Author's response to reviews

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SDHA Loss of Function Mutations in a Subset of Young Adult Wild-type Gastrointestinal Stromal Tumors

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ABSTRACT

Background: KIT/PDGFRA wild-type gastrointestinal stromal tumors (WT GIST) have been associated with alteration of the succinate dehydrogenase (SDH) complex II function. A recent report identified, in two cases of young adults WT GIST, a germline mutation in SDHA encoding the main subunit of the SDH complex II. Methods: Next generation sequencing was applied on five pediatric and one young adult WT GIST, by whole exome capture and SOLiD 3-plus system sequencing. The putative mutations were first confirmed by Sanger sequencing and then screened on a larger panel of 11 pediatric and young adult WT GIST, including 5 in the context of Carney triad. Results: A germline p.Arg31X nonsense SDHA mutation was identified in one of the six cases tested by SOLiD platform. An additional somatic p.D38V missense mutation in SDHA exon 2 was identified by Sanger sequencing in the extended WT GIST patients cohort. Western blotting showed loss of SDHA expression in the two cases harboring SDHA mutations, while expression being retained in the other WT GIST tumors. Results were further confirmed by immunohistochemistry for both SDHA and SDHB, which showed a concurrent loss of expression of both proteins in SDHA-mutant lesions, while the remaining WT tumors showed only loss of SDHB expression.

Conclusion: Germline and/or somatic aberrations of SDHA occur in a small subset of WT GISTs, outside the Carney’s triad and are associated with loss of both SDHA and SDHB protein expression. Mutations of the SDH complex II are more particularly associated with WT GIST occurring in young adults. Although pediatric GIST consistently display alterations of SDHB protein expression, further molecular studies are needed to identify the crucial genes involved in their tumorigenesis.
BACKGROUND

The majority of gastrointestinal stromal tumors (GIST) harbor gain-of-function mutations in KIT or PDGFRA, resulting in the activation of the downstream pathways PI3K/AKT, Ras/MAPK, and JAK/STAT3, and playing a crucial role in tumorigenesis [1, 2]. However, a subset of GIST lack specific KIT or PDGFRA mutations and are designated as KIT/PDGFRA-wild type (WT) GIST. The WT GIST patients form a heterogeneous group, including neurofibromatosis type 1 (NF1), Carney Triad (CT), Carney-Stratakis Syndrome (CSS), pediatric and young adult GIST, and a small proportion (<10%) of sporadic adult GIST [3-7]. The mechanisms of WT GIST tumorigenesis are still poorly understood. Recent studies have indentified defects in cellular respiration and activation of pseudohypoxia signalling pathways as potential crucial events in WT GISTs tumorigenesis. Indeed, WT GIST have been shown to have a consistent loss of succinate dehydrogenase subunit B (SDHB) expression [8]. The succinate dehydrogenase protein complex II (SDH complex II) catalyzes the oxidation of succinate. SDHB is one of four protein subunits forming SDH complex II, the other three being SDHA, SDHC and SDHD. Loss of SDHB expression results in inhibition of the degradation of Hypoxia Inducing Factors (HIF), which in turn impairs apoptosis, and induces angiogenesis and glycolysis [9-12]. Loss of SDHB is observed in WT GIST occurring in the context of CSS with germline mutation of SDHB or SDHC [8]. Such mutations were also found in about 10% of sporadic WT GIST [8]. The mechanisms involved in loss of SDHB expression in WT GISTs without an associated SDHB or SDHC mutations remain unclear. One possible explanation is loss of function mutations in the SDHA gene which have been identified recently in two young adult patients with sporadic WT GIST [13]. The aim of this study was to assess globally by next generation sequencing mutations in the SDH-pathway, as well as
determine the mutational and expression status of SDHA in a series of syndromic and sporadic WT GIST.

**Patients and Methods**

**Patients**

Samples from 17 patients with WT GIST, all gastric in location, were included for analysis. In 6 cases DNA from frozen tissue was available for next generation sequencing; in the remaining cases DNA was available from paraffin embedded material. Thirteen cases were diagnosed in children (≤18 years) and four cases in young adults (defined as older than 18 but younger than age of 30; 3 females and 1 male). Among the 13 pediatric cases (11 females, 2 males), five were diagnosed in the context of CT. The mean age at diagnosis for pediatric patients and young adults was 12 (range 8-18) and 23 years old (range 21-26), respectively. In all cases, KIT (exons 9, 11, 13, and 17), PDGFRA (exon 12, 14, 18) and BRAF (exon 15) genotyping was performed as previously described [14]. The study was approved by the Institutional Review Board (IRB-protocol 02-060) for adult and pediatric patients with GIST to have their tissues investigated for genomic analysis, such as next generation sequencing and targeted sequencing. For pediatric patients, parental consent was obtained for their children.

**Sequencing by Oligonucleotide Ligation and Detection (SOLiD) and Variant Detection**

Six cases, including five children, one of whom in the context of CT, and one young adult were analyzed by the SOLiDTM (Sequencing by Oligonucleotide Ligation and Detection) platform. This next generation sequencing technology interrogates two bases at a time by ligation chemistry and detection of one of four colors associated with those specific two bases. Whole exome capture was performed on 1-3ug of high quality genomic DNA using the
SureSelect Human All Exon Kit, which targets 38 MB of exonic sequences, according to the protocol provided by the manufacturer (Agilent, Santa Clara, CA, USA). Enriched DNA libraries are then sequenced on a SOLiD 3plus system (Applied Biosystems, Carlsbad, CA, USA), generating 68 million reads (50bp). 86% of the targeted region was sequenced at a 10x-coverage.

The colorspace CSFASTA and QUAL files were first converted to double encoded FASTQ files which are then mapped to the target genome (hg19) using BWA with default options plus the colorspace mode option (-c). The output SAM files are tagged with read group ids merge across runs for the same library and then process with MarkDuplicates from Picard. Overlapping paired reads are resolved to remove redundant sequence. Then all the BAM for all samples are merged and process through the GATK toolkit to first Realign in/dels and the base Q scores are recalibrated with Recalibration tool. Paired samples (tumor/normal) are processed in pairs by muTect and we also run the entire cohort through the GATK Unified Genotyper to call both SNPs and in/dels. The variant output file (the VCF file) from the Unified Genotyper was then annotated using the SNPeff program with the UCSC RefSeq HG19 database to annotate the effect of the mutation. The raw output contained approximately 38,000 events. We then used a fairly stringent set of criteria to filter these calls. Only calls marked "PASS" by the Unified Genotype were retained all other events were filtered out. Further we removed events that had a non-reference allele frequency (NRAF) less than 10%. We also then removed any events that were annotated in dbSNP (v132) and removed those not annotated as HIGH IMPACT by SNPeff. This lead to a list of five events. For all five events we manually inspected the read pileups in IGV to look for possible artifacts. Of the five only one had no obvious defects. The other four had either strand bias
issues and/or position bias (the variant reads tended to show the variants at the 3' end of the reads).

**Targeted exon resequencing**

The mutational status of *SDHA* (exon 2, 9 and 13) was assessed by direct Sanger sequencing of genomic DNA. Protocols and primers are available on request. Sequence analysis was performed with Applied Biosystems Sequence ScannerTM v1.0.

**Western Blotting**

Western blotting was performed to assess the expression of SDHA in WT GIST with and without *SDHA* mutation. Frozen tissue from seven WT GIST (two with *SDHA* mutation and five without *SDHA* mutation) were homogenized in RIPA buffer supplemented with protease and phosphatase inhibitors. Electrophoresis and immunoblotting were performed on the protein extracts using 30 µg of protein per sample and the anti-SDHA rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA) was diluted according to the manufacturers’ recommendations. Following hybridization with the secondary anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), the blots were incubated with Immun-Star horseradish peroxidase luminal/enhancer (Bio-Rad, Hercules, CA, USA) and exposed onto Kodak Biomax MR Film (Eastman Kodak Co., Rochester, NY, USA).

**Immunohistochemistry**

Immunohistochemistry (IHC) was applied on all 17 WT GIST cases tested using SDHA (Abcam, Cambridge, UK, 1:1000) and SDHB (Sigma-Aldrich, St. Louis, MO, 1:500), according to manufacturer’s recommendations. One *KIT* exon 11 mutant GIST from a young adult patient was used as control. The immunoreactivity was scored as negative (loss of expression) if the tumor cells were negative but the entrapped normal tissues (endothelial cells) stained positive. Conversely, a positive result (retained expression) was interpreted if the tumor cells
showed the same intensity staining as the internal positive control cells. If the tumor showed a weak intensity of staining, significantly lower than the normal tissue, the result was interpreted as partial loss of expression.

RESULTS

*Mutation of SDHA is a recurrent event in young adults with WT GIST*

Massive parallel next-generation sequencing of six cases of WT GIST (five pediatric and one young adult) revealed that the GIST from the young adult patient (22 year-old man, with multinodular gastric lesions and multiple liver metastases) carried a C to T transition at nucleotide 206 in *SDHA* exon 2, a nonsense mutation resulting in the replacement of arginine with a stop codon at residue 31 of *SDHA*, causing truncation of the peptide chain at residue 30 (p.Arg31X) (fig. 1). This result was then validated by targeted *SDHA* exon 2 Sanger sequencing from the DNA isolated from both tumor and normal tissue, in keeping with a germline mutation. Of note, the *SDHA* sequence electropherogram of the normal DNA revealed equivalent proportion of the wild-type and the mutated allele (T), whereas tumor DNA contained predominantly the mutated allele (T), indicating relative loss of the wild-type *SDHA* allele. This patient is alive with disease 66 months after the initial diagnosis, and was treated with multiple kinase inhibitors with marginal responses, including imatinib, sunitinib, sorafenib and sirolimus, and being presently on regorafenib.

Since one prior report also identified mutations in *SDHA* exons 9 and 13 [13] in WT GIST, we performed targeted *SDHA* exons 2, 9 and 13 sequencing in 11 additional cases of pediatric and young adult WT GIST. By this method, another young adult WT GIST (26 year-old woman with bulky intra-peritoneal and liver metastatic disease) was identified to harbor a missense mutation in *SDHA* exon 2 (p. D38V) (fig. 2). In this latter case, the mutation was observed
only in the tumor and not in the normal tissue DNA tested. This patient was alive with
disease 15 years after the initial diagnosis, preceding the availability for targeted therapy. No
additional mutations in SDHA exons 9 or 13 were identified.

**Mutation of SDHA in WT GIST is associated with concomitant loss of both SDHA and SDHB proteins expression**

To confirm the functional impact of SDHA mutation, we assessed SDHA protein expression
by western blotting in seven WT GISTs, including three samples from the two patients with
SDHA mutation and four without SDHA mutation (fig. 3). We found that SDHA expression
was absent in the two cases harboring a mutation of SDHA and present in the other cases. In
addition, immunohistochemistry for SDHA and SDHB was performed. The WT GIST
associated with a germline SDHA mutation showed complete loss of both SDHA and SDHB
protein (fig. 4 A,B), while the tumor with a somatic, heterozygous SDHA mutation showed
significant decreased in SDHA immuno-expression, as well as complete loss of SDHB (fig. 4
C,D). In contrast, strong and diffuse SDHA reactivity was present in all WT pediatric and
young adult GIST tumors tested without detectable SDHA mutations, which matched with a
complete loss of SDHB expression (fig. 4 E,F). In contrast both SDHA and SDHB expression
was preserved in a control case of a young adult GIST carrying a KIT exon 11 deletion (fig. 4
G,H).

**DISCUSSION**

The dysregulation of metabolism in cancer has been established for over 80 years. Indeed,
one of the first identified biochemical hallmarks of cancer cells was a shift in glucose
metabolism from oxidative phosphorylation to aerobic glycolysis [15]. This metabolic
conversion was considered for a long time a consequence rather than a cause of cancer. However, this vision has been recently challenged by the finding that a significant proportion of familial and apparently sporadic paraganglioma and pheochromocytoma are related to germline somatic mutation of genes encoding proteins of SDH complex II [16-20]. This complex is a membrane-bound enzyme complex linked to the respiratory chain and a member of the Krebs cycle. It consists of 4 subunits: the flavoprotein subunit (SDHA), the iron-sulfur protein subunit (SDHB), and the integral membrane protein subunits (SDHC and SDHD). Mutations of one of the gene encoding these subunits impair the activity of this complex and lead to the stabilization and activation of HIF-1α, which in turn activates cell proliferation and angiogenesis [9-12].

In addition to paragangliomas and pheochromocytomas, a number of other solid tumors have been associated with mutations in genes encoding the succinate dehydrogenase complex (SDH) complex II. These include gastrointestinal stromal tumors (GIST) [8, 13], renal tumors [21], thyroid tumors [21-23], testicular seminoma [24]. The best known association between SDH complex II germline mutations and other tumors is represented by the Carney–Stratakis syndrome (or dyad) which is characterized by the occurrence of WT GIST and paraganglioma. This syndrome is associated with germline point mutations or large deletions of the genes encoding the SDHB, SDHC or SDHD subunits [25]. Strikingly, inactivating germline mutations in SDHB or SDHC genes have been also identified in sporadic WT GISTs occurring in patients without a personal or family history of paraganglioma[8].

The SDHA gene encodes the major catalytic subunit of the succinate dehydrogenase complex II. Germline mutations in SDHA are associated with neurodegenerative diseases such as an early-onset encephalopathy, known as Leigh syndrome [26-29] and a late-onset optic
atrophy, ataxia and myopathy [30]. Until recently, no genetic link between SDHA and cancer could be established. However, two recent studies allowed the identification of SDHA germline mutations in at least 3% patients with apparently sporadic cases of paraganglioma or pheochromocytoma [31]. Interestingly, two cases of sporadic WT GIST occurring in young adult patients have also been associated with germline mutation of SDHA [13]. In the present study, we investigated a series of 17 apparently sporadic and Carney’s triad-related WT GIST for SDHA mutation and found an additional two cases with a mutation of this gene. These were exclusively present in apparently sporadic cases occurring in young adults. The p.Arg31X SDHA germline mutation identified in our study leads to a truncated protein [13]. An identical mutation has been previously reported in four Dutch patients with paraganglioma and in one young adult patient with sporadic WT GIST [13, 31]. The second SDHA mutation identified in our study (p.D38V) has been reported as a single nucleotide polymorphism. However, none of the other 16 GIST cases tested showed this change and this mutation was found only in the tumor DNA, but not in corresponding normal DNA of the patient. Furthermore, this tumor showed significant loss of SDHA protein expression by both western blot and IHC, suggesting a functional impact of this genetic alteration. But since only one source of normal DNA was analyzed in this patient, we cannot formally exclude the possibility of germline mosaicism. However, it is interesting to note that an independent somatic mutation (Arg589Trp) was previously reported in one young adult patient with sporadic WT GIST, carrying a germline p.Arg31X mutation [13]. Therefore, we propose that at least for GIST, mutation affecting the SDHA gene can be germline or somatic. Somatic mutation of metabolic enzymes are not unusual in cancer as demonstrated by the recent discovery in several tumor types of somatic mutations in IDH1 and IDH2 genes, encoding isocitrate dehydrogenases 1 and 2 respectively [32-34].
By performing western blotting, we identified a loss of SDHA protein expression in the two mutated cases whereas expression was retained in the non-mutated cases. This result was expected in the tumor with the p.Arg31X mutation because this mutation leads to a truncated SDHA protein. Although the p.D38V missense mutation does not lead to a truncated protein, the SDHA expression was significantly decreased by IHC and not detected by Western blot. This result can be explained by a conformational change of the mutated SDHA protein compromising the antigenic epitope for the antibody. Another explanation is that the p.D38V mutation leads to SDHA protein instability. Further investigations are needed to address this point.

The persistent expression of SDHA protein in SDHA non-mutated GIST is in accordance with previous studies which showed consistent SDHA protein expression in SDHB-, and SDHD-mutated paraganglioma [20, 31]. Previous studies demonstrated that SDHB-, SDHC-, and SDHD-related paragangliomas and GIST all show loss of SDHB immunohistochemical expression [8, 31]. It was suggested that absence of functional SDHC or SDHD leads to impairment of SDH complex II formation and degradation of SDHB. Our results, showing absence of SDHB expression in SDHA-mutated GIST, are in accordance with this explanation. In contrast, whereas SDHB expression was not detected in all the cases of WT GIST included in our series, all these tumors (except the two with a mutation of the SDHA gene) displayed expression of SDHA. These findings suggest that the SDHB protein is degraded when the SDH complex II is disrupted, whereas the SDHA protein remains intact.

By pooling our results with those of previous studies [8, 13, 35], it appears that the majority of mutations of genes encoding subunits of the SDH complex II identified in apparently sporadic WT GIST occurred in young adults (8 out of 11 patients). However, the majority of
sporadic or syndromic WT GIST occurring in the pediatric or adult setting display loss of SDHB protein [36], suggesting that defects in cellular respiration is a crucial event even in cases without mutation of the SDH complex II. Therefore, further investigation are needed to identify the mechanism involved in the alteration of the SDH complex II function in cases without mutation of SDHA, -B, -C or -D.

In conclusion, this study provides additional evidence that SDHA is another important gene involved in the tumorigenesis of WT GISTs. Although the number of identified mutation carriers is still low, current observations suggest that mutations of the succinate dehydrogenase complex II are more particularly associated with WT GIST occurring in young adults, outside the Carney’s triad. Although pediatric GIST consistently display alterations of SDHB protein expression, further molecular studies are needed to identify the crucial genetic events involved in their tumorigenesis. Altogether, the results of this study confirm that at least the young adult patients with KIT/PDGFRA wild-type GISTs should be screened for germline or somatic mutations in the subunits of the SDH complex II. Indeed, this may impact the follow-up of patients with germline mutation who have a potential increased risk of developing paragangliomas and additional GIST and also the future development of therapies targeting the hypoxia pathway in this specific subset of GIST.

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References


FIGURE LEGENDS

Figure 1. Sanger sequencing validated a nonsense SDHA exon 2 p.Arg31X mutation in both tumor and normal DNA. The SDHA sequence electropherogram of the tumor DNA contained predominantly the mutated allele (T), indicating relative loss of the wild-type SDHA allele, compared to the normal DNA.

Figure 2. ABI sequencing showing a somatic mutation in SDHA exon 2 p. D38V. The normal DNA extracted from this case showed a wild-type sequence for this locus.

Figure 3. Western blot showing loss of SDHA expression in the young adult GIST carrying a germline SDHA exon 2 mutation p.Arg31X (GIST318, primary gastric tumor; GIST353, liver metastasis), as well as in the young adult GIST carrying a somatic SDHA exon 2 mutation (GIST118, peritoneal metastasis). Remaining WT GIST (GIST#289, 491, 507, 517) lacking SDHA mutations showed preserved SDHA protein expression.

Figure 4. Immunohistochemistry showed concomitant loss of SDHA and SDHB expression in SDHA-mutant GIST. Complete loss of expression for SDHA and SDHB was noted in the germline SDHA-mutant GIST (A,B), while normal liver showed preserved reactivity. In the somatic SDHA-mutant GIST there was partial loss of SDHA expression, while SDHB staining was absent (C,D). SDHA expression was retained in a WT pediatric GIST without SDHA mutation, while SDHB reactivity was lost (E,F). In the control KIT exon 11-mutant GIST from a young adult both SDHA and SDHB protein expressions were preserved (G,H).
**Competing interest section.** The authors declare that they have no competing interests.

**Authors’ contributions section.** AI, CLC and SYS carried out the molecular genetic studies (targeted Sanger sequencing, immunoblots) and drafted the manuscript. NDS carried out the next generation sequencing pipeline data analysis. SS, PB and RPD ES participated in the design of the study and drafted the manuscript. CRA conceived of the study, read the immunohistochemistry results and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.