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Loss of expression of SDHA predicts *SDHA* mutations in gastrointestinal stromal tumors

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Gastrointestinal stromal tumors (GISTs) are usually driven by mutations in *KIT* or *PDGFRA*, although 15% of GISTs in adults and >90% in children lack such mutations. The majority of gastric *KIT*/*PDGFRA* wild-type GISTs show distinctive morphological and clinical features and loss of expression of succinate dehydrogenase (SDH) B. Only a small subset of SDHB-deficient GISTs carries loss-of-function mutations in *SDHB*, *SDHC*, or *SDHD*. Because of the complexity of its locus (15 exons) and the presence of three pseudogenes, *SDHA* is rarely analyzed. Recently, mutations in *SDHA* were shown to lead to loss of expression of SDHA in a small group of paragangliomas. We sought to determine whether immunohistochemistry for SDHA could identify GISTs with *SDHA* mutations. Tumors ($n=33$) with pathological features of SDH-deficient GIST were analyzed for expression of SDHA and SDHB by immunohistochemistry, and *SDHA* exons were sequenced from tumors lacking SDHA expression. Exons harboring somatic mutations were examined in DNA from corresponding normal tissue. All 33 tumors showed loss of SDHB expression. A total of 9 out of 33 (27%) tumors also lacked expression of SDHA. SDHA-deficient GISTs affected five men and four women (median age 38 years). SDHA expression was intact in the 24 remaining tumors, including those with known *SDHB* ($n=3$) or *SDHC* ($n=2$) mutations. Nonsense ($n=8$) or missense ($n=1$) mutations in *SDHA* were identified in all SDHA-deficient tumors. Heterozygous mutations were also found in DNA from normal tissues from six patients with available material. Somatic loss of the second allele has been found in seven tumors, five by loss of heterozygosity, one by a 13-bp deletion, and one by a missense mutation. Loss of SDHA expression in GIST reliably predicts the presence of *SDHA* mutations, which represent a relatively common cause of SDH-deficient GIST in adults. Immunohistochemistry for SDHA can be used to select patients for *SDHA*-specific genetic testing.
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The majority of gastrointestinal stromal tumors (GISTs) contain activating mutations in the *KIT* or *PDGFRA* receptor tyrosine kinase genes,[1,2] which not only drive uncontrolled cellular proliferation and survival but also are the basis for effective targeted therapies with the small molecule kinase inhibitors imatinib and sunitinib.[3,4] Approximately 15% of GISTs in adults, and >90% of GISTs in children, do not contain identifiable mutations in *KIT* or *PDGFRA* and were previously lumped into one group referred to as 'wild-type GIST'.[5–7] More recently, these tumors have been subcategorized into genetically defined subgroups, including tumors with activating mutations in *BRAF*,[8–10] loss-of-function mutations in *NF1*, or loss-of-function mutations in components of the inner mitochondrial membrane Krebs cycle enzyme complex succinate dehydrogenase (SDH).[11,12] This latter group of tumors has been designated 'SDH-deficient GIST'.[13,14] The observed distinctions in molecular genotype have important implications for the biological properties of the tumor, as well as sensitivity to targeted therapies.[13,15]

The SDH complex is comprised of or modified by proteins encoded by *SDHA*, *SDHB*, *SDHC*, *SDHD*, and *SDHAF2*.[16] Germline mutations in these genes have been identified in patients with

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paraganglioma,[17–22] renal cell carcinoma,[23,24] and GIST,[11,12] as well as syndromes of multiple tumor types.[11,13] In GIST, alterations in SDH have most commonly been reported in *SDHB*, but also have been found in *SDHC*, *SDHD*, and, recently, *SDHA*.[11,12,25,26] Germline mutations in *SDHB* and *SDHC* have been identified in SDH-deficient GIST but appear to account for $<15\%$ of cases.[12,14] However, mutational analysis of *SDHA* is seldom performed because of the complex structure of the gene with 15 exons and because of the presence of 3 pseudogenes that make sequencing the proper gene challenging.[25] Thus, the prevalence of *SDHA* mutations and consequently the total frequency of any type of SDH subunit gene mutation in GIST are likely underestimated.

SDH-deficient GISTs are characterized by distinctive morphological and clinical features. These KIT-expressing tumors arise in the stomach, are comprised of epithelioid or mixed epithelioid and spindled cells, and show a multinodular and infiltrative appearance.[14,27] Multifocal disease and lymph node metastases are also common, whereas these features are extraordinarily rare in conventional *KIT*-mutant GISTs.[13–15] Furthermore, loss of expression of SDHB by immunohistochemistry is a consistent feature of SDH-deficient GIST, whereas SDHB expression is intact in *KIT*-mutant GISTs.[14,27–29]

A similar genotype/immunophenotype correlation has also been noted in paragangliomas, with mutations in *SDHB*, *SDHC*, or *SDHD*, leading to loss of SDHB expression.[13,30] Recently, loss of expression of both SDHA and SDHB by immunohistochemistry in paragangliomas was shown to correlate specifically with mutations in the *SDHA* gene.[31] In this study, we sought to determine whether immunohistochemistry for SDHA in SDH-deficient GISTs could similarly predict loss-of-function mutations in *SDHA*.

Materials and methods

Archival tumor samples were selected for this study on the basis of morphological features consistent with SDH-deficient GIST (gastric origin, epithelioid or mixed morphology, and multinodular/plexiform architecture), according to Institutional Review Board-approved protocols. All patients in this study were diagnosed at ≥ 18 years of age, because our pathology department and sarcoma oncology group serve an adult patient population.

Immunohistochemistry was performed on 4-μm-thick formalin-fixed paraffin-embedded whole-tissue sections following pressure cooker antigen retrieval (0.001 M citrate buffer; pH 6.0), using a mouse anti-SDHA monoclonal antibody (1:750 dilution; 40 min incubation; clone 2E3GC12FB2AE2; Abcam, Cambridge, MA) and a mouse anti-SDHB monoclonal antibody (1:100 dilution; 40 min incubation; clone 21A11AE7; Abcam). The Envision Plus detection system (Dako, Carpinteria, CA) was used as a secondary antibody. Expression was scored as 'intact' when any granular cytoplasmic staining was observed in tumor cells or 'deficient' when there was a complete absence of granular cytoplasmic staining in tumor cells with positive internal controls. Non-neoplastic cells, such as endothelium, smooth muscle, and epithelium, served as internal positive controls.

In cases of SDHA-deficient GIST, genomic DNA was isolated from tumor and corresponding normal tissue (when available) using a QIAamp DNA FFPE Tissue Kit (Cat. No. 56404, Qiagen, Valencia, CA, USA), according to the manufacturer's recommended protocol. Tumor DNA was amplified using intronic primers flanking each of the 15 exons of *SDHA*, designed to avoid known single-nucleotide polymorphisms and amplification of pseudogene sequences (see Supplementary Information for primer details). The resulting amplicons were bidirectionally sequenced by the Sanger method and compared with genomic repository data. Exons with identified somatic mutations were also analyzed in DNA from corresponding normal tissue (when available) to determine germline status.

Results

A total of 33 cases of GIST were analyzed. The clinical and pathological features of 16 of these cases were previously described.[27,32] As expected based on selection criteria, all 33 cases were deficient for expression of SDHB. Of these, 9 cases (27%) were also deficient for expression of SDHA in tumor cells, whereas expression was maintained in normal epithelial, endothelial, and inflammatory cells in the tissues. Of the patients with SDHA-deficient GISTs, five were male and four were female, with a median age of 38 years (Table 1). None of these patients had a family history of GIST or paraganglioma. SDHA expression was intact in the remaining 24 tumors, including 5 with known mutations in *SDHB* ($n=3$) or *SDHC* ($n=2$) (Figure 1).

Genomic DNA was isolated from the 9 cases of SDHA-deficient GIST, as well as from corresponding normal tissues for which there was adequate material available. PCR amplification and Sanger sequencing of the *SDHA*-coding regions revealed deleterious mutations in all 9 tumors, caused by a single base pair substitution ($n=8$) or a single base pair deletion ($n=1$) (Figure 2). Heterozygous mutations were also found in DNA from normal tissue from all six patients with available material. Somatic loss of the second allele was identified in seven out of nine tumors, by loss of heterozygosity in five cases, a 13-bp deletion in one case, and a second somatic mutation in one case (see Figure 2 and Table 2).

Table 1 Clinical and pathological features of SDHA-deficient gastrointestinal stromal tumors

Patient	*Age at diagnosis*	*Gender*	*Tumor size (cm)*	*Multifocal*	*Cytology*	*Vascular invasion*	*Lymph node metastases*
1	31	M	4.6	No	Mixed	Yes	No
2	38	F	6.8	No	Epithelioid	Yes	Yes
3	39	F	2	No	Mixed	No	No
4	22	M	7.2 (largest)	Yes	Mixed	No	No
5	53	M	8.5	No	Epithelioid	No	No
6	41	M	12.5	No	Epithelioid	No	Yes
7	53	F	3	No	Mixed	No	No
8	35	F	8.5	No	Epithelioid	Yes	No
9	19	M	12.5	No	Mixed	Yes	No

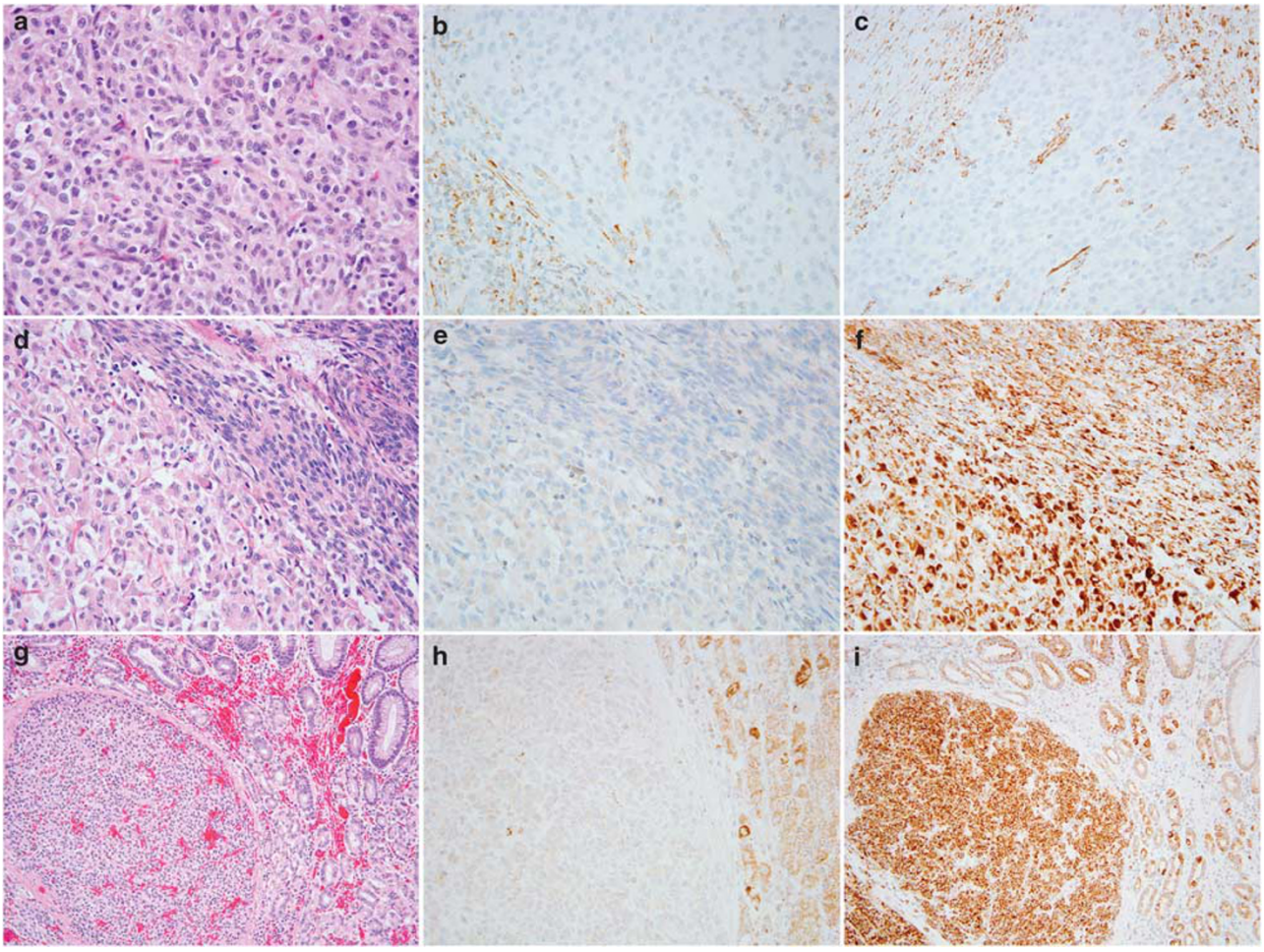

Figure 1 SDH-deficient GISTs of the stomach. (**a–c**) Epithelioid GIST (**a**, H&E) showing loss of expression of both SDHB (**b**) and SDHA (**c**) by immunohistochemistry. Note the intact granular cytoplasmic staining in endothelial cells in intratumoral blood vessels, adjacent muscularis propria, and inflammatory cells. This tumor was found to harbor an *SDHA* mutation. (**d–f**) *SDHB*-mutant GIST with mixed epithelioid and spindle cell morphology (**d**, H&E) showing loss of expression of SDHB (**e**) but intact staining for SDHA (**f**). (**g–i**) *SDHC*-mutant epithelioid GIST (**g**, H&E) showing loss of expression of SDHB (**h**) but intact staining for SDHA (**i**). Note the strong cytoplasmic staining in the epithelial cells in the adjacent mucosa.

Discussion

Loss of expression of the SDHB subunit and SDH activity are universal features of a subset of GISTs characterized by distinctive clinical and morphological features, as well as an absence of mutations in the *KIT* and *PDGFRA* genes.[11–13,28,29] This group of tumors was formerly referred to as 'type 2' or 'pediatric-type' GIST because of their distinctive pathological features, wild-type *KIT* and *PDGFRA* status, and prevalence among GISTs arising in patients <18 years of age.[29,32] Because this type of GIST also arises in adult patients (overall accounting for the majority of such tumors), the term

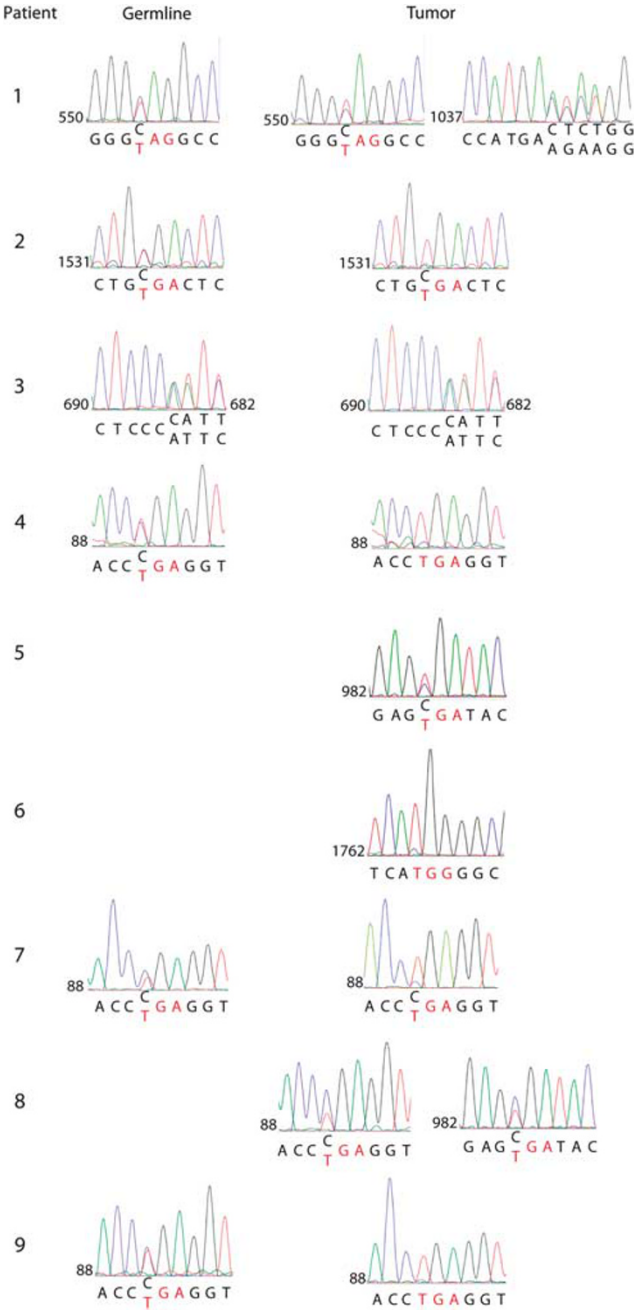

Figure 2 Sequencing chromatograms of selected *SDHA* exons from normal (germline) and tumor tissue. Numbers represent positions of indicated nucleotides. For patient 3, the reverse complement strand is shown for clarity of chromatogram depiction. See Table 2 for mutation details.

'SDH-deficient GIST' is increasingly applied, reflecting the underlying biochemical and, in some cases, genetic lesions.[12–14]

In one report, germline mutations in *SDHB* or *SDHC* were identified in 12% of 34 SDH-deficient tumors, without pathogenic mutations identified in *SDHD*.[12] Of note, no mutations in *SDHB*, *SDHC*, or *SDHD* were identified in another recent study, although only a subset of exons was sequenced.[14] Mutations in *SDHA* have also recently been found in several unselected patients with *KIT/PDGFRA* wild-type GIST.[25,26] However, the *SDHA* gene is not routinely interrogated for mutations both because of the perception that it is infrequently mutated and also because of the technical challenges in sequencing a gene with 15 exons and 3 pseudogenes.[12,25]

Although the diagnosis of SDH-deficient GIST can be made on the basis of morphological features and loss of SDHB expression,[14,27] characterization of the particular genotype has important implications for screening for additional tumor types as well as for genetic counseling and identification of similarly affected family members. Differing SDH subunit mutations are associated with location-specific paraganglioma (in particular, *SDHD* and head and neck tumors, and *SDHB* and thoracoabdominal tumors) and renal cell carcinoma (especially *SDHB*), for example, and therefore screening for occult tumors may be influenced by the particular genetic alterations.[13,24,33–35] Of note, unlike other SDH subunits, there does not appear to be a site predilection for *SDHA*-mutant paragangliomas, as there is a wide reported anatomic distribution (abdominal, bladder, thoracic, vagal, and carotid body paragangliomas, as well as a pheochromocytoma).[31] Additionally, identification of the precise mutation in a proband would facilitate screening of family members. However, it is notable that germline mutations in *SDHA*, unlike mutations in other SDH subunit genes, have not thus far been associated with a familial tumor syndrome. The reported patients with *SDHA*-mutant paragangliomas have had apparently sporadic tumors,[31] and none of the patients with *SDHA*-mutant GISTs in our current study had a family history of either paraganglioma or GIST. Furthermore, no patients with germline *SDHA* mutations and both GIST and paraganglioma have yet been reported (in contrast to the Carney–Stratakis syndrome with germline mutations in *SDHB*, *SDHC*, or *SDHD*). The same *SDHA* mutations reported in paragangliomas have also been identified at a low rate in healthy donors;[31] it therefore seems likely that there is a low penetrance of both GISTs and paragangliomas in patients with germline *SDHA* mutations. Until additional follow-up and complete family history are obtained on a larger cohort of patients with *SDHA*-mutant GISTs, the familial implications of identifying a germline *SDHA* mutation remain somewhat uncertain.

In our study, we have found that expression of SDHA is lost in 27% of SDH-deficient GISTs, and that, similar to paraganglioma,[31] loss of SDHA expression in tumors reliably predicts the presence of *SDHA* mutations in tumor cells and associated germline material. Thus, immunohistochemistry can help focus germline testing on *SDHA*. Interestingly, the most common mutation in our patient cohort (c.91C>T; p.R31X), found in four tumors, was previously reported in one of four patients with *SDHA*-mutant GIST[26] and is also the most frequently reported *SDHA* mutation in paragangliomas.[31] Of

Table 2 Germline and somatic mutations identified in nine patients with SDHA-deficient gastrointestinal stromal tumor

Sample	*Tumor tissue mutation(s)*			*Normal tissue mutation*		
	SDHA exon	*Nucleotide*	*Amino acid*	*SDHA exon*	*Nucleotide*	*Amino acid*
1	Exon 5 Exon 8	c.553C>T c.1043-1055del	p.Q185X Frameshift	Exon 5	c.553C>T	p.Q185X
2	Exon 11 Loss of heterozygosity	c.1534C>T	p.R512X	Exon 11	c.1534C>T	p.R512X
3	Exon 8 None detected	c.688delG	Frameshift	Exon 8	c.688delG	Frameshift
4	Exon 2 Loss of heterozygosity	c.91C>T	p.R31X	Exon 2	c.91C>T	p.R31X
5	Exon 8 None detected	c.985C>T	p.R329X	Not available		
6	Exon 13 Loss of heterozygosity	c.1765C>T	p.R589W	Not available		
7	Exon 2 Loss of heterozygosity	c.91C>T	p.R31X	Exon 2	c.91C>T	p.R31X
8	Exon 2 Exon 8	c.91C>T c.985C>T	p.R31X p.R329X	Not available		
9	Exon 2 Loss of heterozygosity	c.91C>T	p.R31X	Exon 2	c.91C>T	p.R31X

note, three patients from our study with confirmed *SDHA* mutations underwent clinical sequencing of *SDHB*, *SDHC*, and *SDHD*, all of which were negative. The prevalence of detected *SDHA* mutations in our cohort suggests that alterations in this gene are likely more common than those in *SDHB*, *SDHC*, and *SDHD*,[12] although this finding needs to be confirmed in a larger series. Although these studies together suggest that only 35–40% of SDH-deficient GISTs contain mutations in an SDH subunit, undetected mutations, deletions or epigenetic alterations of SDH subunit genes, or possibly mutations in genes encoding other cofactor proteins may account for the remaining cases of SDH deficiency. Further identification of the genetic mechanisms leading to SDH-deficient GIST will facilitate genetic classification of these tumors and will help guide mutation-specific cancer screening and genetic counseling.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)