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IMMUNOHISTOCHEMICAL LOSS OF SUCCINATE DEHYDROGENASE SUBUNIT A (SDHA) IN GASTROINTESTINAL STROMAL TUMORS (GISTS) SIGNALS SDHA GERMLINE MUTATION

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Abstract

A subset (7–10%) of gastric GISTs is notable for the immunohistochemical loss of succinate dehydrogenase (SDH) subunit B (SDHB), which signals the loss of function of the SDH-complex consisting of mitochondrial inner membrane proteins. These SDH-deficient GISTs are known to be *KIT/PDGFRA* wild type, and most patients are young. Some of these patients have germline mutations of SDH-subunits B, C, or D, known as Carney-Stratakis syndrome when combined with paraganglioma. More recently, germline mutations in SDH-subunit A (*SDHA*) have been also reported in few patients with KIT/PDGFRA wild type GISTs. In this study we examined immunohistochemically 127 SDHB-negative and 556 SDHB-positive gastric GISTs and 261 SDHB-positive intestinal GISTs for SDHA expression using a mouse monoclonal antibody 2E3 (Abcam). Cases with available DNA were tested for *SDHA*, B, C, and D gene mutations using a hybridization-based custom capture next-generation sequencing assay. A total of 36 SDHA-negative GISTs (28%) were found among 127 SDHB-negative gastric GISTs. No SDHB-positive GIST was SDHA-negative. Among 7 SDHA-negative tumors analyzed, there were 7 *SDHA* mutants, most germline. A second hit indicating biallelic inactivation of SDHA was present in 6 of those cases. These patients had no other SDH subunit mutations. Among the 25 SDHA-positive, SDHB-negative GISTs analyzed, we identified 3 *SDHA* mutations (one germline), and 11 *SDHB*, *SDHC* or *SDHD* mutations (mostly germline), and 11 patients with no SDH mutations. Compared with patients with SDHA-positive GISTs, those with SDHA-negative GISTs had an older median age (34 vs. 21 years), lower female to male ratio (1.8 vs. 3.1) but similar mitotic counts and median tumor sizes, with a slow course of disease in most cases, despite a slightly higher rate of liver metastases. SDHA-negative GISTs comprise approximately 30% of SDHB-negative/SDH-deficient GISTs, and SDHA loss generally correlates with *SDHA* mutations.

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INTRODUCTION

A small subset of gastric GISTs (7–10%) have the loss of function of the succinate dehydrogenase (SDH) complex of inner mitochondrial membrane.[1–4] This complex of multiple proteins, all coded by chromosomal DNA, participates in the Krebs cycle and electron transport of oxidative phosphorylation and is normally ubiquitously present in mitochondria in all normal nucleated eukaryotic cells.[5] Loss of function of the SDH-complex in these tumors is signaled by immunohistochemical loss of succinate dehyrogenase subunit B (SDHB). SDHB-negative (SDH-deficient) GISTs comprise a great majority of gastric GISTs of children and young adults and a small portion of gastric GISTs in older adults. Although still incompletely understood, their oncologic pathogenesis is believed to be related to HIF1-α mediated pseudohypoxia signaling triggered by succinate accumulation by SDH-loss and not to KIT/PDGFRA gain-of-function mutations, which are absent in these GISTs.[1–4] Instead, oncogenic signal in these tumors is likely mediated via activated insulin-like growth factor-1 receptor signaling as a consequence of HIF1-α overexpression.[1]

A minority of patients with SDH-deficient GISTs have found to harbor germline mutations of *SDHB*, *SDHC*, or *SDHD*, known as the Carney-Stratakis syndrome when combined with paraganglioma.[1,6,7] However, the mechanism of SDH protein losses in most cases of SDH-deficient GISTs remains unclear. Recently, a small number of GISTs have been found associated with germline loss-of-function mutations of *SDHA* gene encoding the key catalytic component of the SDH-complex.[8,9] Such mutations have been previously detected in an abdominal catecholamine secreting paraganglioma.[10] In as patient with Leigh syndrome, a severe neurodegenerative disease, there was a compound heterozygous germline SDHA-mutation (one allele with nonsense and another with missense mutation).[11] A significant portion of paraganglioma patients has mutations in the other SDH subunits, *SDHB*, *SDHC*, and *SDHD*.[12,13] In this study, we report 36 SDHA-negative gastric GISTs among 127 SDHB-negative/SDH-deficient GISTs and examine *SDHA* and other SDH-subunit mutations, and pathology and prognosis of these tumors.

MATERIALS AND METHODS

Tissue material and immunohistochemistry

A total of 127 SDHB-negative (SDH-deficient) gastric GISTs were studied for SDHA expression using a primary mouse monoclonal antibody to SDHA (Clone 2E3, Abcam, Cambridge, Massachusetts, diluted at 1:1000). Also tested were 556 gastric GISTs and 230 small intestinal and 31 colorectal GISTs positive for SDHB, determined immunohistochemically with a mouse monoclonal antibody 21A11 (Abcam, diluted at 1:1000). All immunostainings were performed on a Leica BondMax autostainer (Leica Microsystems, Bannockburn, IL) using the BondMax avidin biotin free polymer-based detection system preceded by heat-induced epitope retrieval with Leica retrieval solution (high-pH buffer), for 25 min. Diaminobenzine was used as the chromogen. All cases tested for SDHA and SDHB were validated as informative by showing a positive internal control (fibrovascular, lymphoid, smooth muscle, or mucosal elements). Immunohistochemistry for SDHA was analyzed blindly without knowing the SDHB expression or SDH-subunit mutation status.

In addition, SDHB-negative GISTs with available material were evaluated for KIT (polyclonal antibody A4502, Dako Cytomation, Carpinteria, CA), diluted 1:500, and anoctamin1/DOG1 (monoclonal antibody, clone K9, Leica, diluted 1:300, Desmin (clone D33, Dako), diluted 1:300, and CD34 (Dako Cytomation, clone QBEnd/10, dilutes 1:150), using a similar epitope retrieval and automation procedure as described above. Smooth

muscle actin (clone 1A4, Sigma Chemicals, St. Louis, MO, diluted 1:1000) was evaluated similarly but without epitope retrieval. All SDHB-negative tumors tested were positive for KIT (n=125) and Ano-1/DOG1 (n = 80), and 87/111 cases were positive for CD34. Only 1/105 cases was focally positive for SMA.

Genetic studies

DNA was obtained from microdissected formalin-fixed, paraffin-embedded tumor tissue. SDH-subunit genes *SDHA*, *SDHB*, *SDHC*, and *SDHD* were evaluated for mutations using a hybridization-based custom capture reagent (Agilent, Inc., Santa Clara, CA) followed by sequencing on the GAIIx instrument (Illumina, Inc., San Diego, CA) according to manufacturer's protocols. The Illumina raw sequence data was aligned with the Burrows-Wheeler Aligner (BWA) against human genome hg19 and then variant calling was performed with the Samtools mPileup algorithm. The reference sequence for *SDHA* was NM_004168; *SDHB*: NM_003000; *SDHC*: NM_003001; and *SDHD*: NM_003002. A subset of SDHA mutations was further validated by TaqMan assay (Invitrogen/Life Technologies, Carlsbad, CA). Mutations were verified germline by showing their presence in normal tissue or peripheral blood, but in the absence of these tissues in some cases, the nature of mutations (germline vs. somatic) remained indeterminate. Comparative genomic hybridization was performed using a 180K feature array (Agilent) in selected cases to examine the copy number status of chromosome 5p, which includes the *SDHA* locus.

RESULTS

Pathology of SDHA-negative GISTs

A total of 36 of 127 gastric GISTs (28%) that were also verified as SDHB-negative (SDHB-deficient GISTs), were immunohistochemically SDHA-negative (Table 1). These cases showed granular cytoplasmic SDHA-immunoreactivity only in the cellular components of the fibrovascular septa, lymphoid, or smooth muscle elements (Fig. 1).

Data on SDHA-negative GISTs are summarized in Tables 1 and 2, and representative histologic images are illustrated in Fig. 2. *SDHA* mutations were detected in all 7 SDHA-negative tumors with available sequencing data, and 6 of them were germline mutations. Three of these were truncating mutations, 3 were missense mutations, and 1 one a splice site mutation (Table 3). Six of these cases had a 2^{nd} hit in the *SDHA*-locus: 3 LOH in the 5p15 region, 2 somatic mutations, and 1 5p deletion. There were no mutations in *SDHB*, *SDHC*, and *SDHD* in these patients.

The tumor size varied from 1.2–21.5 cm (median, 5.0 cm). Mitotic rate varied from 0–26 per 50 high power fields (median mitotic count, 4/50 HPFs). Histologically typical of these tumors was multinodularity, often a plexiform muscularis propria involvement, and predominantly epithelioid hypercellular histology, as typically seen in the SDHB-negative (SDH-deficient) GISTs. A minority of cases showed spindle cell histology, usually as a focal finding. Marked nuclear atypia was detected in only one case, and no tumor showed overt coagulative necrosis. Peritumoral lymphovascular invasion was commonly detected (Fig. 2).

Clinical features of SDHA-negative GISTs

The clinical features are summarized in Table 2. The patient group with SDHA-negative GISTs consisted of 23 women and 13 men of median age of 34 years (range, 8–83 years). There were only 3 children ≤16years, and 13 patients were older than 40 years. None of the patients were known to have neurologic deficit syndrome, paraganglioma or pulmonary chondroma.

Follow-up data were available of 20 patients (Table 2). Five patients were alive with no evidence for disease 8.5–17 years after first surgery, 8 were alive with liver metastases at 2–28.1 years, 4 died with liver metastases at 2.2, 3.3, 15.7, and 21.5 years, and 3 died of unrelated causes at 8–21 years.

SDHA-positive, SDHB-negative GISTs

A total of 91 of 127 (72%) SDHB-negative gastric GISTs retained SDHA and showed granular cytoplasmic positivity for SDHA, the normal pattern (Fig. 3). Twenty-five of these cases were available for mutation testing. Most (22/25, 88%), contained wild type SDHA sequences. However, 3 patients had *SDHA* missense mutations, 1 of them germline (Table 3). Eleven patients had mutations in other SDH-subunit genes, most of them germline: 5 in *SDHB*, 5 in *SDHC*, and 1 in *SDHD* (Table 3). No SDH-subunit mutations were detected in tumors from the remaining 11 SDHA-positive cases tested.

These tumors did not significantly differ from SDHA-negative GISTs histologically, and they had similar median mitotic rate and tumor size, but there was a slightly higher percentage of tumors > 10cm (Table 2). Of the 59 patients with follow-up, 33 were alive without disease 1.5–42 years (median, 16.3 years), 16 were alive with disease (most with known liver metastases) 2–43 years after first surgery (median, 8.8 years), 9 died of disease at 1.5–35.6 years (median survival, 8.8 years). One patient died of unrelated disease at 15.7 years. Four patients had also paragangliomas, and one of these patients had an *SDHB*-mutation and therefore fulfilled the criteria of Carney-Stratakis syndrome. Two others had pulmonary chondromas (Carney triad).

All 433 gastric, 53 duodenal, 177 small intestinal (jejunal/ileal), and 31 colorectal SDHB-positive GISTs were SDHA-positive.

DISCUSSION

In this study, we examined the frequency, genetic correlation, and clinicopathologic features of gastric GISTs with immunohistochemical succinate dehydrogenase subunit A (SDHA) loss. These tumors form a subgroup of SDHB-negative (SDH-deficient) GISTs comprising 28% (36/127) of all such cases in our patients. Like other SDHB-negative GISTs in general, SDHA-negative GISTs are restricted to gastric location and they have a predilection to young patients, although they are less common in children and have a higher patient median age.

As an immunohistochemical marker, SDHA is analogous to SDHB. For practical interpretation, it is critical to observe the presence of immunoreactivity in non-neoplastic components to validate the immunostaining as technically adequate. The general principle in interpretation of negative result is to observe a contrast between the negative tumor and the positive internal control: cellular components of the fibrovascular septa, smooth muscle, lymphoid, or epithelial elements. Most cells retaining SDHA show granular cytoplasmic immunostaining consistent with the mitochondrial location of this antigen.

Considering that SDHB-loss destabilizes the SDH-complex rendering it non-functional[12,13] and that A subunit is anchored by subunit B[5], it is somewhat unexpected that SDHA expression is still retained in a majority of cases with SDHB loss. However, the immunohistochemical presence of SDHA, the main catalytic unit of succinate dehydrogenase[5], does not mean that this subunit is functional. Also, the complex is anyway non-functional with the loss of SDHB, the iron sulphur protein participating in electron transfer of oxidative phosporylation.

There was a strong correlation between immunohistochemically observed loss of SDHA and *SDHA* mutations, which were detected in all 7 patients analyzed, while only present in 3 of 25 patients with SDHA-positive, SDHB-negative GISTs. On the other hand, none of these patients with SDHA-negative GISTs had *SDHB*, *SDHC*, or *SDHD* mutations. Therefore, SDHA mutations (mostly verified as germline here) are the apparent cause for SDHA loss and destabilization of the SDH-complex, especially because bi-allelic changes with losses or somatic mutations in SDHA-locus were common and detected in most cases in this study. A previous study also showed *SDHA* germline mutations coupled with somatic *SDHA* mutations in the tumor in some cases.[9] Therefore, these *SDHA* alterations seem to follow a classic two hit hypothesis of tumor suppressor genes, as has been previously found for other SDH-subunit gene mutations in paragangliomas.[12,13]

Both truncating and missense *SDHA* germline mutations were associated with the loss of protein expression. This is not surprising in view of most SDH-subunit loss-of-function mutations in SDHB-negative paragangliomas also being missense mutations.[14] Loss of function has also been resulting from missense mutations in other tumor suppressor proteins, such as merlin, the NF2 gene product in schwannoma, and TSC1 in transitional cell carcinoma. Based on those studies, such missense mutations are deleterious causing protein instability and premature degradation.[15,16] Abnormal trafficking of subunit proteins or problems in assembly of the SDH-complex containing one mutant protein could be additional explanations.

Immunohistochemical loss of SDHA can pinpoint an *SDHA* germline mutation in most cases, but a minority of such mutant proteins seems to retain immunoreactivity. Therefore, mutation analysis will be necessary to definitively determine *SDHA* mutation status and type, but detection of SDHA loss allows focusing the mutation analysis specifically to *SDHA* gene. Although detection of an *SDHA* germline mutation may not have an immediate clinical significance, its presence raises the question for need of family studies and associated genetic counseling. It may also become a factor in treatment selection in the future. SDHB-negativity in GIST is not effective in determining the specific SDH-genotype, as SDHB expression is lost following the loss of any SDH-subunit proteins, based on our studies and the previous ones in paragangliomas.[12,13]

According to our observations, *SDHA* mutations in SDHB-negative (SDH-deficient) GISTs seem to be as common as other SDH-subunit mutations together. However, *SDHA* is the only mutated subunit gene in SDHA-negative GISTs. A caveat in the comparison of the relative frequencies of SDH-subunit gene mutations is that large deletions can escape detection in our custom capture mutation search. Although such deletions have been reported in paraganglioma patients, they are a minority of all SDH-mutations and have not been reported in *SDHA* (TC Cycle Gene Mutation Database).[14]

There are some clinicopathologic differences between SDHA-negative and SDHA-positive, SDHB-negative (SDH-deficient) GISTs. SDHA-negative gastric GISTs are rare in children and have a later onset and a lesser female predominance than SDHA-positive, SDHB-negative GISTs. They have similar mean tumor sizes and mitotic rates but a slightly lower frequency of tumors >10 cm. Both groups have a slow course of disease with a relatively low rate of mortality (15–20%), but patients with SDHA-negative tumors seem to have a higher frequency of liver metastasis. However, the number of cases is small, so that definitive assessment of possible prognostic difference requires additional data. Even patients with liver metastases often survive for years with a slow if any disease progression, as is known for SDHB-negative GISTs in general, so that appearance of liver metastasis is not necessarily a relevant endpoint in prognostic analysis for these patients. To what extent this long survival with metastases is a factor of given targeted and other new therapies, is

unknown. However, our groups were not biased for any particular treatment regimen. A previous study of SDHB-negative GISTs found that 7 of 66 patients died of disease in long-term follow-up.[4] Paraganglioma does not seem to be as commonly associated with SDHA-negative as with SDHA-positive, SDH-deficient GISTs, and pulmonary chondromas were not detected, so that none of the patients with SDHA-negative GISTs fulfilled the criteria of Carney-Stratakis syndrome or Carney triad.

In summary, we detected immunohistochemical loss of SDHA in 28% of patients with SDHB-negative GISTs. This loss was associated with *SDHA*-germline mutations and unassociated with any other SDH subunit gene mutations. Therefore, immunohistochemical analysis of SDHA expression may indirectly assist in SDH-genotyping. Compared with other SDH-deficient GISTs, SDHA-negative GISTs occur in older patients, have a lesser female predominance, but they seem prognostically essentially similar to the other SHD-deficient GISTs. Further studies are needed to establish the biologic correlation and clinical significance of these findings.

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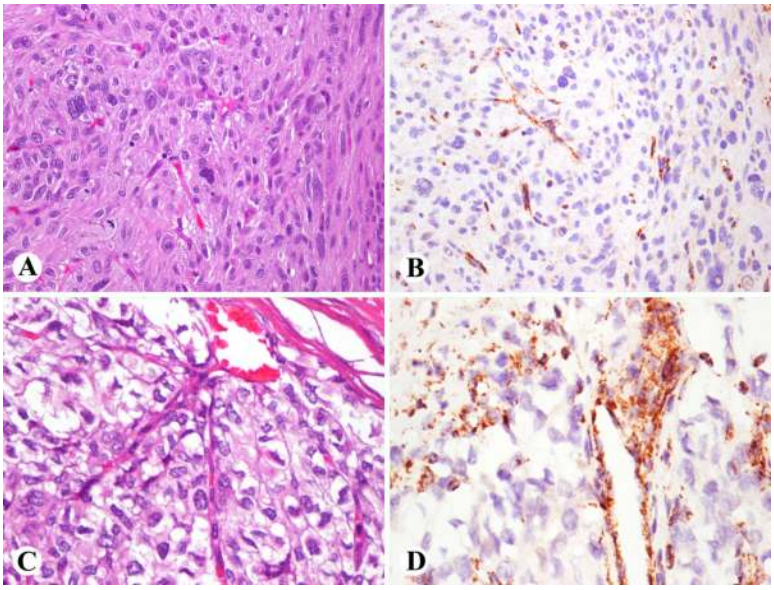

Fig. 1.
Paired hematoxylin and eosin stains and SDHA-immunostains of two examples of SDHA-negative GISTs. A, C. Note focal pleomorphism and epithelioid morphology. B, D. The tumor cells are negative for SDHA, but the blood vessels walls and smooth muscle elements are positive.

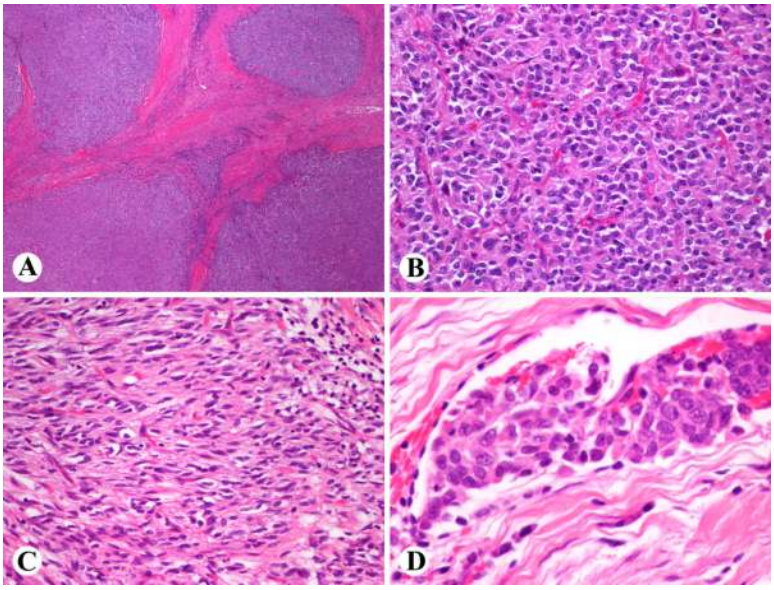

Fig. 2.

Typical histological features of SDHA-negative GISTs. A. Multinodular, “plexiform” muscularis propria involvement. B. Epithelioid hypercellular histology with back-to-back tumor cells with eosinophilic cytoplasm was the most common histologic pattern, sometimes with vague nesting. C. Spindle cell histology was an uncommon, usually focal finding. D. Lymphovascular invasion was a common feature.

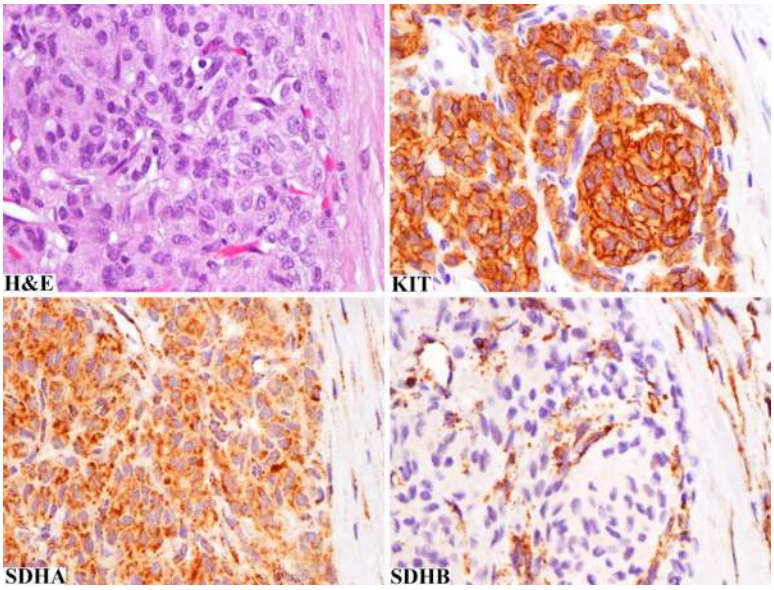

Fig. 3.
An example of an SDHB-negative and SDHA-positive GIST. This tumor is also KIT-positive. Note that SDHB immunostaining is restricted to smooth muscle cells (to the right) and blood vessel walls, whereas SDHA is present in all cells.

Table 1

Summary of SDHA expression in different subcategories of GIST related to SDHB expression, SDH subunit mutations, and tumor location.

Category	SDHA loss	% with SDHA loss
SDHB-negative gastric GISTs (SDH-deficient GISTs)	36/127	28
SDHA germline mutants (n = 7)	6/7	86
SDHA mutants, unknown whether germline or somatic (n = 3)	1/3	33
SDHB, SDHC, and SDHD mutants (n = 11)	0/11	0
No SDH subunit mutations (n =11)	0/11	0
SDHB-positive GISTs	0/817	0
Gastric GISTs	0/556	0
Duodenal GISTs	0/53	0
Small intestinal sporadic GISTs (jejunal and ileal)	0/169	0
Small intestinal GISTs, NF-1 associated	0/8	0
Colorectal GISTs	0/31	0

Table 2

Comparison between 36 SDHA-negative and 91 SDHA-positive, SDHB-negative (SDH-deficient) GISTs. The total in each line refers to patients with data available.

Parameter	SDHA-negative GISTs (n = 36)	SDHA-positive GISTs (n = 91)
Median age yrs. (range)	34 (8–83)	21 (8–77)
Number of patients ≤16 yrs	3/36 (8%)	29/91 (32%)
Number of patients > 40 yrs	13/36 (36%)	11/91(12%)
Female:Male ratio	1.8 (23:13)	3.1 (69:22)
Median tumor size (range)	5.0 cm (1.2 – 21.5 cm)	5.0 cm (1–21 cm)
Cases with tumor ≥10 cm	3/29 (10%)	12/73 (16%)
Median mitotic count per 50 HPFs, 5 mm^2 (range)	4 (0–26)	5 (0–102)
Cases with ≥10 mitoses/50 HPFs	8/35 (23%)	28/84 (33%)
Patients alive without disease	5/20 (25%) Median follow-up, 14 yrs	33/59 (56%) Median follow-up, 16 yrs
Patients alive with metastases	8/20 (40%)	16/59 (27%)
Patients dead of disease	4/20 (20%)	9/59 (15%)
Patients dead of unrelated causes	3/20 (15%)	1/59 (2%)

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Table 3

Detected SDH subunit mutations in SDH-defidcient GISTs with corresponding cDNA changes and predicted amino acid sequences. Germline mutations are indicated in the right column. The unmarked cases in that column are indeterminate whether somatic or germline.

Case	Gene	Exon	Mutation in corresponding cDNA	Predicted amino acid sequence	Germline mutation
1–7	SDHA-negative GISTs				
1–3	SDHA	2	c.C91T	R31X	Yes
4	SDHA	6	c.C767T	T256I	Yes
5	SDHA	10	c.C1334T	S445L	
6	SDHA	13	c.G1794C	K598N	Yes
7	SDHA	14	c.G(1795–1)T	Exon 14 5' splicing	Yes
8–21	SDHA-positive GISTs				
8	SDHA	5	c.C562T	R188W	Yes
9	SDHA	7	c.C818T	T273I	
10	SDHA	10	c.C1361A	A454E	
11	SDHB	1	c. 17_dup26GTCG{dup26}GCCA	A15HfsX4	Yes
12	SDHB	2	c.G137A	R46Q	Yes
13	SDHB	3	c.T274A	S92T	Yes
14	SDHB	4	c.T380G	I127S	Yes
15	SDHB	6	c.G600T	W200C	Yes
16	SDHC	1	c.6delT	A2fs	Yes
17	SDHC	1	c.A1G	M1V	Yes
18	SDHC	4	c.G224A	G75D	
19	SDHC	4	c.A380G	H127R	
20	SDHC	5	c.C397T	R133X	Yes
21	SDHD	4	c.352delG	D118fs	Yes