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## Over-expression of IGF1R and Frequent Mutational Inactivation of *SDHA* in Wild-type *SDHB*-negative Gastrointestinal Stromal Tumors

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### Abstract

Approximately 15% of gastrointestinal stromal tumors (GISTs) in adults and 85% in children lack mutations in *KIT* and *PDGFRA* and are known as wild type GISTs. Wild type GISTs from adults and children express high levels of insulin-like growth factor 1 receptor (IGF1R) and exhibit stable genomes compared to mutant GISTs. Pediatric wild type GISTs, GISTs from the multi-tumor Carney-Stratakis syndrome and the Carney triad share other clinico-pathological properties (e.g. early-onset, multifocal GISTs with epithelioid cell morphology) suggesting a common etiology. Carney-Stratakis is an inherited association of GIST and paragangliomas caused by germline mutations in succinate dehydrogenase (SDH) genes. The connection between defective cellular respiration and GIST pathology has been strengthened by the utilization of *SDHB* immunohistochemistry to identify SDH deficiency in pediatric GISTs, syndromic GISTs, and some adult wild type GISTs. *SDHB* and IGF1R expression was examined in 12 wild type and 12 mutant GIST cases. Wild type GISTs were screened for coding-region alterations in *SDH* genes and for chromosomal aberrations using genome-wide SNP and MIP arrays. *SDHB*-deficiency, identified in 11/12 wild type GIST cases, was tightly associated with over-expression of IGF1R protein and transcript. Biallelic inactivation of the *SDHA* gene was a surprisingly frequent event, identified in 5/11 *SDHB*-negative cases, generally due to germline point mutations accompanied by somatic *SDHA* allelic losses. As a novel finding, inactivation of the *SDHC* gene from a combination of a heterozygous coding-region mutation and hyper-methylation of the wild type allele was found in one *SDHB*-negative case.

### Introduction

Gastrointestinal stromal tumors (GISTs) are mesenchymal neoplasms of the gastrointestinal tract that are thought to arise from the interstitial cells of Cajal or from less differentiated mesenchymal precursor cells (Kindblom et al., 1998; Sircar et al., 1999). Most GISTs express the type III receptor tyrosine kinase (RTK) *KIT* (CD117) (Medeiros et al., 2004;

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Tarn et al., 2005), and approximately 85-90% of sporadic adult GISTs possess gain-of-function mutations in *KIT* or in the related receptor *PDGFRA* (Hirota et al., 1998; Rubin et al., 2001; Tarn et al., 2005). Recently, activating mutations in the serine-threonine kinase *BRAF* have been identified in a small number of GIST cases (Agaram et al., 2008b; Agaimy et al., 2009; Belinsky et al., 2009; Hostein et al., 2010). Therapy with the tyrosine kinase inhibitor imatinib is initially effective for most *KIT/PDGFRA* mutant GISTs (Debiec-Rychter et al., 2006; Heinrich et al., 2008) but is generally less effective for the ~ 10-15% of GISTs that are “wild type” for these receptors (Heinrich et al., 2008; Janeway et al., 2009).

GISTs also occur in children, and ~ 85% of these tumors are wild type for *KIT* and *PDGFRA* (Janeway et al., 2007; Agaram et al., 2008a). Wild type GISTs from adults and children express higher levels of the insulin-like growth factor 1 receptor (*IGF1R*) than mutant GISTs (Prakash et al., 2005; Agaram et al., 2008a; Belinsky et al., 2008; Tarn et al., 2008; Pantaleo et al., 2009), and *IGF1R* may be a potential therapeutic target in wild type GIST (Tarn et al., 2008). Overexpression of *IGF1R* may be indicative of a more generalized gene expression profile common to wild type GISTs (Prakash et al., 2005; Agaram et al., 2008a; Pantaleo et al., 2011b), potentially characterized by expression of genes restricted to neural tissue (Pantaleo et al., 2011b). Wild type GISTs from adults and children also exhibit relatively stable genomes as compared to mutant GISTs (Janeway et al., 2007; Belinsky et al., 2009; Astolfi et al., 2010).

Several multitumor syndromes involving *KIT/PDGFRA*-wild type GIST have been described. Carney triad, a non-familial association of GIST with paragangliomas, pulmonary chondromas, or other tumors (Carney et al., 1977; Carney 1999; Zhang et al., 2010), affects mainly young females, and the GISTs present almost exclusively in the stomach as multifocal growth with frequent lymph node metastasis. Carney-Stratakis, an inherited syndrome pre-disposing towards the development of multifocal gastric GIST and paragangliomas, is caused by germline mutations in component genes of the Krebs cycle complex succinate dehydrogenase (*SDH*) (McWhinney et al., 2007). *SDH* deficiency in tumorigenesis is thought to result in stabilization of hypoxia-inducible factor 1-alpha (*HIF1A*) and activation of pseudohypoxia signaling (Dahia et al., 2005; King et al., 2006; Koivunen et al., 2007). Several cases of *SDHx* gene mutations in non-Carney-Stratakis GISTs have recently been reported (Janeway et al., 2011; Pantaleo et al., 2011a; Pantaleo et al., 2011c), and a more generalized defect of the *SDH* pathway, indicated by a lack of immunohistochemical expression of the *SDHB* protein, has been identified in patients with the Carney triad or Carney-Stratakis syndrome, in sporadic pediatric GISTs, and in a small percentage of sporadic adult wild type cases (Gill et al., 2010; Gaal et al., 2011; Janeway et al., 2011; Miettinen et al., 2011).

In this study, the expression level of *IGF1R* and of several neural markers, as well as the presence of *SDHB* protein, were characterized in a series of 12 wild type and 12 mutant GIST cases. Wild type GIST cases were also evaluated for germline and somatic coding-region mutations in all *SDH* subunit genes. Chromosomal aberrations in these tumors were identified using genome-wide single-nucleotide polymorphism (SNP) and molecular inversion probe (MIP) arrays.

## Materials and Methods

### Preparation of Genomic DNA and Total RNA from Tumor Samples

All tumor samples and normal blood were obtained following informed consent under the Fox Chase Cancer Center Institutional Review Board guidelines. Tumor specimens obtained from surgically treated GIST patients were snap-frozen in liquid nitrogen and stored at -80°C until use. Frozen samples were embedded in optimal cutting temperature (OCT)

medium, and sections cut and stained with hematoxylin/eosin to evaluate tumor cellularity. Genomic DNA was isolated from OCT sections or from patient blood using the Easy-DNA kit (Life Technologies), according to manufacturer's instructions. DNA concentration was determined using the NanoDrop 1000 (ThermoFischer Scientific), and agarose-gel electrophoresis used to check the integrity of DNA preparations. Total RNA was isolated from cut OCT sections using TRIzol® reagent (Life Technologies), quantified, and the RNA integrity verified using agarose-gel electrophoresis.

### Immunohistochemical Analysis

Immunohistochemical (IHC) staining for KIT and IGF1R (Tarn et al., 2008) and SDHB (Gill et al., 2010b) was performed on 5 µm slides as described. All IHC evaluation was performed in a blinded manner by one author (DF). The following criteria were used to assess SDHB expression: positive score denotes discrete granular cytoplasmic staining (mitochondrial), whereas negative score indicates lack of mitochondrial staining with presence of internal positive controls (adjacent normal tissue, stromal cells, endothelial cells). IGF1R was scored based on distribution and intensity of positive tumor cell staining. **Distribution:** Absent tumor cell staining was scored as 0, <10% of positive tumor cells staining were scored as 1, 10% to 50% of cells staining were scored as 2, 50% to 90% of cells staining were scored as 3, and >90 of cells staining were scored as 4. **Intensity:** Absent staining in tumor cells was scored as 0, equivocal was scored as 1, clearly positive was scored as 2, and strong positive staining was scored as 3. **Scoring:** The results for intensity and distribution were summed and a "score" assigned as follows: sum of 0, no staining (score 0); sum of 1 to 3, slight staining (score 1); sum of 4 to 5, moderate staining (score 2); and sum of 6 to 7, marked staining (score 3).

### qRT-PCR Analysis

Random-primed cDNA was prepared from 2 µg total RNA using the High Capacity cDNA Reverse Transcription KIT (Life Technologies). RNA expression was measured by real-time PCR on an ABI PRISM 7900 HT Sequence Detection System using fluorescein phosphoramidite (FAM) primer/probe sets (Applied Biosystems). Primer-probe sets are described in Supplementary Table 2. RNA expression data for IGF1R, CDH2, ELAVL3, and SDH subunit genes were normalized to beta actin. For cases with more than one analyzed GIST, normalized expression values of the individual tumors were averaged for case reporting.

### Mutational Analysis

Relevant exons and surrounding intronic regions were PCR-amplified and subjected to Sanger sequencing (Beckman Coulter Genomics). Mutational screening for *KIT* (exons 9, 11, 13, 17), *PDGFRA* (exons 12, 14, 18), and *BRAF* (exons 11,15) was as previously described (Belinsky et al., 2009). Primers for *SDHA*, designed to avoid amplification of pseudogenes on chromosomes 3 (*SDHAP1*, *SDHAP2*) and 5 (*SDHAP3*), have been previously described (Burnichon et al., 2010). Primers for *SDHB*, *SDHC*, and *SDHD* have been described (Wood et al., 2007). Primer sequences for amplification of cDNA regions of the *SDHA* and *SDHC* genes (Supplementary Table 3) were designed using Primer3 (Rozen and Skaletsky 2000). Mutation nomenclature conforms to the recommendations of the Human Genome Variation Society (den Dunnen and Antonarakis 2000).

### SDHC Bisulfite Sequencing

Genomic DNA samples were bisulfite modified using the EZ DNA Methylation-Direct™ Kit (Zymo Research), as per manufacturer's instructions. Primers for amplification of bisulfite-modified DNA from the *SDHC* gene (Supplementary Table 3) were designed using

MethPrimer (Li and Dahiya 2002). Amplicons were cloned into the vector pCR2.1 using a TA cloning kit (Life Technologies) and sequenced using vector-based primers. Universal Methylated DNA Standard (Zymo Research) and normal blood DNA were used as positive and negative controls, respectively, for methylation of CpG dinucleotides.

### SNP and MIP Copy Number Analysis

Chromosomal copy number analysis and allele-specific genotyping using SNP arrays were performed as described (Belinsky et al., 2009). Briefly, genomic DNA isolated from macrodissected OCT-embedded tumor sections was digested with restriction endonucleases, adaptor-ligated and PCR-amplified. Purified PCR products were fragmented, biotin-labeled and hybridized to Affymetrix Genome-Wide Human SNP Array 6.0, or GeneChip® Human Mapping 50K Xba Arrays. Genotyping and copy number analysis for the 6.0 arrays was done using Affymetrix Genotyping Console 2.1 and CNAT 5.0, respectively, using default configurations. For the 50K arrays Affymetrix GeneChip® Genotyping Analysis Software 4.1 and CNAT 4.0, with default settings were used for genotyping and copy number analysis. For MIP analysis, genomic DNA was extracted from GIST samples and processed using OncoScan™ FFPE Express (Affymetrix, Inc., Santa Clara, CA). This assay utilizes MIP technology based on SNP microarrays and has been described previously in detail, including its ability to provide high quality copy number and loss of heterozygosity (LOH) data on formalin-fixed paraffin-embedded (FFPE) clinical samples (Wang et al., 2005; Absalan and Ronaghi 2007; Wang et al., 2007; Ji and Welch 2009; Wang et al., 2009; Wang et al., (in press)). The samples were prepared and run on this assay as previously described (Schiffman et al., 2009; Schiffman et al., 2010; Schiffman et al., 2011; Jahromi et al., in press). The data was analyzed with Nexus Copy Number v5.1 software (BioDiscovery, Inc., El Segundo, CA). Nexus settings for this analysis included Quadratic Wave Correction with SNP-FASST2 Segmentation, with the following settings: High gain 1.2, Gain 0.3, Normal 0, Loss -0.3, Big Loss -1.2 with a significance threshold of 5.0E-7 and call requirement including a minimum of 3 probes per segment. All probes were initially filtered prior to analysis to include only those with standard deviation < 0.2 and a call rate > 90%. All described regions for SNP and MIP assays are based on NCBI build 36.

## Results

### Clinical and Pathological Features of *KIT/PDGFR/BRAF* Wild Type GISTs

12 *KIT/PDGFR/BRAF* mutation-negative GIST cases and 12 mutant cases were examined in this study (Table 1). The 12 wild type cases included 11 adults with a median age of onset of 43 years (range 20-57) and a pediatric case (case 11). Three of the wild type cases (cases 7, 8 and the pediatric case) were Carney triad cases, having manifested extra-adrenal paragangliomas and/or pulmonary chondromas along with multiple GISTs. Nine of the wild type cases were females, 10 cases presented with gastric GIST, and 11 were of epithelioid or mixed epithelioid/spindle-cell morphology. One wild type GIST (case 12) originated in the small bowel and exhibited spindle-cell morphology. The kinase-mutant cases included 10 cases with *KIT* mutations and 2 with *PDGFR* mutations. These 12 patients, including 7 males and 5 females, presented with a median age of onset of 64 years (range 37-86), with 7/12 of gastric and 3/12 small bowel origin, and with spindle-cell morphology in 10/12. All cases were Fox Chase cases except wild type cases 4 and 5, which correspond to cases 38 and 48, respectively, from a recent clinical trial from the Radiation Therapy Oncology Group (Rink et al., 2009).

### IGF1R is Over-expressed in Wild Type SDHB-negative GISTs

IHC for both SDHB and IGF1R was performed to investigate the relationship between IGF1R expression and expression of SDHB in wild type and mutant GISTs. All GISTs were

also stained for KIT expression. Representative IHC results are shown in Figure 1 and summarized in Table 2. All wild type and mutant GISTs were KIT-positive by IHC. IGF1R expression level was evaluated using a scoring system combining distribution and intensity of staining (Tarn et al., 2008). Eleven of 12 wild type GISTs exhibited marked staining for IGF1R (score = 3), while all 12 mutant cases exhibited slight or moderate staining (score = 1-2, Table 2). SDHB protein staining, on the other hand, was absent in wild type cases 1-11 but clearly present in all mutant GISTs and in wild type case 12. Tumors were denoted SDHB-negative when tumor cell negativity was accompanied by positive staining in internal controls such as adjacent normal tissue or endothelial cells (upper and middle panels in Figure 1). IGF1R expression varied strongly either as a function of kinase genotype ( $P \sim 9.6 \times 10^{-6}$ , Fisher exact probability, two-tailed) or SDHB status ( $P \sim 4.0 \times 10^{-7}$ ).

IGF1R expression was further quantified using qRT-PCR. SDHB-negative wild type GISTs expressed significantly higher levels of IGF1R RNA (~ 69-fold higher,  $P < 0.0001$ ) as compared to SDHB-positive GISTs (Figure 2). Transcript levels of two markers of neural tissue, CDH2 (cadherin-2, type 1, neuronal) and ELAVL3 (embryonic lethal, abnormal vision, Drosophila-like 3) were also examined. Both genes were over-expressed in SDHB-negative wild type GISTs, although differences in expression were not as great as for IGF1R (CDH2~ 18-fold, ELAVL3 ~ 28-fold). In contrast, RNA expression analysis for SDHA, B, C and D revealed no significant differences in expression of the SDH subunits between SDHB-negative and SDHB-positive GISTs (data not shown).

### Germline Mutations and Somatic Inactivation of *SDHA* may be Common in Adult SDHB-negative Wild Type GIST

All 12 wild type GISTs were examined for defects in *SDH* subunit genes by sequencing all exons and bordering intronic regions in the *SDHA*, *SDHB*, *SDHC*, and *SDHD* genes. Tumor DNA was sequenced in all cases and in patient blood DNA when available. Genome-wide copy-number analysis and allele-specific genotyping was carried out using genome-wide single-nucleotide polymorphism (SNP) arrays. As tumor samples for case 10 were available only as formalin-fixed paraffin-embedded (FFPE) tissue, these samples were evaluated for chromosomal aberrations using molecular inversion probe (MIP) technology, which provides high quality results on FFPE specimens. Chromosomal copy number changes have previously been reported for some of the GIST cases (Belinsky et al., 2009) as noted in Supplementary Table 1.

Surprisingly, germline and/or somatic mutations in *SDHA* were identified in 5/11 SDHB negative cases (1-5), while an *SDHC* mutation was identified in case 6. Chromatograms illustrating these mutations are shown in Figure 3 and summarized in Table 3, along with relevant findings from the SNP analysis. Case 1 harbored a germline heterozygous missense mutation in *SDHA* exon 7 (*SDHA* c.818C>T) and a somatic missense mutation in exon 10 (*SDHA* c.1357G>A) (Figure 3A). The resulting amino acid changes (Table 3) are predicted to be pathological based on amino acid conservation and functional annotation (Calabrese et al., 2009; Adzhubei et al., 2010). Case 2 harbors a heterozygous 3 base deletion (*SDHA* c.457-2\_c457delAGC) in the germline that spans the IVS4/exon 5 junction; this deletion is nearly homozygous in tumor DNA (Figure 3B). Sequencing of this region in cDNA prepared from the patient's GIST reveals the use of a cryptic splice acceptor site within exon 5 and the deletion of 4 ribonucleotides, which would predict a frame-shift and a resulting stop-codon downstream of the deletion (Figure 3B, Table 3). The SNP array analysis identified copy number loss at 5p15.33 in the tumor, a region that includes the *SDHA* gene (Table 3, Supplementary Figure 1). Case 3 harbors a heterozygous germline nonsense mutation (*SDHA* c.91C>T, p.Arg31\*) in exon 2. The germline mutation is accompanied by copy-number neutral loss-of-heterozygosity (LOH) throughout most of the short arm of chromosome 5 in the GIST; as a result the C>T mutation is nearly homozygous in the tumor

(Figure 3C, Table 3). Two homozygous nonsense mutations were identified in cases 4 and 5: *SDHA* p.Arg31\* in exon 2 and *SDHA* p.Glu491\* in exon 11 (Figure 3D,E; Table 3). Although germline DNA was not available for these cases, the SNP analysis suggested loss of the wild type allele in these tumors via chromosome 5p LOH (case 4) and copy-number loss (case 5) (Table 3, Supplementary Figure 1). Case 6 was the only case harboring an *SDHC* mutation, a heterozygous p.Gly75Asp (c.224G>A) mutation in exon 4. Interestingly, although no additional *SDHC* gene mutations or allelic losses were detected, sequencing through this region in the tumor cDNA suggests that only the mutant form is expressed (Figure 3F). To investigate the mechanism of inactivation of the wild type *SDHC* allele in this tumor, primers for amplification of bisulfite-modified DNA (Supplementary Table 4) were designed for a previously described CpG island in the 5' region of the *SDHC* gene (Huang et al., 2009). Amplified DNA from the GIST (along with control methylated DNA and un-methylated DNA) was cloned into a plasmid vector (see Materials and Methods). DNA sequencing identified clones from the GIST that were either nearly fully methylated at CpG nucleotides, or fully unmethylated in this region (Figure 4), presumably corresponding to the silenced wild type isoforms and the expressed mutant isoforms, respectively. Of 10 sequenced clones, 5 displayed methylation at 10 or 11 of 12 CpG di-nucleotides, while 5 displayed methylation on 0/12 CpGs (data not shown). Clones derived from the positive and negative controls (n = 5 each) exhibited CpGs that were either fully methylated or unmethylated (data not shown).

Genome-wide chromosomal copy number changes were determined in 29 individual tumors from the 24 GIST cases (Supplementary Table 1). Wild type GIST samples exhibited a remarkably low-level of genomic instability, with most cases displaying 0-3 regions of copy number change. The exception to this is case 12, which exhibits a high degree of genomic instability. This case is the single *SDHB*-positive wild type case, and other properties such as small bowel location, spindle-cell morphology and a lower *IGF1R* IHC score distinguish this case from the other wild type cases in this study. The kinase-mutant cases, many of which have been previously reported (Belinsky et al., 2009), exhibit 2-19 regions of chromosomal aberrations and show the usual propensity towards copy-number loss at chromosomes 1p (58%), 14q (83%), 15q (50%), and 22q (42%).

## Discussion

12 wild type and 12 mutant GISTs were investigated for *IGF1R* and *SDHB* expression, and the wild type tumors screened for mutations in *SDHx* genes. Notably, 11/12 wild type tumors exhibited complete lack of *SDHB* staining. *SDHB*-negative GIST includes GISTs from the Carney-Stratakis syndrome (in association with *SDHx* mutation), Carney triad GISTs, as well as some apparently sporadic wild type GISTs (Gill et al., 2010; Gaal et al., 2011; Janeway et al., 2011; Miettinen et al., 2011). The current study included 3 Carney triad cases and 8 sporadic *SDHB*-negative GISTs. Immunological *SDHB* deficiency in sporadic GISTs has a predilection for younger patients (Janeway et al., 2011; Miettinen et al., 2011). In support of this observation the 8 sporadic *SDHB*-negative GIST cases in this study presented with an average age of ~ 40 years, 20 years younger than the median age of a GIST diagnosis. The *SDHB*-negative cases were mostly females (8/11) and presented with gastric GISTs with epithelioid or mixed cell morphology, consistent with what has been described for “*SDHB*-negative” GISTs (Gill et al., 2010).

Strong immunohistochemical staining for *IGF1R* was seen exclusively in the 11 *SDHB*-negative GISTs, in agreement with a recent report (Chou et al., 2012). The one *SDHB*-positive wild type sample in this analysis (case 12) showed both weaker *IGF1R* protein staining and lower *IGF1R* RNA expression than the other wild type samples. The small bowel presentation, spindle-cell morphology, and multiple chromosomal aberrations of this

GIST also distinguish this case from the other wild type cases. While no *KIT*, *PDGFRA*, or *BRAF* mutations were identified in case 12, we do not rule out the possibility that this case harbors a mutation in another kinase. Strong IGF1R staining in the present study correlates well with high steady-state IGF1R RNA levels. Over-expression of IGF1R RNA may be part of a global RNA expression profile for wild type GIST (Prakash et al., 2005; Agaram et al., 2008a; Pantaleo et al., 2011b). The study by Pantaleo et al describes high expression of a set of neural markers (including IGF1R) in a small group of wild type GISTs. Over-expression of two of these markers (CDH2 and ELAVL3) was validated in the wild type cases in the current study. The phenomenon of IGF1R over-expression in SDH-negative wild type GIST may reflect derivation from an earlier stage of ICC development (Pantaleo et al., 2011b). Alternatively, IGF1R over-expression as a potential consequence of stress due to SDH deficiency and mitochondrial dysfunction has also been suggested (Chou et al., 2012). Whether IGF1R over-expression in wild type GIST is a consequence of the observed SDH deficiency or is a feature of the un-identified cell of origin for these GISTs, IGF1R remains an attractive therapeutic target for wild type GIST.

This study convincingly demonstrates that germline mutations in *SDHA* accompanied by somatic allelic losses may be a common occurrence in sporadic SDHB-negative wild type GIST. Germline mutations in the *SDHB-D* genes, originally reported in Carney-Stratakis families (McWhinney et al., 2007; Pasini et al., 2008), are notably absent in non-familial Carney triad cases, although chromosomal losses in the region surrounding the *SDHC* gene (1q23.3) as well as 1p losses surrounding the *SDHB* gene have been described in Carney triad GIST (Matyakhina et al., 2007). In a panel of 34 patients with apparently sporadic wild type GIST, several mutations were found in *SDHB* (n = 3) and in *SDHC* (n = 1), all in pediatric and young adult cases (Janeway et al., 2011). In a panel of 66 SDHB-negative gastric GISTs, no mutations in a limited panel of exons from the *SDHB*, *SDHC*, and *SDHD* genes were reported (Miettinen et al., 2011). For the most part these studies did not focus on *SDHA* gene sequencing. *SDHA* was originally implicated as a tumor suppressor gene in paraganglioma (Burnichon et al., 2010), and, very recently, inactivating mutations in *SDHA* were reported in sporadic GISTs from three adults and a pediatric case (Pantaleo et al., 2011a; Pantaleo et al., 2011c). In the current study, biallelic inactivating *SDHA* gene mutations were identified in 5/11 SDHB-negative GIST cases, a finding that buttresses the case for *SDHA* as a tumor suppressor gene in GIST. The predominant scenario appears to be the presence of a germline *SDHA* point mutation or micro-deletion accompanied by somatic loss of the wild type allele, although in case 1 the second hit was a point mutation as well. In contrast, compound heterozygous point mutations were the mechanism of inactivation in 3 of 4 previously described GIST cases with *SDHA*-mediated SDH deficiency (Pantaleo et al., 2011c). Finally, the finding of a genetic lesion in an *SDHC* allele accompanied by epigenetic inactivation of the second allele adds a novel layer of complexity to the model of GIST tumorigenesis through SDH inactivation. The identification of additional mutations in *SDH* genes underscores the need for patients with wild type GIST to undergo genetic counseling for assessment of germ line *SDHx* mutations.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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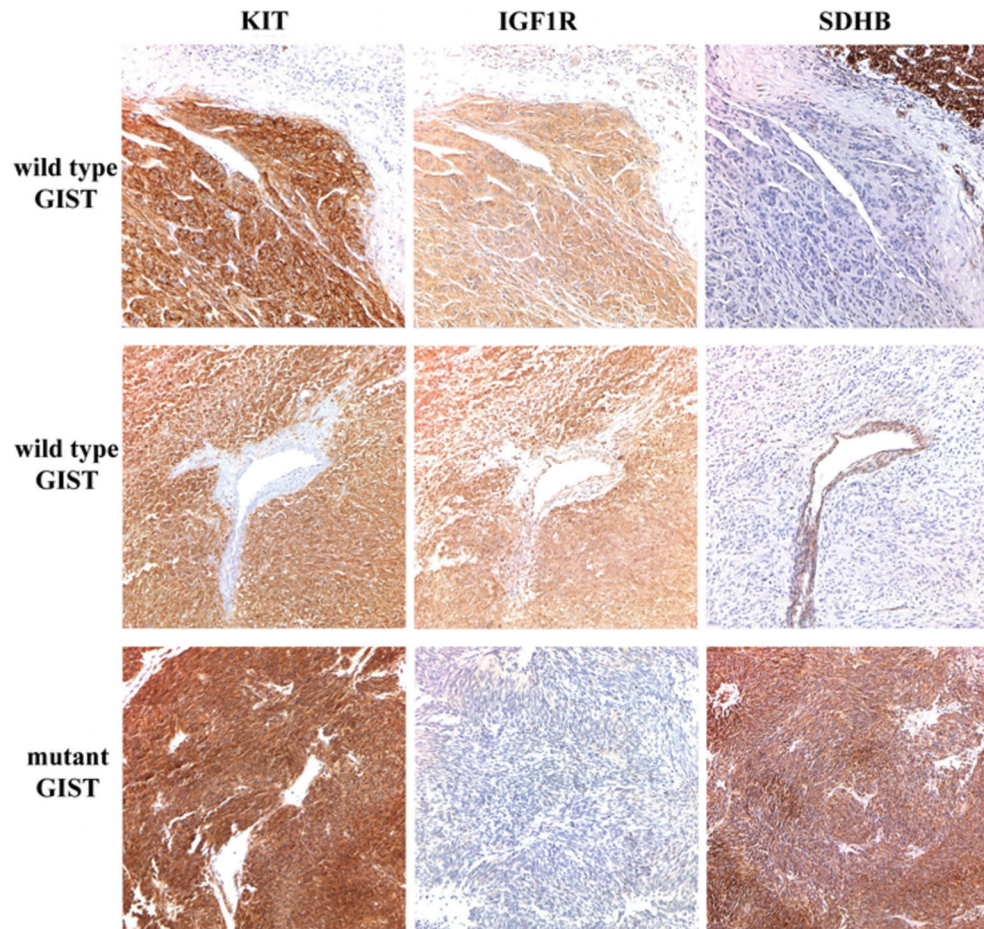
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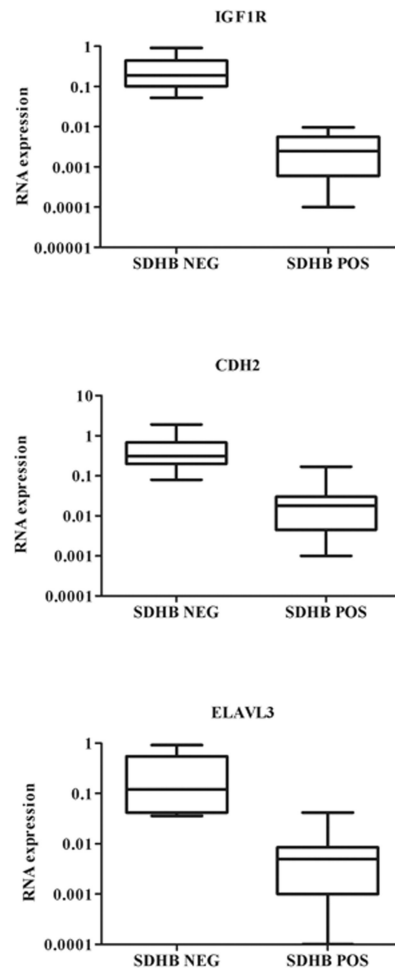
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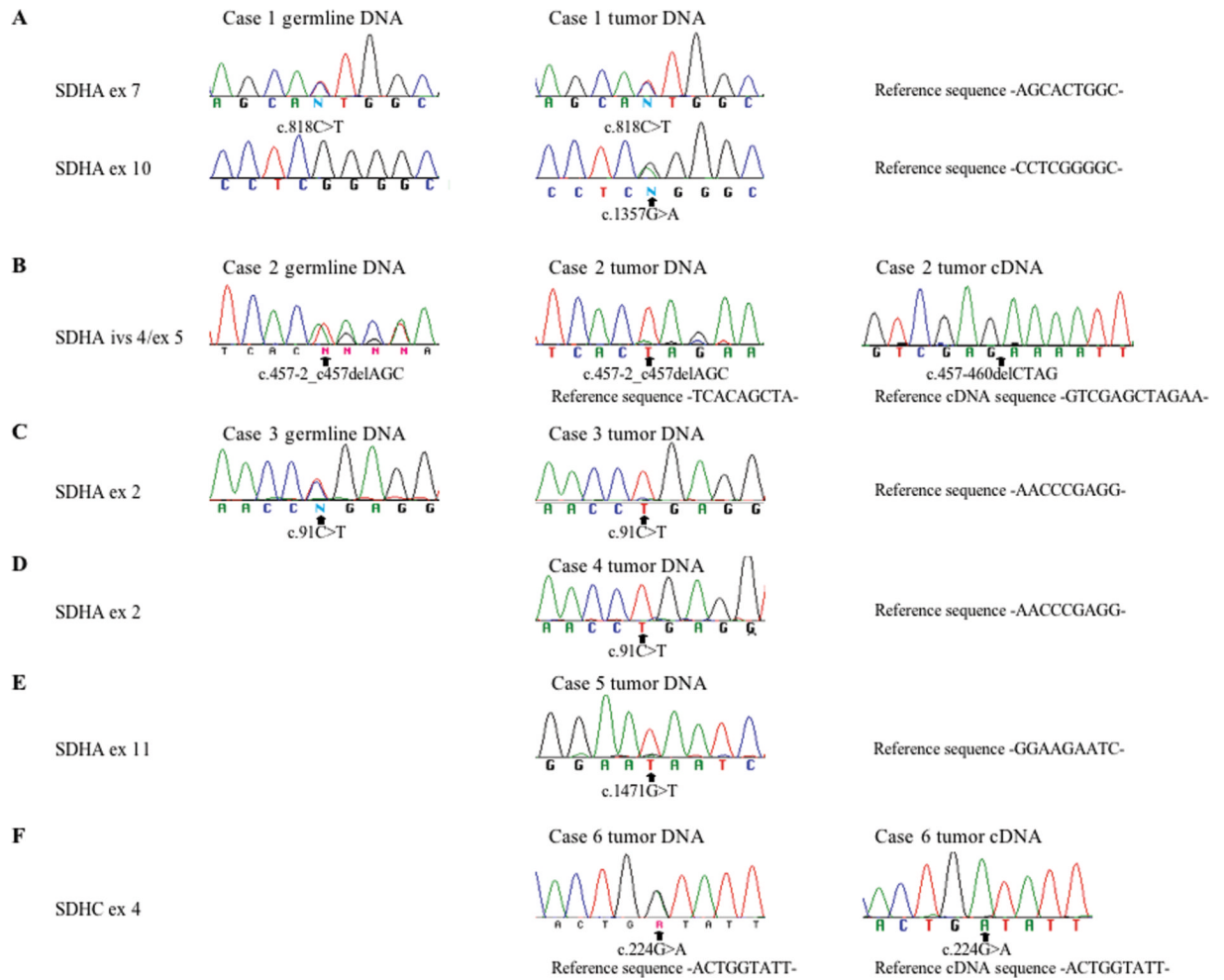
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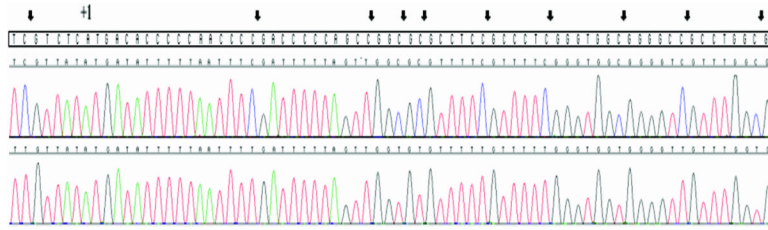
**Figure 1.** KIT, IGF1R and SDHB expression in GIST specimens. IHC expression in an adult wild type (top panel), pediatric wild type (middle panel) and mutant (bottom panel) GIST. Primary antibodies used include KIT (Dako), IGF-1R (Cell Signaling) and SDHB (Abcam). Positive KIT staining is evident throughout tumor tissue in all cases. Strong staining for IGF1R is seen in the wild type GISTs, while SDHB staining is evident in the mutant GIST and in the adjacent normal tissue and epithelial cells in the wild type cases, but absent in wild type GISTs.



**Figure 2.** RNA expression of IGF1R, CDH2, and ELAVL3 in SDHB-negative and SDHB-positive GISTs as determined by qRT-PCR. Expression levels were normalized to beta-actin. Statistical significance as calculated by the Mann-Whitney test was as follows: IGF1R:  $P < 0.0001$ ; CDH2:  $P \sim 0.002$ ; ELAVL3:  $P \sim 0.003$ .



**Figure 3.** Partial sequence chromatograms from amplified genomic DNA from patient blood, and from genomic DNA and cDNA from GISTs. Nucleotide numbering is from the coding regions for *SDHA* (NM\_004168.2) and *SDHC* (NM\_003001.3).



**Figure 4.**

Partial chromatogram of cloned PCR products from the 5' end of the *SDHC* gene. Tumor genomic DNA from case 6 was bisulfite-treated prior to amplification and cloning. Arrows show the location of 10 (of 12) CpG dinucleotides (seen in the boxed reference sequence) in the predicted CpG island. The upper chromatogram shows a cloned product with methylation of 11/12 CpGs (the third CpG is un-methylated), while the lower panel shows a fully un-methylated cloned product. The ATG site is indicated as +1.

Table 1

Demographic and clinical description of GIST cases

Case	Kinase genotype <sup>a</sup>	Age	Sex	Primary Site	Morphology
1	wild type	39	F	stomach	epithelioid/spindle
2	wild type	52	F	stomach	epithelioid/spindle
3	wild type	33	F	stomach	epithelioid/spindle
4	wild type	43	F	stomach	epithelioid/spindle
5	wild type	48	M	not available	epithelioid
6	wild type	53	M	stomach	epithelioid
7 <sup>b</sup>	wild type	51	F	stomach	epithelioid
8 <sup>b</sup>	wild type	37	M	stomach	epithelioid
9	wild type	35	F	stomach	epithelioid
10	wild type	20	F	stomach	epithelioid
11 <sup>b</sup>	wild type	12	F	stomach	epithelioid
12	wild type	57	F	small bowel	spindle
13	<i>KIT</i> : p.Asn567_Leu576delinsLysGlu	71	M	colon	spindle
14	<i>KIT</i> : p.Met552_Tyr553del	66	F	small bowel	spindle
15	<i>KIT</i> : p.Ala502_Tyr503dup	43	M	small bowel	spindle
16	<i>KIT</i> : p.Gln556_Lys558delinsAsn	60	F	stomach	spindle
17	<i>KIT</i> : p.Trp557_Val559delinsPhe	63	M	rectum	spindle
18	<i>KIT</i> : p.Tyr823Asp	81	F	stomach	mixed
19	<i>KIT</i> : p.Lys642Glu	47	M	small bowel	spindle
20	<i>PDGFRA</i> : p.[Asn659Tyr(:)p.Tyr849Cys]	37	M	stomach	epithelioid
21	<i>KIT</i> : p.Val559Gly	70	F	stomach	spindle
22	<i>KIT</i> : p.Val560Asp	56	F	stomach	spindle
23	<i>KIT</i> : p.Tyr823Asp	84	M	stomach	spindle
24	<i>PDGFRA</i> : p.Asp842Val	86	M	stomach	spindle

<sup>a</sup> Amino acid numbering from NP\_000213.1 (*KIT*) and NP\_006197.1 (*PDGFRA*)



*b* Camey triad cases

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**Table 2**

IGF1R expression and SDHB status in GISTs

Case	IGF1R score	SDHB status
1	3	negative
2	3	negative
3	3	negative
4	3	negative
5	3	negative
6	3	negative
7	3	negative
8	3	negative
9	3	negative
10	3	negative
11	3	negative
12	2	positive
13	2	positive
14	1	positive
15	2	positive
16	2	positive
17	1	positive
18	2	positive
19	1	positive
20	2	positive
21	2	positive
22	2	positive
23	2	positive
24	2	positive

Table 3

*SDH* mutations<sup>a</sup> and allelic losses

Case	<i>SDH</i> mutations (DNA)	<i>SDH</i> mutations (protein)	Germline DNA	GIST DNA	<i>SDH</i> allelic losses in GIST
1	<i>SDHA</i> c.818C>T <i>SDHA</i> c.1357G>A	<i>SDHA</i> p.Thr273Ile <i>SDHA</i> p.Gly453Arg	Heterozygous Absent	Heterozygous Heterozygous	None identified
2	<i>SDHA</i> c.457-2_c457delAAGC	<i>SDHA</i> p.Leu153Lysfs*71	Heterozygous	Homozygous	arr: 5p15.33(152,441-3,564,616)x1
3	<i>SDHA</i> c.91C>T	<i>SDHA</i> p.Arg31*	Heterozygous	Homozygous	arr: 5p15.33,p13.2(261,369-93,515,804)x2 hnz
4	<i>SDHA</i> c.91C>T	<i>SDHA</i> p.Arg31*	Not available	Homozygous	arr: 5p15.33,p15.2(173,508-23,417,765)x2 hnz
5	<i>SDHA</i> c.1471C>T	<i>SDHA</i> p.Gln491*	Not available	Homozygous	arr: 5p15.33(134,437-1,173,438)x1
6	<i>SDHC</i> c.224G>A	<i>SDHC</i> p.Gly75Asp	Not available	Heterozygous	None identified

<sup>a</sup>Nucleotide and amino acid numbering from the coding regions for *SDHA* (NM\_004168.2) and *SDHC* (NM\_003001.3)