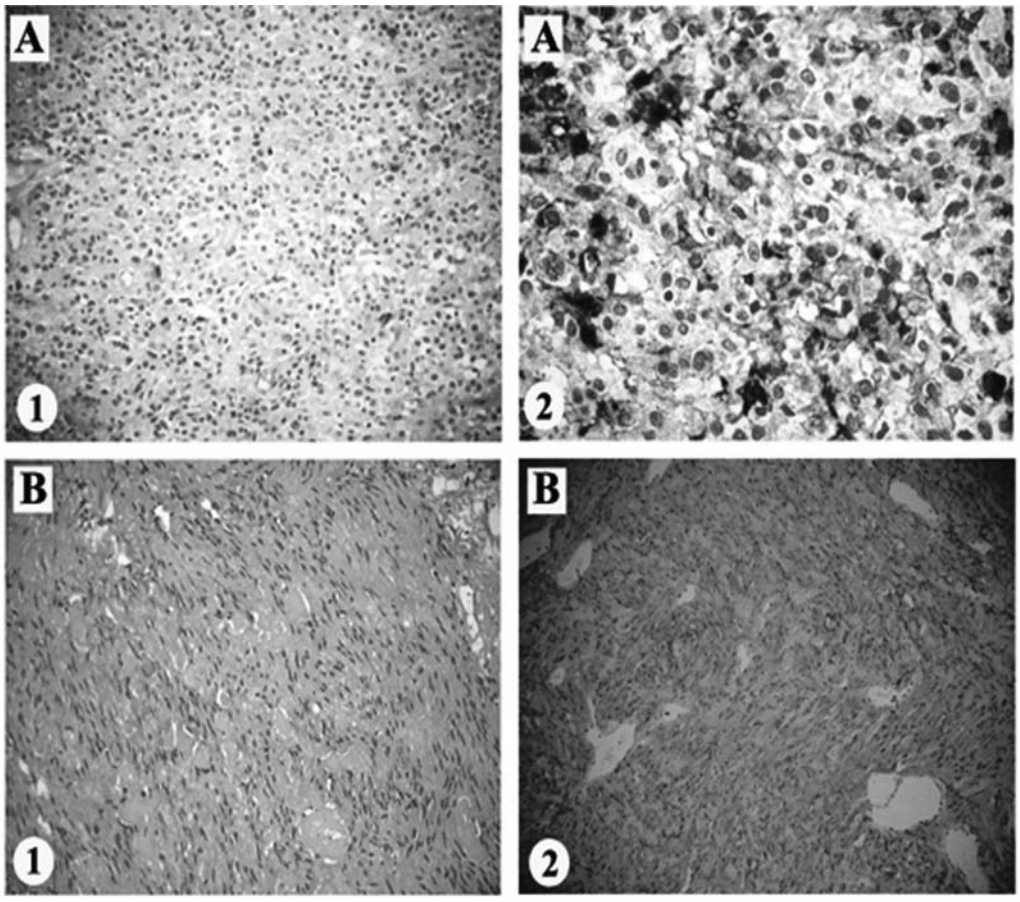

Figure 1. *A: Epithelioid gastrointestinal stromal tumor with exon 12 PDGFRA mutation (S582_E585del): Hematoxylin and eosin (HE) staining (1) and a weak and focal cytoplasmic staining for KIT (2). B: Spindle cell gastrointestinal stromal tumor with exon 11 KIT mutation (E562V): HE staining (1) and strong and diffuse cytoplasmic staining for KIT (2).*

patients were treated with imatinib for recurrent disease. No patient was treated with a tyrosine kinase inhibitor before developing recurrence.

KIT and PGDFRA mutations. The complete list of *KIT* and *PDGFRA* mutations is shown in Table II. *KIT* mutations were identified in 41 out of 55 tumors (74.5%). *KIT* mutations were found at the extracellular (exon 9) and at the juxtamembrane domains (exon 11), in two (3.6%) and 39 (70.9%) cases, respectively. No mutations were found in exons 13 and 17, which encode for the intracellular catalytic domains. In exon 9, both cases possessed the same Ala^{502} – Tyr^{503} in-frame insertion. Both mutations occurred in tumors originating from the small intestine. One of the tumors had metastasized at the time of surgery and it was considered as overtly malignant tumor. The other tumor was of low-risk at the time of the resection. At exon 11, a total of 23 in-frame deletions, 9 missense mutations, one in-frame insertion and six complex mutations (five concomitant in-frame deletions and insertions and one in-frame deletion plus missense mutation) were identified. Most of the mutations were located between codons 557 and 566 (29 out of 39 cases with exon 11 abnormalities), mainly involving codons 557-558 (25 out of 29 cases with mutations located between codons 557 and 566). In-frame deletions or concomitant in-frame deletion and insertion (deletion-insertion) affecting codons 557-558 occurred in 22 out of 39 (56.4%) cases with exon 11 mutations.

We found *PDGFRA* mutations in 4 out of 55 (7.3%) cases being 3 missense and 1 in-frame deletion. Three out of four mutations (75.0%) were located in exon 12 and no mutations were found in exon 14. All tumors with *PDGFRA* mutations were epithelioid and originated in the stomach. All four cases having *PDGFRA* mutations showed moderately positive staining for KIT by immunohistochemistry (Figure 1A2) in contrast with cases that harbored *KIT* activating mutations, which predominantly showed intense positive staining for *KIT* (32/39) (p=0.010) (Figure 1B2).

Table III. *Prognostic significance of genetic alterations, risk, anatomic site, tumor cell type, age and sex of patients with GISTs.*

		Survival			
Variables	N	Median (months)	Median (months)	5-Year (%)	*P*-value
Age, years	≤65	40	43	46.0	0.098
	>65	15	NR	75.0	
Gender	Male	22	NR	58.9	0.246
	Female	33	43	48.5	
Site	Stomach	26	NR	52.6	0.722
	Other	29	NR	52.6	
Risk	Low and very low	23	NR	76.7	**0.007**
	Intermediary	12	43	36.4	
	High and overtly malignant	20	19	31.9	
Histological subtype	Spindle	38	NR	54.5	0.444
	Mixed	7	19	33.3	
	Epithelioid	9	NR	66.7	
KIT	Wild-type	14	NR	66.7	0.259
	Mutation	41	49	47.9	
Exon 11	Wild-type	16	NR	64.6	0.333
	Exon 11 mutation	39	49	47.5	
Exon 11	Deletion	23	NR	50.6	0.473
	Other	16	43	43.0	
Exon 11	W557_K558del	12	47	42.9	0.845
	Other	27	49	49.5	
Exon 11	557_558del	18	47	36.1	0.384
	Others	21	NR	57.4	
Exon 11	557_558del or del-ins	22	30	28.9	**0.023**
	Other	17	NR	73.1	
Exon 11	557_558del-ins	4	12	0	**0.003**
	Other	35	NR	53.7	
PDGFRA	Wild-type	51	NR	48.4	0.090
	Mutation	4	NR	100	

del: Deletion; del-ins: deletion-insertion; NR: not reached.

Prognostic value of tumor characteristics, KIT and PDGFRA mutations. The median follow up was 60 months. Recurrent disease after surgery was seen in 23 (41.8%) patients. The 5-year DFS rate for patients overall was 52.8%. As expected, we observed a significant correlation between malignant behavior and 5-year DFS rates (p=0.007). The high-risk and overtly malignant group had a lower 5-year DFS rate (31.9%) than did the intermediate (36.4%) or low- and very low-risk groups (76.7%) (p=0.007) (Table III and Figure 2A).

Regarding *KIT* mutations, we did not observe any association between the 5-year DFS and the presence of mutation (mutated *KIT*, 47.9% *versus* wild-type *KIT*, 66.7%; p=0.259). Moreover, we did not find any impact on 5-year DFS rates for the presence of exon 11 *KIT* mutations (47.5%) *versus* wild-type exon 11 (64.6%) (p=0.333) nor for the type of mutation affecting exon 11 (deletions, 50.6% *versus* others exon 11 *KIT* mutations, 43.0%; p=0.473) (Table III). However, the 5-year DFS rate was significantly lower in patients with tumors having in-frame deletions or concomitant in-frame deletions and insertions affecting codons 557-558 (28.9%) than in patients with tumors having other exon 11 *KIT* mutations (73.1%, p=0.023; Table III and Figure 2B). Conversely, when the patients (n=4) with concomitant deletion-insertion mutations affecting codons 557-558 were excluded from the analysis, deletions involving codons 557-558 had no influence on 5-year DFS rates (p=0.384). The four patients with concomitant deletion-insertion mutations affecting codons 557-558 developed recurrent disease at 2 (n=1), 12 (n=2) and 24 (n=1) months after surgery.

In our cohort, *PDGFRA* mutations had no influence on 5-year DFS rate (p=0.090; Table III). Finally, age, sex, anatomical site of tumors and histological subtype had no influence on DFS for the whole group (Table III).

Discussion

Gain-of-function mutations in *KIT* and *PDGFRA* have been implicated in GIST carcinogenesis and have become increasingly important as predictors of clinical response to inhibitors of tyrosine kinase receptor. However, the value of

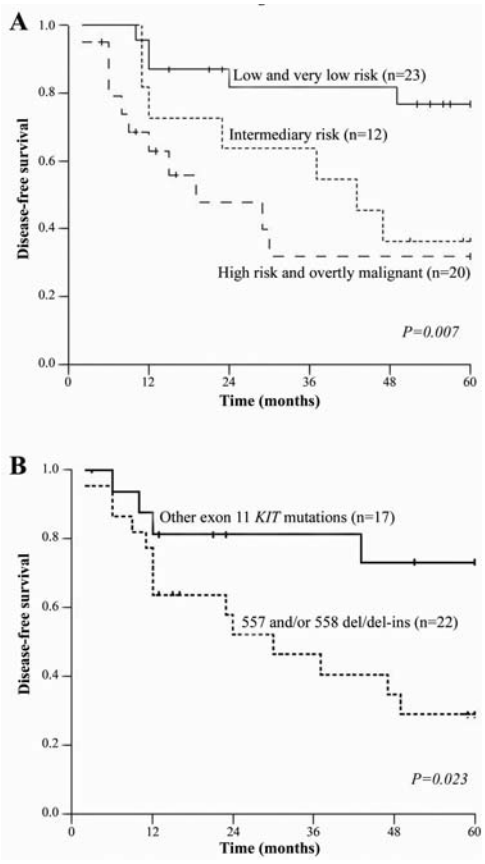

Figure 2. *A: Kaplan-Meier curve for GIST patients according to index of risk. B: Kaplan-Meier curves for GIST patients with deletion or deletion-insertion (del-ins) involving codons 557-558 of KIT exon 11 and with other KIT exon 11 mutations.*

KIT and *PDGFRA* mutation for predicting recurrence after primary tumor resection is still unclear. In the present study, we selected a Brazilian population-based series with completely resected primary GIST and complete follow up information to correlate mutational status of *KIT* exons 9, 11, 13 and 17 and *PDGFRA* exons 12 and 18 with DFS. Recently, Lopes *et al*. characterized a large group of 513 Brazilian GISTs for different clinicopathological and molecular aspects. Among them, 74 cases were analyzed for mutations of *KIT* exons 9 and 11 and *PDGFRA* exon 18 but their prognostic value was not investigated (14).

Previous studies have shown *KIT* exon 11 mutations in 20 to 92% of patients (15). The disparity between studies regarding the prevalence of mutations might be explained by the use of different approaches in the detection of mutations, it being suggested that the use of indirect detection methods such as single-strand conformational polymorphism leads to an under-representation of the mutation prevalence (16). Therefore, to avoid false-negative results, we analyzed all tumors by direct sequencing. *KIT* mutations were identified in 74.5% of cases, being predominantly localized in exon 11 (70.9%). Only 3.6% of *KIT* mutations were localized in exon 9, which is consistent with previous results (range of 3.3% to 18.1% (17-19)). Regarding *PDGFRA* gene, interestingly, in contrast to previous studies, the region encoding the juxtamembrane domain (exon 12) was the most common site of mutation in the present study (20, 21). There are very few data indicating possible differences in *PDGFRA* mutation profiles between different populations (22). Further studies should be carried out to better address this issue.

Previous studies have demonstrated that the presence of *KIT* mutation was an independent factor for prognosis in patients with localized GIST (23, 24). In contrast, DeMatteo *et al*. showed that the presence of any *KIT* mutation did not predict recurrence by univariate analysis (25). In our series, we did not demonstrate any association between the 5-year DFS and the presence of *KIT* mutation.

Few studies have also described adverse impact of different types of *KIT* exon 11 mutations (26-28). Andersson *et al*. indicated that the finding of *KIT* exon 11 deletions is an independent adverse prognostic factor in patients with GIST and Wardelmann *et al*. described the prognostic impact of deletions specifically involving Trp557 and/or Lys558 (27, 28). The latter authors demonstrated an association between a specific *KIT* exon 11 mutation affecting codons 557-558 (K557-W558del) and unfavorable GIST clinical outcome (27). DeMatteo *et al*. demonstrated a significant correlation between *KIT* exon 11 deletion at codons 557 or 558 and DFS on univariate analysis but were unable to find any independent correlation on multivariate analysis (25). In our cohort, the prognostic value of deletions involving codons 557-558 was only shown when complex mutations (concomitant deletion and insertion) were included in the analysis. Although W557-K558del represents the majority of in-frame deletions involving codons 557-558, they had no impact on 5-year DFS in our cohort. However, patients with tumors having these complex mutations (concomitant deletion and insertion) involving codons 557-558 had an unfavorable clinical outcome. K558delinsNP was one of the complex mutations involving 557-558 found in our cohort. Such mutation was previously reported and has been shown to cause constitutive KIT tyrosine phosphorylation and sensitivity to imatinib *in vitro* (4). In addition, Lasota *et al*. recently showed that short insertions exclusively involving *KIT* codon 558, including K558delinsNP, are rare but might indicate an increased risk for malignant behavior in gastric GIST (29). The biological potential and sensitivity to imatinib of tumors bearing the other deletion-insertion mutations found in the present study still need to be investigated.

In conclusion, with respect to DFS, in-frame deletion and in-frame deletion–insertion affecting codons 557-558 in *KIT* was associated with poor prognosis in GIST. Once data are confirmed on multivariate analysis, this information can be used to select patients appropriate for adjuvant therapy.

Acknowledgements

We thank MEF Haussmann (Instituto Nacional de Câncer) for technical assistance. We are grateful to Dr. José Cláudio Casali da Rocha for fruitful discussion on part of the data presented in this manuscript. DA Braggio was recipient of Training Program Awards of the Ministry of Health at the Instituto Nacional do Câncer.

Conflict of Interest Statement

The study was supported by an unrestricted grant from Novartis Brasil

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Received October 16, 2009
Revised April 27, 2010
Accepted April 30, 2010