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

Katherine A. Janeway,a,b,1,2 Sy Young Kimb,1 Maya Lodishc, Vanía Nosed, Pierre Rustint, José Gaal, Patricia L. M. Dahiat, Bernadette Lieglb, Evan R. Balla, Margarita Raygad, Angela H. Laix, Lorna Kellyj, Jason L. Hornickk, NIH Pediatric and Wild-Type GIST Clinical Program, National Cancer Institute, Bethesda, MD 20892; Section on Endocrinology and Genetics, Program on Developmental Endocrinology Genetics, The Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892; Department of Pathology, University of Miami Miller School of Medicine, Miami, FL 33136; Inserm U676 and Université Paris 7, Faculté de Médecine Denis Diderot, IRFR2 Paris, France; Department of Pathology, Josephine Neffens Institute, Erasmus MC—University Medical Center, 3000 CA, Rotterdam, The Netherlands; Departments of Medicine and Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, TX 78229; Institute of Pathology, Medical University, 8036 Graz, Austria; Program on Developmental Endocrinology Genetics, The Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892; National Children’s Research Centre, Our Lady’s Children’s Hospital, Crumlin, Dublin 12, Ireland; Department of Pathology, Brigham and Women’s Hospital, Boston, MA 02115; Department of Sarcoma Medical Oncology, MD Anderson Cancer Center, Houston, TX 77030; Department of Oncology, St. Jude Children’s Research Center, Memphis, TN 38105; Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, PA 19111; Histology Laboratory, Our Lady’s Children’s Hospital, Crumlin, Dublin 12, Ireland; Department of Medical Oncology, and Ludwig Center, Dana-Farber/Harvard Cancer Center, Boston, MA 02115; and Departments of Pathology, and Surgery, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

*Edited by Stuart H. Orkin, Children’s Hospital and the Dana Farber Cancer Institute, Harvard Medical School and Howard Hughes Medical Institute, Boston, MA, and approved November 23, 2010 (received for review June 30, 2010)

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 SDH expression by immunohistochemistry and Western blotting and for complex II activity. For comparison, SDHB expression was also determined in wild-type GIST (WT) tumors occurring in children and 15% of GISTs occurring in adults lack KIT or PDGFRA mutations (termed wild-type or WT GISTs) (45–6). 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 SDHD, -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, SDHA, that interacts with and flavinates SDH subunit A (SDHA) was very recently described. Loss of function mutations in SDHA also result in destabilization of the SDH complex (16), and germline loss of function mutations in SDHA are associated with familial paraganglioma (17). This study was performed to test the hypothesis that germline loss of function mutations in SDHA are present in sporadic GIST.


The authors declare no conflict of interest.

K.A.J. and S.Y.K. contributed equally to this work.

1To whom correspondence should be addressed. E-mail: kjaneaway@partners.org.


This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1009199108/-/DCSupplemental.
complex and loss of complex II activity, and SDHAF2 germline mutations 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 SDHD, -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 PDGFRα. Three of the remaining tumors were confirmed to be WT in at least four of the commonly mutated KIT and PDGFRα 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 12%, and the remainder included the stomach, colon, pancreas, and peritoneum. Sex and age at diagnosis were comparable to those reported in prior studies (22, 23). A total of 50 patients (37 with confirmed WT GIST, 12 with a GIST-like diagnosis) had a personal or family history of paraganglioma, and were left untested for germline mutations.

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 exons-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 identical 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, we compared 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exon</th>
<th>Mutation</th>
<th>Amino acid change</th>
<th>Reference</th>
<th>Mutation type</th>
<th>Age at diagnosis (y)</th>
<th>Sex</th>
<th>SDHB IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDHB 3</td>
<td>c. 274 T &gt; A/T</td>
<td>p. Ser-92 Thr</td>
<td>No previous reports</td>
<td>Missense</td>
<td>18</td>
<td>M</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>SDHB 6</td>
<td>c. 600 G &gt; G/T</td>
<td>p. Trp-200 Cys</td>
<td>27</td>
<td>Missense</td>
<td>22</td>
<td>M</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>SDHB 7</td>
<td>c. 725 G &gt; A/G</td>
<td>p. Arg-242 His</td>
<td>12</td>
<td>Missense</td>
<td>21</td>
<td>F</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>SDHC Intron 5</td>
<td>c. 405+1 G &gt; A</td>
<td>20</td>
<td>Splice site</td>
<td>16</td>
<td>F</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDHD 1</td>
<td>c. 34 G &gt; A</td>
<td>p. Gly-12 Ser</td>
<td>24</td>
<td>Polymorphism</td>
<td>7</td>
<td>F</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>SDHD 2</td>
<td>c. 34 G &gt; A</td>
<td>p. Gly-12 Ser</td>
<td>24</td>
<td>Polymorphism</td>
<td>58</td>
<td>F</td>
<td>1+</td>
<td></td>
</tr>
</tbody>
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phosphate dehydrogenase + complex III), antimycin-sensitive decylubiquinol-cytochrome c reductase (OCCR; 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 SDH 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 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, 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 SDHB 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
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 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 SDHA 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).
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+, 5–15% 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. Immunorecognition was detected by using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) and captured with the Fuji LAS1000-plus imaging system. Blots were stained with antibodies to SDHB, PKCα (Santa Cruz Biotechnology), KIT (DAKO), and actin (Sigma).

SNP Array. We evaluated SDH subunit gene deletions in WT GST 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, 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).