

ARTICLE

# Molecular alterations and expression of succinate dehydrogenase complex in wild-type *KIT/PDGFR A/BRAF* gastrointestinal stromal tumors

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Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal neoplasms of the gastrointestinal tract, disclosing somatic *KIT*, *PDGFRA* and *BRAF* mutations. Loss of function of succinate dehydrogenase (SDH) complex is an alternative molecular mechanism in GISTs, namely in carriers of germline mutations of the SDH complex that develop Carney–Stratakis dyad characterized by multifocal GISTs and multicentric paragangliomas (PGLs). We studied a series of 25 apparently sporadic primary wild-type (WT) *KIT/PDGFR A/BRAF* GISTs occurring in patients without personal or familial history of PGLs, re-evaluated clinicopathological features and analyzed molecular alterations and immunohistochemistry expression of SDH complex. As control, we used a series of well characterized 49 *KIT/PDGFR A/BRAF*-mutated GISTs. *SDHB* expression was absent in 20% and *SDHB* germline mutations were detected in 12% of WT GISTs. Germline *SDHB* mutations were significantly associated to younger age at diagnosis. A significant reduction in *SDHB* expression in WT GISTs was found when compared with *KIT/PDGFR A/BRAF*-mutated GISTs. No significant differences were found when comparing *DOG-1* and c-*KIT* expression in WT, *SDHB*-mutated and *KIT/PDGFR A/BRAF*-mutated GISTs. Our results confirm the occurrence of germline *SDH* genes mutations in isolated, apparently sporadic WT GISTs. WT *KIT/PDGFR A/BRAF* GISTs without *SDHB* or *SDHA/SDHB* expression may correspond to Carney–Stratakis dyad or Carney triad. Most importantly, the possibility of PGLs (Carney–Stratakis dyad) and/or pulmonary chondroma (Carney triad) should be addressed in these patients and their kindred.

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## INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal neoplasms of the gastrointestinal tract.<sup>1</sup> Somatic gain-of-function mutations of v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (*KIT*) or of platelet-derived growth factor receptor and alpha polypeptide (*PDGFRA*) genes are the most prevalent molecular alterations occurring in GISTs<sup>2–4</sup> and observed in a mutually exclusive manner.<sup>2</sup> *KIT* mutations are described in 41–92% GISTs<sup>5–10</sup>, whereas *PDGFRA* mutations are present in 5–8% GISTs.<sup>11</sup> *KIT* protein expression is reported in >95% GISTs, including wild-type (WT) tumors and most *KIT*- and *PDGFRA*-mutated GISTs.<sup>12</sup>

Treatment of *KIT*- and *PDGFRA*-mutated GISTs with the tyrosine kinase inhibitor (TKI) imatinib mesylate has shown a great efficiency in patients,<sup>13</sup> but GISTs without *KIT/PDGFR A* somatic mutations may be resistant to treatment with imatinib.<sup>14,15</sup> Sunitinib was approved as an alternative TKI for the treatment of patients with resistance or intolerance to imatinib.<sup>16</sup> This resistance and/or intolerance to imatinib reinforce the concept that other molecular events rather than *KIT* or *PDGFRA* mutations may be implicated in GIST tumorigenesis.

The first studies indicating an alternative pathway involved in GIST tumorigenesis were related to a molecular alteration occurring in the mitogen-activated protein kinase pathway: *BRAF*<sup>V600E</sup> somatic mutation was reported in 3–7% *KIT/PDGFR A* WT GISTs<sup>17–19</sup> and in one *KIT*-mutated GISTs.<sup>20</sup>

Recently, loss of function of succinate dehydrogenase (SDH) complex was proposed as another alternative molecular mechanism in *KIT/PDGFR A/BRAF* WT GISTs.<sup>21–27</sup> In Carney–Stratakis dyad, patients develop multifocal GISTs and paraganglioma (PGL),<sup>28,29</sup> and GISTs are known to display deficient SDH protein expression.<sup>22</sup> Further molecular studies addressing Carney–Stratakis syndrome patients disclosed the presence of germline mutations of the SDH subunits B (*SDHB*), C (*SDHC*) and D (*SDHD*).<sup>25,27</sup> These GIST patients do not harbor *KIT* or *PDGFRA* mutations.<sup>25</sup> In the Carney triad (PGLs, GISTs and pulmonary chondromas)<sup>30</sup> patients also present SDH-deficient GISTs<sup>22,23</sup> but *SDHA*, *-B*, *-C* and *-D* mutations have not been described so far.<sup>31,32</sup>

We studied a series of 25 apparently sporadic WT primary *KIT/PDGFR A/BRAF* GISTs occurring in patients without personal or familial history of PGLs and pulmonary chondromas, re-evaluated

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their clinicopathological features and evaluated protein expression and molecular alterations of the SDH complex.

## MATERIALS AND METHODS

From a series of 78 primary GISTs previously characterized by our group,<sup>33</sup> we selected 25 WT *KIT/PDGFR $\alpha$ /BRAF* GISTs (WT GISTs) with tumors located in the stomach ( $n=16$ ), small intestine ( $n=7$ ), colon ( $n=1$ ) and in undetermined location ( $n=1$ ). Two WT GISTs (cases 4 and 22) had multifocal presentation, and one (case 22) had metastasis in the colon and omentum. Follow-up of the patients was updated and tumors were re-evaluated according to their clinicopathological features (Table 1). The risk behavior of GIST recurrence was evaluated according to the National Institutes of Health (NIH) risk classification<sup>1</sup> and the National Comprehensive Cancer Network (NCCN) risk classification.<sup>34</sup>

Three patients with WT GISTs were treated with imatinib mesylate. One (case 2) was treated with imatinib mesylate after recurrence of primary tumor, but the patient died due to unrelated comorbidity. Adjuvant imatinib was used in another patient (case 3) that is alive without evidence of recurrence at last follow-up. Neoadjuvant imatinib was used in another patient (case 12) submitted to complete tumor surgical resection after maximal response, and the patient is alive without evidence of recurrence at last follow-up.

Immunohistochemistry (IHC) for SDHA and SDHB proteins (Figure 1a and b) was performed in representative tumor tissue sections of all 25 WT GISTs, as previously described.<sup>21</sup> Negative and positive controls were used simultaneously to ensure specificity and reliability of the staining process. Previously tested positive cases of oncocyctic variant of papillary thyroid carcinoma were used as positive controls. Omission of the primary antibody was used as negative control.

IHC for DOG-1 protein was performed in 23 WT GISTs. Briefly, tissue sections with 2  $\mu$ m thickness were deparaffinized, rehydrated and pre-treated with 1  $\times$  Epitope Retrieval Solution pH 9 (Tris/EDTA-based buffer containing surfactant) (E7119; Leica Microsystems, Newcastle upon Tyne, UK) in a pressure cooker at 98 °C for 1 min, and at 125 °C for 5 min. Immunohistochemical staining was performed with the Novocastra Novolink Polymer Detection System (RE7140-CE; Leica Microsystems), according to the manufacturer's instructions. The tissue sections were incubated 1 h at room temperature with DOG-1 primary antibody (clone K9, mouse, 1:100 dilution; NCL-L-DOG-1; Leica Microsystems). Detection was performed with Novocastra Novolink Polymer Detection Systems, according to the manufacturer's instructions, and the samples were developed with DAB chromogen. The slides were mounted using a Richard-Allan Scientific Mounting Medium (Fisher Scientific GmbH, Schwerte, Germany), after counterstaining with hematoxylin. Expression of DOG-1 was evaluated and compared with the expression of KIT in matched tumor sections. The data regarding KIT expression was reported previously by our group<sup>33</sup> in a study comprising the tumors from the present series.

IHC for SDHA, SDHB and DOG-1 protein was performed also in 49 *KIT/PDGFR $\alpha$ /BRAF*-mutated GISTs from our previously reported series<sup>33</sup> that were used as control. IHC evaluation was performed independently by two observers (JML and VM). For SDHA and SDHB protein expression, an IHC score was established, which corresponded to the sum of the intensity of expression (negative = 0, faint/moderate = 1, strong = 2) with the extent of each tumor protein expression (0–5% = 0; 5–25% = 1, 26–50% = 2, 51–75% = 3, > 75% = 4). Expression of SDHA and SDHB was classified as negative, low, moderate and high, when the sum of IHC score was 0,  $\leq 2$ , 3–4 and > 4, respectively.

Tumor DNA was extracted from all 25 WT GISTs as previously described,<sup>21</sup> and all the cases were evaluated by PCR and DNA sequencing for the presence of *SDHB*, *SDHC* and *SDHD* point mutations and deletions of exon 1 in *SDHB*, as previously described.<sup>35,36</sup> Briefly the PCR mixture, 25  $\mu$ l in total, contained 100 ng of genomic DNA, 1  $\mu$ l dNTP's (5 mM each; Bioron GmbH, Ludwigshafen, Germany), 1  $\mu$ l of each primer (10  $\mu$ M),<sup>36</sup> 5  $\mu$ l 5  $\times$  Green GoTaq Flexi Buffer, 2.5  $\mu$ l MgCl<sub>2</sub> (25 mM) and 0.15 U GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA). The respective PCR product was purified using the GFX PCR DNA and gel band purification kit (GE Healthcare, Buckinghamshire, UK). The purified PCR product was

subjected to automatic sequencing (ABI PRISM 3100 genetic analyzer; Applied Biosystems, Foster City, CA, USA), using the BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems). Sense and antisense sequencing was performed. All mutations were further verified by PCR and sequencing from a new DNA template.

In GISTs with mutations of *SDHB*, *SDHC* or *SDHD*, matched adjacent non-tumor tissue DNA was also obtained. Additionally, germline DNA from peripheral blood lymphocytes was obtained from patients in cases 1–5 by the standard proteinase K-SDS digestion and saline precipitation.<sup>37</sup> In patients (cases 4 and 5) with SDHA- and SDHB-negative tumors, *SDHA* mutations were screened in germline DNA of the peripheral blood lymphocytes, as previously described.<sup>38</sup> The presence of mutations was validated by a second PCR followed by direct sequencing.

Given that GIST cases 2 and 3 carried *SDHB* germline mutation, the loss of the other WT allele of the *SDHB* gene was evaluated. Tumor DNA was analyzed for intragenic deletions using multiplex-ligation-dependent probe amplification (MLPA) assay (SALSA MLPA KIT, P226SDHD, MRC-Holland b.v., Amsterdam, The Netherlands), according to the manufacturer's instructions. MLPA fragments were discriminated in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems), and the resulting data was analyzed using Coffalyser software (MRC-Holland b.v.). All MLPA results were reproduced at least three times.

Fisher's exact test, non-parametric Mann–Whitney *U* test and parametric independent samples *t*-test were used for the statistical analysis of the results. Specific survival time analysis was determined by Kaplan–Meyer and log-rank tests with SPSS for Windows, V.19.0 (SPSS Inc., Chicago, IL, USA). Values were considered significantly different when  $P < 0.05$ .

## RESULTS

### Clinicopathological features of WT GISTs

We studied 25 apparently sporadic primary WT *KIT/PDGFR $\alpha$ /BRAF* GISTs (WT GISTs) whose clinicopathological features are summarized in Table 1. The gender ratio of the patients (male:female) was 1.8:1. The median age of the patients was 62 (range 26–82) years, the median size of the tumors was 6 cm, and the median mitotic index (mitoses per 50 high-power fields) was 4. The tumors were located in the stomach ( $n=16$ ), small intestine ( $n=7$ ), colon ( $n=1$ ) and undetermined site ( $n=1$ ). Eight percent (2/25) of the patients had multiple GISTs and one patient (4%–1/25) had metastases in the colon and omentum at presentation. Histologic morphology classification of the tumors revealed epithelioid, spindle and mixed (epithelioid/spindle) GISTs in 20% (5/25), 68% (17/25) and 12% (3/25) of the cases, respectively.

Tumors located in the stomach displayed a significantly higher mean mitotic index (mean 6) than tumors of the small intestine (mean 2) ( $P=0.022$ ). However, when SDHB-negative WT *KIT/PDGFR $\alpha$ /BRAF* GISTs (all located in stomach) were excluded from the analysis, no significant difference was detected in the mitotic index between WT *KIT/PDGFR $\alpha$ /BRAF* GISTs located in the stomach and those located in the small intestine ( $P=0.094$ ).

The size (19 cm) of the single metastatic tumor was significantly ( $P=0.04$ ) larger than the mean sizes of the other non-metastatic primary WT GISTs ( $6.99 \pm 0.78$  cm) at clinical presentation. Tumors from patients with macroscopic residual tumor (R2; non-curative surgery) revealed significantly ( $P=0.037$ ) larger mean dimensions ( $15.75 \pm 3.25$  cm) than tumors from patients with no macroscopic residual tumor (R0; potentially curative surgery) ( $6.79 \pm 0.89$  cm).

Patients with high risk of recurrence according to the NIH classification were significantly ( $P=0.049$ ) younger ( $53.0 \pm 5.4$  years) than patients of lower risk groups ( $65.6 \pm 3.3$  years). According to the risk NCCN classification, patients with high risk of recurrence were also younger ( $52.1 \pm 7.5$  years) than patients of lower recurrence risk

**Table 1 Clinicopathological features of 25 patients with *KIT/PDGRFA/BRAF* wild-type GIST**

Case	Gender	Age (years)	Primary site	Tumor largest size (cm)	Histologic cell subtype	Mitosis/50 HPF	NIH risk classification <sup>b</sup>	NCCN risk classification <sup>b</sup>	Molecular alterations SDHB, SDHC, SDHD	SDH IHC expression		Curative surgery -R classification	Follow-up (months/status)
										SDHA	SDHB		
1	M	26	Stomach	6	Spindle	16/50	High	High	Pos (germline) <sup>c</sup> c.1282A, p.Ile44Asn	High	Negative	R0	104/ANED
2	M	30	Stomach	10	Epithelioid	8/50	High	High	Pos (germline) <sup>d</sup> SDHB c.1-10413_73-3866del	High	Negative	R0	156/DUC
3	M	42	Stomach	3	Spindle	12/50	High	Moderate	Pos (germline) <sup>e</sup> SDHB c.1-10413_73-3866del	High	Negative	R0	94/ANED
4	M	49	Stomach	6.5	Epithelioid	7/50	High	High	Neg <sup>f</sup>	Negative	Negative	R0	271/ANED
5	F	58	Stomach	3.5	Epithelioid	1/50	Low	Very low	Neg <sup>f</sup>	Negative	Negative	R1	198/ANED
6	M	75	Duodenum	>3	Spindle	1/50	NA	NA	Pos (somatic) SDHB c.1-10413_73-3866del	Moderate	Moderate	NR	9/DOD
7	F	77	Small intestine	6	Spindle	1/50	Intermediate	Moderate	Neg	High	High	R0	132/DUC
8	M	74	Stomach	3	Mixed	5/50	Low	Very low	Neg	High	High	R0	120/DUC
9	F	72	Stomach	8	Spindle	4/50	Intermediate	Low	Neg	High	High	R1	35/ANED
10	F	53	Small intestine	3.5	Spindle	4/50	Low	Low	Neg	High	Moderate	R0	124/ANED
11	M	62	Stomach	6.5	Spindle	5/50	Low	Low	Neg	High	Low	R0	111/ANED
12	M	73	Small intestine	6	Spindle	3/50	Intermediate	Moderate	Neg	High	High	R0	106/ANED
13	F	66	Stomach	14	Spindle	5/50	High	Moderate	Neg	Moderate	Low	R0	129/ANED
14	F	63	Small intestine	6.5	Mixed	2/50	Intermediate	Moderate	Neg	High	Moderate	R0	270/ANED
15	M	52	Small intestine	9.5	Spindle	4/50	Intermediate	Moderate	Neg	High	Moderate	R0	213/ANED
16	M	55	Small intestine	4	Spindle	1/50	Low	Low	Neg	High	Moderate	R0	66/DUC
17	F	50	Stomach	5.6	Spindle	6/50	High	High	Neg	High	Moderate	R1	198/ANED
18	F	82	Stomach	7	Epithelioid	5/50	Intermediate	Low	Neg	Low	Low	R0	80/DUC
19	M	81	Stomach	3.3	Spindle	1/50	Low	Very low	Neg	Moderate	Moderate	R0	75/DUC
20	M	81	Colon	12.5	Epithelioid	12/50	High	High	Neg	High	Moderate	R2	0/DOD
21	M	60	ND	13	Mixed	4/50	High	High	Neg	High	Low	NR	48/DOD
22	M	69	Stomach	19	Spindle	8/50	High	High	Neg	High	Moderate	R2	4/DOD
23	M	76	Stomach	6	Spindle	2/50	Intermediate	Low	Neg	Moderate	Low	R0	105/ANED
24	F	41	Stomach	4.5	Spindle	2/50	Low	Very low	Neg	High	Moderate	R0	94/ANED
25	M	57	Stomach	17	Spindle	2/50	High	Moderate	Neg	Moderate	Moderate	R0	107/ANED

Abbreviations: ANED, alive, no evidence of disease; DUC, dead of unrelated causes; DOD, dead of disease; F, female; GIST, gastrointestinal stromal tumor; HPF, high-power field; IHC, immunohistochemistry; M, male; NA, not applicable; NCCN, National Comprehensive Cancer Network; ND, not determined; Neg, negative; NIH, National Institutes of Health; NR, no resection; Pos, positive; R0, no macroscopic residual tumor (potentially curative surgery); R1, microscopic residual tumor; R2, macroscopic residual tumor (non-curative surgery); SDH, succinate dehydrogenase.

<sup>a</sup>NIH risk classification<sup>b</sup> for primary GIST.

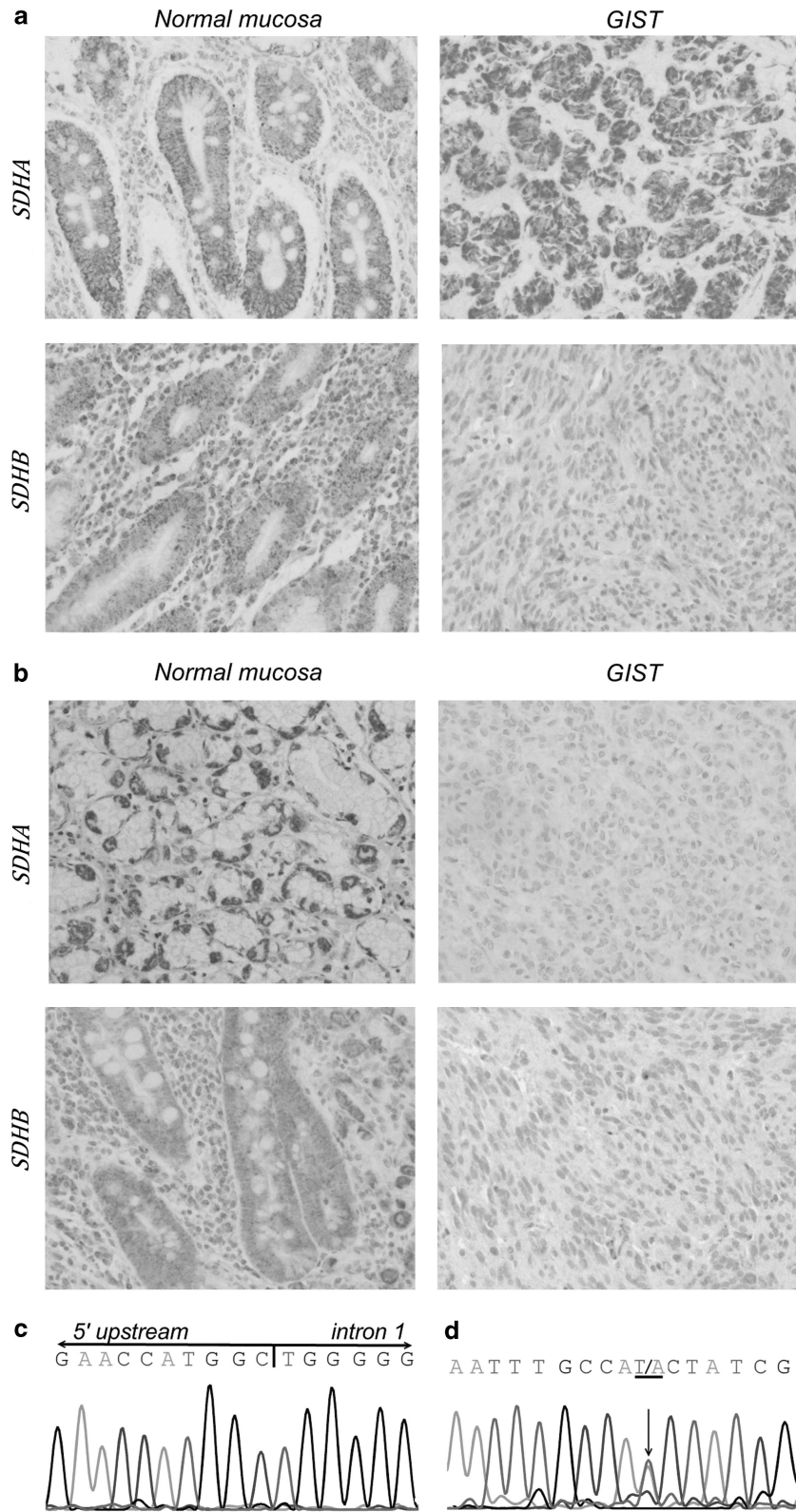
<sup>b</sup>NCCN risk classification<sup>c</sup> for primary GIST.

<sup>c</sup>Loss of heterozygosity (LOH) of the *SDHB* normal allele in the tumor.

<sup>d</sup>Tumor with complete loss of *SDHB* promoter and exon 1 (MLPA analysis).

<sup>e</sup>Tumor with complete loss of *SDHB* promoter and loss heterozygosity (LOH) exon 1 (MLPA analysis).

<sup>f</sup>Screening of mutations in *SDHA* exons performed in germline DNA of the patient peripheral blood lymphocytes.



**Figure 1** SDHA and SDHB protein expression in the adjacent non-tumor gastric mucosa and GIST tumor cells of case 2 (a) and case 4 (b). Note the negative expression of SDHB in the tumor cells of case 2, and the negative expression of SDHB and SDHA in the tumor cells of case 4. Original magnification:  $\times 200$ . Electropherogram representative of *SDHB* c.1-10413\_73-3866del (c) and *SDHB* c.T282A mutation (d).

groups ( $63.8 \pm 3.1$  years), although not reaching statistical significance ( $P = 0.1$ ).

The mean follow-up of the patients was  $114 \pm 15$  months. At the last follow-up (December, 2011), 15 out of 25 (60%) patients were alive, with no evidence of disease, and 10 patients (40%) were dead. In four patients, deaths were due to GIST progression, and in the remaining by unrelated tumor patient comorbidities. The 5-year specific disease survival of the 25 patients was 83.8%. In the univariate analysis, patients with high-risk tumors (NIH and NCCN classifications) displayed significantly poorer prognosis ( $P = 0.032$  and  $P = 0.004$ , respectively) than patients with lower risk WT GISTs.

It is noteworthy that apart from the aforementioned metastatic lesions, no other tumors (pulmonary chondromas, PGLs) were found in clinical, pathologic and imaging (X-ray and CT) evaluations.

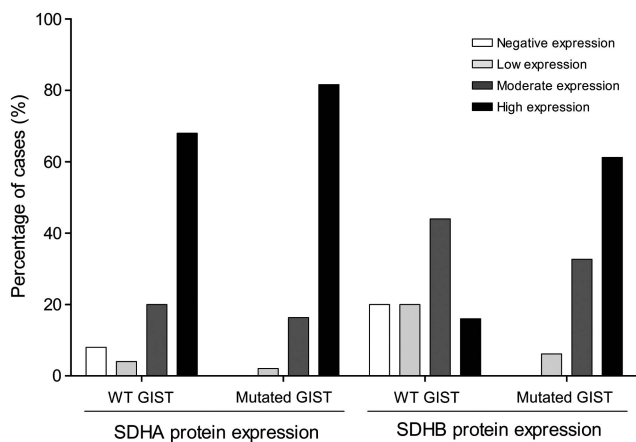
### SDHA, SDHB and DOG-1 protein expression in GISTs

WT GISTs displayed high (16%), moderate (44%), low (20%) and negative (20%) SDHB expression; and high (68%), moderate (20%), low (4%), and negative (8%) SDHA expression (Figure 2). Five out of the 25 (20%) tumors did not express SDHB (Table 1, Figure 2). Two out of the 5 (40%) SDHB-negative WT GISTs were also negative for SDHA expression.

Patients with SDHB-negative tumors were significantly ( $P = 0.002$ ) younger ( $41.0 \pm 5.9$  years) than patients with SDHB-positive tumors ( $66.0 \pm 2.6$  years). The mitotic index of GISTs without SDHB expression (mean 9) was significantly ( $P = 0.05$ ) higher than GISTs positive for SDHB expression (mean 4). Absence of SDHB expression was more frequent ( $P = 0.038$ ) in tumors composed exclusively by epithelioid cells.

We evaluated SDHA and SDHB expression in 49 cases of *KIT/PDGFR $\alpha$ /BRAF*-mutated GISTs previously described by our group,<sup>33</sup> and all were positive for both proteins: high (61%), moderate (33%) and low (6%) expression of SDHB; and, high (82%), moderate (16%) and low (2%) expression of SDHA (Figure 2).

The absence of SDHB expression was significantly ( $P < 0.001$ ) associated to WT GISTs when compared with *KIT/PDGFR $\alpha$ /BRAF*-mutated GISTs. Even excluding WT GISTs negative for SDHB and SDHA/SDHB expression, we found a significant ( $P = 0.003$ )



**Figure 2** Frequencies (%) of wild-type and mutated *KIT/PDGFR $\alpha$ /BRAF* GISTs with negative, low, moderate and high tumor cell expression of SDHA and SDHB proteins.

reduction of SDHB expression in WT GISTs when compared with *KIT/PDGFR $\alpha$ /BRAF*-mutated GISTs.

In addition, no differences were found in DOG-1 and c-KIT expression when comparing WT, *SDHB*-mutated and *KIT/PDGFR $\alpha$ /BRAF*-mutated GISTs. Overall, 93% (69/74) of GISTs expressed KIT and 90% (65/72) expressed DOG-1. In WT GISTs, 92% (23/25) and 83% (19/23) of the tumors expressed KIT and DOG-1, respectively, and 94% (46/49) *KIT/PDGFR $\alpha$ /BRAF*-mutated GISTs expressed both KIT and DOG-1. Expression of KIT was significantly ( $P = 0.045$ ) associated with DOG-1 expression in this cohort of GIST patients.

### SDH molecular alterations in WT GISTs

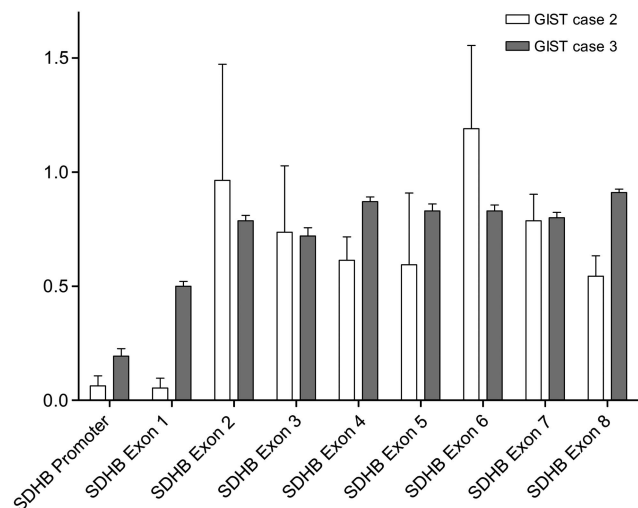
Four out of 25 (16%) WT GISTs displayed *SDHB* mutations (Table 1). No somatic or germline *SDHC* and *SDHD* mutations were detected in these four cases.

Three patients (12%–3/25) were carriers of germline *SDHB* mutations (Table 1): two (case 2 and 3) carried a germline deletion encompassing the promoter region and exon 1 of *SDHB* (c.1-10413\_73-3866del; Figure 1c) and one (case 1) carried a germline point mutation in the *SDHB* (c.T282A; Figure 1d). Case 1 was previously reported by our group.<sup>21</sup> None of the germline-mutated cases expressed SDHB in the tumors.

One patient (case 6) presented a tumor with a somatic deletion of promoter and exon 1 of *SDHB* (c.1-10413\_73-3866del). This deletion was absent in the DNA from adjacent non-tumor tissue. SDHB and SDHA expression was detected in the tumor cells of this patient.

Tumor MLPA analysis revealed complete loss of the *SDHB* promoter and of the exon 1 in the tumor of case 2 (Figure 3). In case 3, MLPA analysis also revealed a complete loss of the *SDHB* promoter; in addition, we found loss of heterozygosity (LOH) of *SDHB* in exon 1 in this tumor (Figure 3). In our previous report,<sup>21</sup> MLPA analysis indicated LOH of the WT allele in the tumor of case 1.

*SDHA* mutations were not found in the germline or tumoral DNA of cases 4 and 5, which did not express SDHA in their GISTs.



**Figure 3** MLPA quantification of each *SDHB* exon in DNA extracted from GIST tissue of cases 2 and 3; *SDHB* promoter and exon 1 display an almost complete loss in the tumor of case 2; in case 3, *SDHB* promoter displays also an almost complete loss whereas *SDHB* exon 1 displays LOH in the tumor. Data were normalized using five genomic DNA control samples isolated from normal human tissue. The bars represent the average of three experimental replicates.

Patients with germline mutations in *SDHB* were significantly ( $P=0.001$ ) younger ( $32.7 \pm 4.8$  years) than patients without germline mutations in *SDHB* ( $65.6 \pm 2.6$  years). The mitotic index of GISTs from patients carrying *SDHB* germline mutations (mean 12) was significantly ( $P=0.002$ ) higher than that of patients without germline *SDHB* mutations (mean 4). However, the presence of germline *SDHB* mutation was not significantly associated with high risk, according to the NIH ( $P=0.059$ ) and NCCN ( $P=0.194$ ) classifications.

## DISCUSSION

In the present study, we evaluated the presence of *SDH* mutations in a series of 25 WT *KIT/PDGFR/RAF* GISTs of patients with no apparent personal or familial history of PGLs and/or pulmonary chondroma. We detected *SDHB* germline mutations in 12% of the patients. No germline mutations were found in *SDHA*, *SDHC* or *SDHD*. Our results fit with a series recently reported by Janeway *et al*<sup>24</sup> who also identified germline mutations of *SDH* genes in 12% of seemingly sporadic WT GIST patients.

Twenty percent of WT *KIT/PDGFR/RAF* GISTs did not express *SDHB* or *SDHA/SDHB*. Three of the five patients carried germline *SDHB* mutations. Specifically, one patient (case 1) carried the germline *SDHB*<sup>T282A</sup> (p.Ile44Asn) mutation, which was previously reported by our group, and the tumor tissue presented features consistent with LOH of the WT *SDHB* allele.<sup>21</sup> The two remaining patients (cases 2 and 3) carried a germline deletion in the promoter and exon 1 of *SDHB* (c.1-10413\_73-3866del);<sup>35</sup> this deletion had previously been identified in PGLs, but this is the first report of its association with the development of GISTs. Case 2 presented complete loss of the promoter and exon 1 of *SDHB* in the tumor, and case 3 showed complete loss of *SDHB* promoter and features consistent with LOH of exon 1 of *SDHB* in the tumor. Altogether, our data fit with the classical two 'hit' tumor-suppressor inactivation model in cancer.<sup>21,27,38</sup>

Carney–Stratakis dyad is a familial condition with an apparently autosomal dominant inheritance pattern with incomplete penetrance, characterized by GISTs associated with PGLs.<sup>28,29</sup> In this dyad, germline mutations in *SDHB*, *SDHC* or *SDHD* have been described in the absence of somatic or germline mutations in *KIT/PDGFR/RAF*.<sup>25,27</sup>

The Carney triad is a non-familial condition characterized by GISTs, PGLs and pulmonary chondroma.<sup>30,39</sup> So far, no somatic or germline *KIT/PDGFR/RAF* mutations, *SDHB*, *SDHC* and *SDHD* mutations, have been described in this triad.<sup>31,32</sup> None of our patients presenting GISTs with loss of *SDHB* or *SDHB/SDHA* expression revealed clinical-imaging evidence of other tumors (eg, pulmonary chondromas and/or PGLs).

So far, GISTs from patients with Carney triad and Carney–Stratakis dyad have been reported as negative for *SDHB* expression.<sup>22</sup> In a previous study, we screened for the germline *SDHB*<sup>T282A</sup> mutation in case 1 patient family<sup>21</sup> and found the same germline mutation in the patient's mother. As the patient did not show evidence of PGLs, neither his mother of GISTs or PGLs, we hypothesized that this case may represent an incomplete phenotype of the Carney–Stratakis dyad or a rather distinct entity. On the whole, our results raise the issue of whether GIST patients carrying germline mutations in *SDHA*, *-B*, *-C*, or *-D* are Carney–Stratakis dyad cases with reduced penetrance and/or expressivity of the disease, or if they represent a new hereditary GIST syndrome.

In our series, WT GIST patients with germline mutations in *SDHB* were significantly younger than WT GIST patients without germline mutations in *SDHB*. Notably, WT *KIT/PDGFR/RAF* patients with *SDHB*- or *SDHA/SDHB*-negative GISTs were also significantly

younger than patients with *SDHA/SDHB*-positive GISTs. Such association between *SDH* protein complex defects and younger age of patients has been also described in previous reports.<sup>22–24,26,40</sup>

A significant higher mitotic index was observed in WT *KIT/PDGFR/RAF* GIST negative for *SDHB* or *SDHA/SDHB* expression, when compared with WT *KIT/PDGFR/RAF* GISTs with *SDHB/SDHA* expression. This significant higher mitotic index was even more pronounced in WT *KIT/PDGFR/RAF* GISTs of patient carriers of *SDHB* germline mutations. Thus, it is possible that loss of *SDH* complex function may confer a proliferative advantage to GISTs.

In our series, WT *KIT/PDGFR/RAF* GISTs negative for *SDHB* or *SDHA/SDHB* expression were all located in the stomach. Such observations has been reported by Gill *et al*,<sup>23</sup> who additionally suggested that negative *SDHB* GISTs should be classified as type 2. Recently, Doyle *et al*<sup>41</sup> also reported similar observations, although none of the studies performed the molecular characterization of tumors and patients. We observed that WT *KIT/PDGFR/RAF* GISTs located in the stomach showed higher mitotic index than those of the small intestine. However, when WT *KIT/PDGFR/RAF* GISTs with negative expression for *SDHB* (all located in stomach) were excluded from the analysis, this association was lost suggesting that the mitotic index was associated with *SDHB* expression. A larger series should be considered to clarify this issue.

The WT GISTs negative for *SDHB* or *SDHA/SDHB* displayed epithelioid and spindle cell type morphologies. These findings do not fit with the previously reported exclusive epithelioid or mixed (epithelioid/spindle) cell morphology of GISTs negative for *SDHB*.<sup>22,23,40,41</sup> Our findings however concur with those from Miettinen *et al*<sup>26</sup> and indicate that tumor cells from *SDHB*-deficient GISTs can display epithelioid, spindle and mixed morphology.

It is noteworthy that we found two WT *KIT/PDGFR/RAF* GISTs with loss of *SDHA/SDHB* expression and without *SDHA*, *SDHB*, *SDHC* and *SDHD* mutations, as described in Carney triad.<sup>22</sup> However, we did not find evidence of pulmonary chondromas and PGLs in any of these patients. Important, the cases reported in literature with Carney's triad exhibited variable expression of GISTs, pulmonary chondromas and PGLs.<sup>23</sup>

Concerning the clinicopathological features of our series, we found a lower gender ratio of the patients with WT GISTs than in previous reports, in which at least half of patients were female.<sup>24,26,40,42</sup> Patients with high risk of recurrence of primary WT *KIT/PDGFR/RAF* GISTs, according to the NIH classification,<sup>1</sup> were significantly younger than patients of lower recurrence WT GIST risk groups.

All *KIT/PDGFR/RAF*-mutated GISTs used as controls expressed *SDHB* and *SDHA* proteins, indicating that molecular alterations in *SDH* complex do not seem to have a major role in the pathogenesis of *KIT/PDGFR/RAF*-mutated GISTs. Furthermore, when WT-negative *SDHB* GISTs were excluded from the analysis, WT GISTs displayed decreased *SDHB* protein expression compared with *KIT/PDGFR/RAF*-mutated GISTs. Our results highlight the role of the *SDH* complex deregulation as an additional molecular mechanism in WT *KIT/PDGFR/RAF* GISTs,<sup>21–27</sup> albeit diverse processes may drive its deregulation. As far as we are aware there are no reports of concomitant germline *SDHx* genes mutation in *KIT/PDGFR/RAF*-mutated GISTs, which fits with the positive staining for *SDHA/SDHB* in *KIT/PDGFR/RAF*-mutated GISTs. However, we cannot rule out the existence of somatic *SDHx* mutations and further studies should be performed in a series of *KIT/PDGFR/RAF*-mutated GISTs to evaluate (somatic and germline) genetic alterations in *SDH* complex.

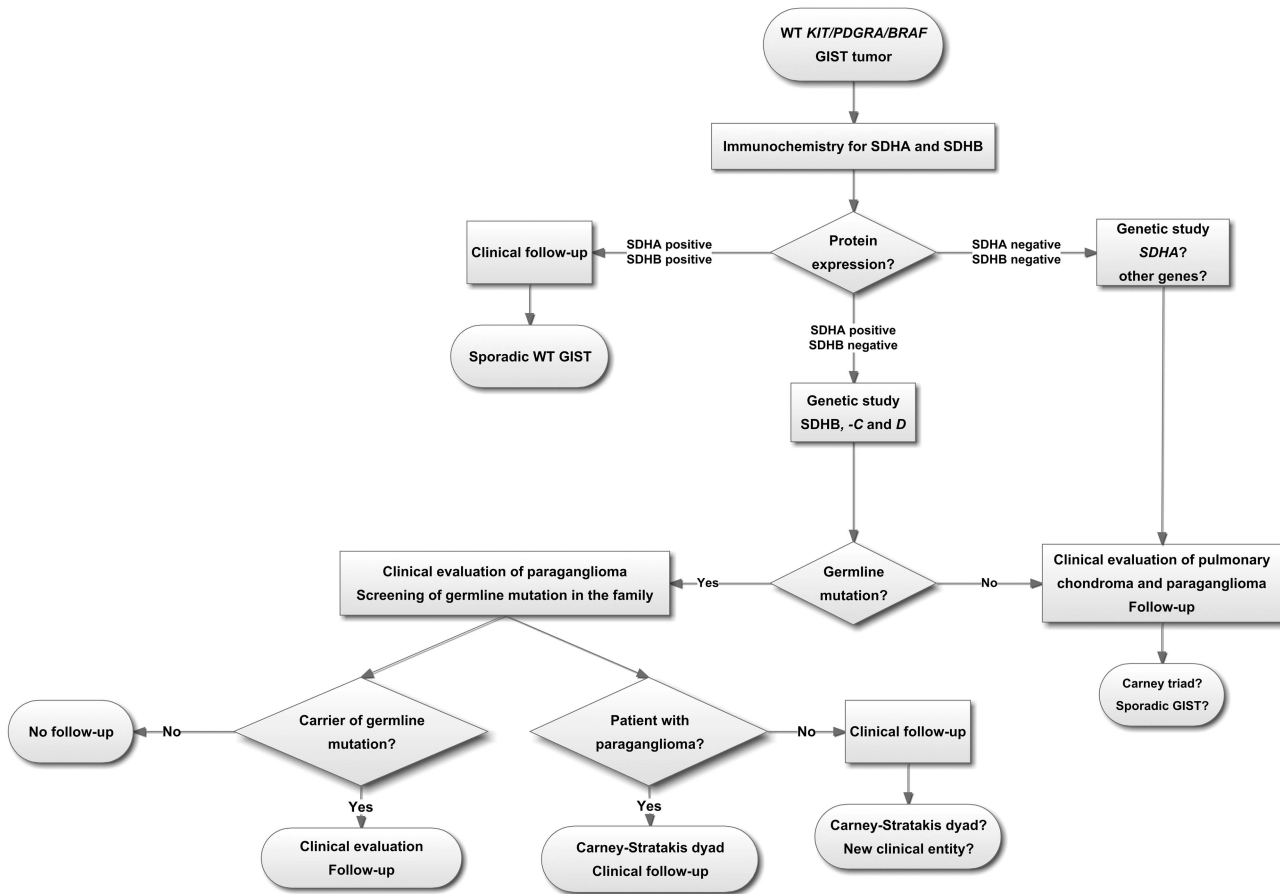


Figure 4 Flowchart for the management of patients with *KIT/PDGFRA/BRAF* wild-type GISTs.

Positive expression of DOG-1 was found in most of the evaluated GISTs, and there were no significant differences in KIT and DOG-1 expression when comparing WT GISTs to *KIT/PDGFRA/BRAF*-mutated GISTs in our cohort. Expression of KIT in GISTs was significantly associated to DOG-1 expression, suggesting that DOG-1 may be an useful additional marker for the diagnosis of GISTs.<sup>43</sup>

To summarize, in our series we found that 20% of primary WT *KIT/PDGFRA/BRAF* GISTs did not express SDH proteins and 12% carried *SDHB* germline mutations, which were particularly associated with patient's younger age. The results obtained in our study underline the importance of SDHB and SDHA immunohistochemical screening, particularly in young patients ( $\leq 45$ -year-old) harboring WT *KIT/PDGFRA/BRAF* GISTs. In negative SDHB and SDHA/SDHB GISTs, patients should be screened for germline mutations of *SDHA*, *-B*, *-C*, and *-D* genes. Primary GISTs without SDHB or SDHA/SDHB expression may represent a distinctive group that should be considered in the management decisions of these patients (Figure 4). Most importantly, the possibility of coexistent PGLs (Carney–Stratakis dyad) and/or pulmonary chondroma (Carney triad) should be addressed in WT GIST patients and their kindred.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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