Defects in succinate dehydrogenase in gastrointestinal stromal tumors lacking *KIT* and *PDGFRA* mutations

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Carney-Stratakis syndrome, an inherited condition predisposing affected individuals to gastrointestinal stromal tumor (GIST) and paraganglioma, is caused by germline mutations in succinate dehydrogenase (SDH) subunits B, C, or D, leading to dysfunction of complex II of the electron transport chain. We evaluated the role of defective cellular respiration in sporadic GIST lacking mutations in *KIT* or *PDGFRA* (WT). Thirty-four patients with WT GIST without a personal or family history of paraganglioma were tested for *SDH* germline mutations. WT GISTs lacking demonstrable *SDH* genetic inactivation were evaluated for SDHB expression by immunohistochemistry and Western blotting and for complex II activity. For comparison, SDHB expression was also determined in *KIT* mutant and neurofibromatosis-1–associated GIST, and complex II activity was also measured in *SDH*-deficient paraganglioma and *KIT* mutant GIST; 4 of 34 patients (12%) with WT GIST without a personal or family history of paraganglioma had germline mutations in *SDHB* or *SDHC*. WT GISTs lacking somatic mutations or deletions in *SDH* subunits had either complete loss of or substantial reduction in SDHB protein expression, whereas most *KIT* mutant GISTs had strong SDHB expression. Complex II activity was substantially decreased in WT GISTs. WT GISTs, particularly those in younger patients, have defects in SDH mitochondrial complex II, and in a subset of these patients, GIST seems to arise from germline-inactivating *SDH* mutations. Testing for germline mutations in *SDH* is recommended in patients with WT GIST. These findings highlight a potential central role of SDH dysregulation in WT GIST oncogenesis.

genetic predisposition | sarcoma | pediatric

Gastrointestinal stromal tumor (GIST), the most common mesenchymal neoplasm of the gastrointestinal tract, is resistant to conventional cytotoxic chemotherapy (1). Oncogenic mutations in *KIT* or *PDGFRA* have been identified as central tumor-initiating events in many GISTs (2, 3). However, 85% of GISTs occurring in children and 15% of GISTs occurring in adults lack *KIT* or *PDGFRA* mutations (termed wild-type or WT GISTs) (4S–6). The tumor-initiating event(s) in these WT GISTs is not known. Imatinib and sunitinib, small-molecule inhibitors of the mutant KIT and PDGFRA receptor tyrosine kinases, significantly prolong survival in patients with GIST (7, 8). However, imatinib is less effective against WT tumors (9, 10), and initial studies suggest

that sunitinib only rarely results in objective responses in WT GIST (10, 11). Identification of the pathogenetic mechanism in WT GIST will facilitate the identification of drug targets in these tumors.

The succinate dehydrogenase (SDH)–ubiquinone complex II, a component of the Krebs cycle and the respiratory chain, is a heteroligomer composed of subunits A, B, C, and D. The familial paraganglioma syndromes 1, 3, and 4 are caused by germline-inactivating mutations in the genes coding for SDH subunits D (*SDHD*), C (*SDHC*), or B (*SDHB*), respectively. An additional 12–16% of patients with apparently sporadic paraganglioma carry germline-inactivating mutations in *SDHB*, *-C*, or *-D* (12, 13). Germline mutations in *SDHB* have also been associated with pheochromocytoma, especially malignant forms, and renal cell carcinoma. In familial paraganglioma, *SDH* acts as a classic tumor suppressor (14): germline-inactivating mutations in one allele combined with somatic inactivation of the remaining normal allele lead to tumor development. Inactivation of any one of the three commonly mutated SDH subunits results in destabilization of the SDH complex and loss of enzymatic function (15). An additional SDH–ubiquinone complex II component, SDHAF2, that interacts with and flavinates SDH subunit A (SDHA) was very recently described. Loss of function mutations in *SDHAF2* also result in destabilization of the SDH

complex and loss of complex II activity, and *SDHAF2* germline mutation is a rare cause of familial paraganglioma (16–18).

Carney-Stratakis syndrome is an inherited predisposition to GIST and paraganglioma (19) that is caused by inactivating germline mutations in *SDHB*, *-C*, or *-D* (20, 21). Sporadic WT GIST occurring in patients without a personal or family history of paraganglioma is more common than Carney-Stratakis syndrome, but the causative oncogenic events in these WT GISTs remain unknown. We sought to evaluate the role of defective cellular respiration in sporadic WT GISTs.

Results

Subjects Were Identified Through the National Institutes of Health Pediatric and WT GIST Clinic.
The National Institutes of Health (NIH) Pediatric and WT GIST Clinic, a biannual collaborative effort between clinicians, researchers, support groups, and patients, was established in 2008 to further the investigation of the clinical features and oncogenic mechanisms underlying WT GIST (www.pediatricgist.cancer.gov). After meeting with a geneticist and a genetic counselor, all patients attending the clinic were offered testing for germline mutations in *SDHB*, *-C*, and *-D*. At the time that this study was conducted, 37 patients had attended the NIH Pediatric and WT GIST Clinic. Thirty-four patients had confirmed WT GIST, had no family or personal history of paraganglioma, and consented to participation in genetic testing. Thirty of 34 tumors were confirmed to be WT in exons 9, 11, 13, and 17 of *KIT* and exons 12 and 18 of *PDGFRA*. Three of the remaining tumors were confirmed to be WT in at least four of the commonly mutated *KIT* and *PDGFRA* exons. One tumor was confirmed to be WT only in exons 9 and 11 of *KIT*. One patient had a diagnosis of neurofibromatosis type 1 (NF-1). In this group of patients, age at GIST diagnosis was 5–58 y (median = 22 y). The primary tumor site was gastric in 82% of patients, small intestine in 9%, and advanced in 9%. Fifty-six percent of primary tumors were multifocal at presentation, and 79% of the patients were female.

Germline *SDH* Mutations Are Present in 12% of Individuals With WT GIST Without a Personal or Family History of Paraganglioma.
SDHB, *-C*, and *-D* exons and exon–intron boundaries were sequenced from genomic DNA isolated from whole blood of the 34 patients with confirmed WT GIST. Four (12%) patients had germline mutations in *SDHB* or *-C* (Table 1 and Table S1). Three mutations were identified in *SDHB* in exons 3, 6, and 7. *SDHB* mutations were missense mutations resulting in changes in amino acids that are highly conserved across species. Two of the *SDHB* mutations have previously been reported in familial paraganglioma (22). The other *SDHB* mutation, S92T, resulted in a substitution at a highly conserved amino acid, which is expected—based on in silico analysis—to inactivate SDHB function [position-specific independent count (PSIC) score of 2.231]. One splice site mutation was identified in *SDHC* at position +1 of intron 5. A mutation at this site, previously reported in both paraganglioma and Carney-Stratakis syndrome, causes deletion of exon 5, and it results in a frame shift and premature termination (20, 23).

Two patients (6%) had an *SDHD* germline sequence change with questionable pathogenicity (c. 34 G > A) that has previously been reported to be present in patients with pheochromocytoma

(24), hereditary paraganglioma (25), and Cowden syndrome (26). In Cowden syndrome, the resulting amino acid change, G12S, was associated with a twofold increase in AKT and MAPK activity and an increase in reactive oxygen species. However, this *SDHD* sequence alteration has also been seen in control populations, with an incidence ranging from 0% to 2.5% (24, 26).

To confirm the functional impact of these germline mutations, we performed SDHB immunohistochemistry (IHC) on paraffin-embedded GIST tumor samples, when available, from patients with *SDH* subunit germline mutations. SDHB protein expression was evaluable in two of three patients with germline *SDHB* mutations, and in both, expression was absent (Fig. 1). SDHB protein expression was 1+ in the one patient with germline *SDHD* sequence change in which there was sufficient tumor for analysis.

Patients with *SDHB* mutations were all young adults, diagnosed at 18, 21, and 22 y of age. The patient with the *SDHC* mutation was 16 y at diagnosis. The sex distribution of patients with *SDH* mutations was 50% male and 50% female. All patients with *SDH* mutations had multifocal GIST, but 50% of the patients without *SDH* mutations also had multifocal GIST.

WT GISTs Have Either Complete Loss or Substantial Reduction in SDHB Protein Expression.
To determine whether loss of SDHB protein expression was a general feature of WT GISTs, 30 WT GIST tumors without associated SDH mutations were evaluated for SDHB protein expression by IHC ($n = 22$), Western blotting ($n = 4$), or both Western blotting and IHC ($n = 4$). Eighteen of the WT GISTs used in these studies were classified as pediatric (age of diagnosis ≤ 18 y); 12 were classified as adult. In 25 of 30 WT GISTs, absence of an associated *SDH* mutation was confirmed by sequence analysis using germline or tumor DNA. For the remaining five WT GISTs, there was neither germline DNA nor tumor DNA available to confirm a lack of an associated *SDH* mutation (Table S1). Furthermore, 250,000 SNP analyses, performed in 7 of 31 GISTs, showed absence of *SDHB*, *SDHC*, or *SDHD* deletions in 6 GISTs (Fig. S1), whereas one tumor had a loss of most of 1p, a common abnormality in *KIT* mutant GISTs, resulting in an *SDHB* deletion.

SDHB protein expression was absent in 18 of 18 (100%) pediatric WT GISTs evaluated for SDHB expression by IHC (Fig. 1) or Western blotting (Fig. 2), including four cases that were negative by both methods (Fig. 2, cases marked with an asterisk had an SDHB IHC score of 0). SDHB protein expression was absent in 8 of 12 (67%) and was weak (SDHB IHC score of 1+) in 4 of 12 (33%) of the adult WT GISTs. By comparison, only 1 of 18 (6%) of the *KIT* mutant GISTs and 0 of 5 NF-1–associated GISTs lacked SDHB expression.

WT GIST Has Markedly Decreased SDH (Complex II) Activity.
Loss of SDHB expression has previously been shown to be highly correlated with SDH or complex II inactivation in paraganglioma (15). However, we did not know whether this would also be true in GIST. Therefore, a detailed spectrophotometric study of the activity of mitochondrial respiratory chain complexes rotenone-sensitive NADH-quinone reductase (NQR; complex I), malonate-sensitive succinate-cytochrome *c* reductase (SCCR; complexes II and III), glycerol-3-phosphate cytochrome *c* reductase (GCCR; glycerol-3-

Table 1. Germline *SDH* mutations identified in 34 patients with WT GIST without a family or personal history of paraganglioma

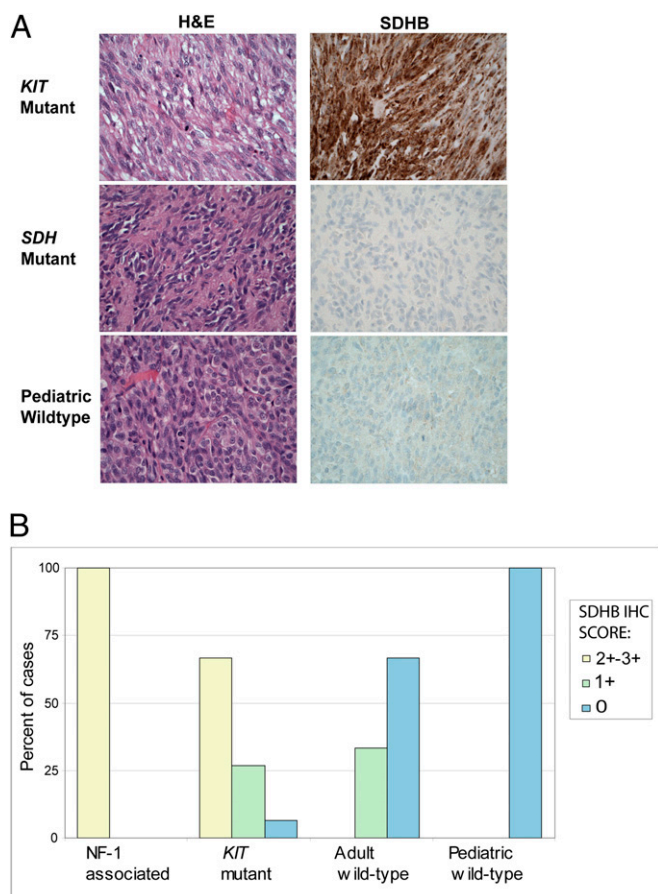| Gene	Exon	Mutation	Amino acid change	Reference	Mutation type	Age at diagnosis (y)	Sex	SDHB IHC
SDHB	3	c. 274 T > A/T	p. Ser-92 Thr	No previous reports	Missense	18	M	Negative
SDHB	6	c. 600 G > G/T	p. Trp-200 Cys	27	Missense	22	M	Negative
SDHB	7	c. 725 G > A/G	p. Arg-242 His	12	Missense	21	F	NA
SDHC	Intron 5	c. 405+1 G > A		20	Splice site	16	F	NA
SDHD	1	c. 34 G > A	p. Gly-12 Ser	24	Polymorphism	7	F	NA
SDHD	1	c. 34 G > A	p. Gly-12 Ser	24	Polymorphism	58	F	1+

Fig. 1. (A) Representative H&E stains (*Left*) and corresponding SDHB IHC (*Right*) in *KIT* mutant (*Top*), *SDH* mutant (*Middle*), and WT GIST (*Bottom*), highlighting strong SDHB immunoexpression in the *KIT* mutant GIST and absence of SDHB expression in the SDH mutant GIST and the WT GIST. (B) Summary of SDHB IHC scores in pediatric (*n* = 14) and adult (*n* = 12) WT GIST, *KIT* mutant GIST (*n* = 15), and NF-1–associated GIST (*n* = 5).

phosphate dehydrogenase + complex III), antimycin-sensitive decylubiquinol-cytochrome *c* reductase (QCCR; complex III), cyanide-sensitive cytochrome *c* oxidase (COX; complex IV), and oligomycin-sensitive ATPase (complex V) was performed in two WT GISTs lacking somatic mutations or deletions in *SDH* subunit genes, a *KIT* mutant GIST, and an *SDH* mutant paraganglioma. Both absolute and relative (to GCCR) SCCR activity, in which the limiting activity is the SDH complex, were markedly reduced in the WT GISTs (Fig. 3). The extent of reduction in SCCR activity seen in the WT GISTs was equal to that seen in an *SDHB* mutant paraganglioma. In *KIT* mutant GIST, SCCR activity was comparable with that seen in normal abdominal tissue.

Discussion

The *SDHB* and *SDHC* germline mutations identified in 12% of patients with WT GIST in this study are highly likely to be
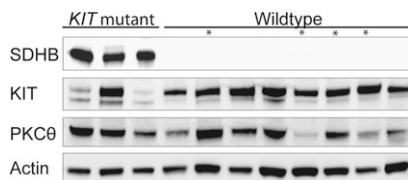

Fig. 2. Western blot of SDHB expression in *KIT* mutant and WT GIST. Cases marked by an asterisk had an SDHB IHC score of 0.

pathogenic, and to have predisposed these patients to the development of GIST. These germline mutations in the *SDH* subunit genes were found in individuals with GIST without a personal or family history of paraganglioma. Three of the four *SDHB* and *SDHC* germline mutations identified in these patients with GIST have previously been reported to occur in individuals with paragangliomas (12, 20, 27, 28). Like the majority of *SDHB* mutations associated with paraganglioma, the identified *SDHB* mutations in these patients with WT GIST are missense mutations in highly conserved amino acids (12, 22). The *SDHC* mutation identified here has previously been shown to result in an inactivating frame shift (20). GIST tumor specimens from two of the patients with *SDHB* germline mutations lacked SDHB protein expression, and the other patient was not evaluable. Absence of SDHB protein expression, as determined by IHC, has recently been shown to have a sensitivity of 100% for the presence of *SDHB*, *SDHC*, or *SDHD* mutations in paragangliomas and pheochromocytomas (15). We have not been able to determine the penetration of the clinical phenotype associated with these mutations, because not all first-degree relatives have undergone germline testing. The *SDHD* base pair change identified here in two patients is likely to be a polymorphism, despite the previously reported associations with pheochromocytoma, paraganglioma, and Cowden syndrome; this is because the c.34A > G nt change has been reported in up to 2.5% of normal controls (24), and the base pair change alters an amino acid that is not conserved across species. Furthermore, a GIST tumor specimen from one of the patients with this *SDHD* sequence change had 1+ SDHB protein expression.

Based on the 12% incidence of *SDH* subunit germline mutations in this series of patients with WT GIST, testing for germline mutations in *SDHB*, *SDHC*, and *SDHD* in all patients diagnosed with WT GIST is recommended, particularly in younger individuals. The incidence of germline mutations in apparently sporadic pheochromocytoma or functional paraganglioma is similar to that seen in GIST (12–16%) (12, 13), and germline testing has been recommended (29) for these patients. The identification of a germline mutation in a patient with WT GIST has the potential for clinical benefit by alerting the treating physician to a presumed increased risk of paragangliomas and additional GISTs. In addition, because *SDHB*-associated paragangliomas and GIST share several features such as PET positivity and intraabdominal location, it is possible for a functional paraganglioma to be mistaken for recurrent GIST. Knowledge of a germline mutation in one of the *SDH* subunit genes could prevent the potentially life-threatening complication of resection of a functional paraganglioma mistaken for a GIST.

This series is not sufficiently large to definitively identify clinical features associated with the presence of *SDH* germline mutations in patients with WT GIST. However, the sex distribution of those patients with germline mutations was 50% male, which is different from the female predominance typical of WT GIST in general and the female predominance of patients seen in the NIH Pediatric and WT GIST Clinic. In fact, two of seven males tested (29%) were found to have germline mutations in *SDH* subunit genes.

The association of germline *SDHB* and *SDHC* mutations and WT GIST suggested that abnormalities of cellular respiration might exist in WT GISTs generally, even in patients without germline mutations in one of the *SDH* subunits. To investigate this possibility, we evaluated SDHB expression and function in WT GISTs without associated *SDH* mutations. SDHB expression is absent in all pediatric WT GISTs and absent or weak in adult WT GISTs, whereas most *KIT* mutant and all NF-1–associated GISTs had strong SDHB expression. The observed lack of SDHB expression is not likely to be explained by somatic mutations in SDHB, -C, or -D in GIST tumors, because SDH mutation analysis was performed from tumor in 13 of the cases lacking SDH protein expression on IHC or Western blot. There has been one prior study of SDHB IHC in GIST (30). It is
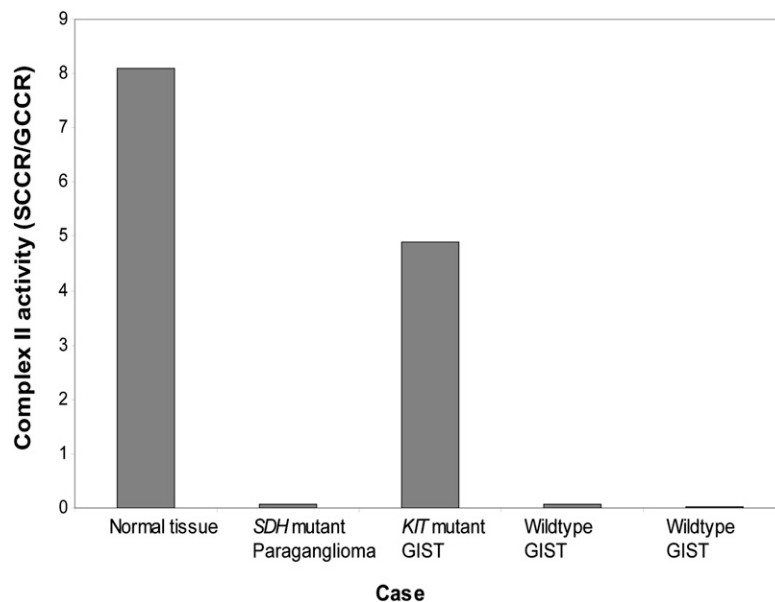
Fig. 3. Assessment of complex II activity in a paraganglioma arising in a patient with a germline *SDH* mutation, in two WT GISTs, and in a *KIT* mutant GIST. Cytochrome *c* oxidase (complex IV), quinol cytochrome *c* reductase (complex III), NADH quinone reducatse (complex I), and ATPase (complex V) activities were normal in these tumors.

somewhat difficult to compare our results with this previously published study, because in the published study, *KIT*, *PDGFRA*, and *SDH* subunit genotype were available for only a limited number of cases. In that study, 97% of sporadic GISTs had positive SDHB IHC. The nine GISTs lacking SDHB expression occurred in patients with either Carney Triad or clinical features suggestive of WT GIST. Therefore, our results are not inconsistent with this previously published study.

KIT and *PDGFRA* sequencing is recommended in suspected WT GIST, because response to standard GIST therapies, imatinib and sunitinib, and natural history differs in WT tumors (9, 11, 31). However, molecular analysis is frequently not performed because of cost. Given the association between SDHB IHC results and genotype, an SDHB IHC score of less than 2+ could be used to identify tumors that are likely to be WT.

Loss of SDHB expression and lack of complex II activity in WT GIST without an associated *SDH* mutation or deletion implicate defects in cellular respiration as a potential central oncogenic mechanism in WT GIST. One possible mechanism for the observed loss of *SDHB* expression and complex II function in the WT GISTs samples analyzed in this study is epigenetic modification resulting in decreased mRNA expression of one of the components of the SDH complex. However, mRNA expression of SDHB, SDHC, and SDHD did not differ significantly between WT and *KIT* mutant GISTs, as evaluated by quantitative RT-PCR (Fig. S2). Another possible explanation is loss of function mutations in S*DHA* or *SDHAF2*, each of which has recently been described to occur in an individual patient and an individual family, respectively, with paraganglioma (16, 17, 32). However, *SDHAF2* mutation analysis was conducted in 42 of the WT GIST cases from this study and an additional 48 WT GISTs, and no mutations were identified. *SDHA* mutation analysis was conducted in four of the WT GIST cases from this study and one additional WT GIST, and no mutations were identified (Table S1). We sequenced *SDHA* in only a small group of WT GISTs because of availability of appropriate material for sequencing, and further investigation of *SDHA* mutations in WT GIST is warranted. Another consideration warranting further study is alterations in other components of cellular respiration such as

isocitrate dehydrogenase (33, 34) or yet to be identified tricarboxylic acid cycle proteins interacting with SDH.

Materials and Methods

Patients and Tumor Samples. Patients were either self-referred or referred by their treating physician to the NIH Pediatric and WT GIST Clinic (www.pediatricgist.cancer.gov). Patients were accepted into the clinic only if they had GIST diagnosed at age 18 y or less (pediatric GIST), prior molecular analysis of their tumor with results consistent with WT GIST, or clinical features highly suggestive of WT GIST. Patients participated in research protocols that were approved by the institutional review boards at the relevant institutions. All participants gave consent or when relevant, assent for participation in the clinic and associated studies, including genetic testing.

For each participant in the NIH Pediatric and WT GIST Clinic, primary medical data, including clinic notes, radiographic studies, surgical reports, and pathology reports, were reviewed by NIH GIST team members. Over a 2.5-d period, participants in the NIH Pediatric and WT GIST Clinic underwent a history, physical examination, consultation with a geneticist, and a session with a genetic counselor. In addition, participants met with physician members of the Consortium for Pediatric and WT GIST Research (CPGR), a consortium of clinicians, researchers, and patient advocates who share the goal of defining the natural history and underlying biology of WT GIST in an effort to develop effective and novel treatment regimens.

Patients' GIST tumors were confirmed to be WT by obtaining the report describing the results of mutation testing. When mutation analysis had not previously been performed, genomic DNA was extracted from the paraffin-embedded tumor, and exons 9, 11, 13, and 17 of *KIT* and exons 12 and 18 of *PDGFRA* were sequenced as previously described (6). Additional tumor samples, not from participants in the NIH Pediatric and WT GIST Clinic used in this study, have been described previously (4, 35, 36). Ten additional pediatric GIST cases were collected from the archives and referral cases of one of the authors (M.O.) for inclusion in the immunohistochemistry portion of this study.

Mutation Analysis. Genomic DNA was isolated from blood or cryopreserved tumor. All exons and exon–intron boundaries of *SDHB*, *SDHC*, and *SDHD* were PCR-amplified and screened for mutation by standard methods at Beckman Coulter Genomics (for cryopreserved tumor; formerly Agencourt) or GeneDx (for germline DNA; www.geneDx.com) or as previously described (for paraffin-embedded tumor) (13, 37, 38). Sequence analysis was performed using the Mutation Surveyor software (SoftGenetics) and based on RefSeq [National Center for Biotechnology Information (NCBI)] for the appropriate gene (for cryopreserved tumor) or as previously described [for germline DNA (20, 21) and paraffin-embedded tumor (13, 37–40)]. Homology was determined based on homologene (NCBI).

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Immunohistochemistry. Immunohistochemistry analysis was performed on 4-μm sections of formalin-fixed tumor as previously described (15, 41). Immunoreactivity was graded semiquantitatively using the following scale: 0, no staining; 1+, less than 5% of tumor cells reactive; 2+, 6–50% of tumor cells reactive; 3+, over 51% of tumor cells reactive.

Western Blotting. Whole-cell lysates of cryopreserved tumors were prepared as previously described (42). Lysates were separated by gel electrophoresis using NuPAGE 4–12% Bis·Tris gels (Invitrogen) and blotted to nitrocellulose membranes. Immunostains were detected using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) and captured with the Fuji LAS1000-plus imaging system. Blots were stained with antibodies to SDHB, PKCθ (Santa Cruz Biotechnology), KIT (DAKO), and actin (Sigma).

SNP Array. We evaluated *SDH* subunit gene deletions in WT GIST using SNP arrays. Genomic DNA isolated from cryopreserved GISTs and four normal control samples was digested with the StyI restriction enzyme. Digested DNA was then ligated to an adaptor before subsequent PCR amplification using AmpliTaq Gold (Applied Biosystems). PCR products were pooled, concentrated, and fragmented with DNase I to a size range of 200–1,100 bp. Fragmented PCR products were then labeled, denatured, and hybridized to Affymetrix 250K *Sty* SNP arrays interrogating ~238,000 SNPs. After hybridization, the arrays were washed on Affymetrix fluidics stations, stained, scanned using Gene Chip Scanner 3000 7G, and interpreted using genotyping software Affymetrix Genotyping Tools Version 2.0. Data analysis was performed as previously described (4).

Mitochondrial Respiratory Chain Activity. The activities of the respiratory chain complexes, rotenone-sensitive NQR (complex I), malonate-sensitive SCCR (complexes II and III), GCCR (glycerol-3-phosphate dehydrogenase + complex III), antimycin-sensitive QCCR (complex III), cyanide-sensitive COX (complex IV), and oligomycin-sensitive ATPase (complex V) were spectrophotometrically measured on homogenates prepared from frozen tumors as previously described (43). Normal abdominal tissue was used as a positive control, and paraganglioma from a patient with a pathogenic *SDH* mutation was used as a negative control. Results are expressed as a ratio of SCCR to GCCR activity.

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