Germline SDHA mutations in children and adults with cancer

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Abstract Mutations in succinate dehydrogenase complex genes predispose to familial paraganglioma-pheochromocytoma syndrome (FPG) and gastrointestinal stromal tumors (GIST). Here we describe cancer patients undergoing agnostic germline testing at Memorial Sloan Kettering Cancer Center and found to harbor germline SDHA mutations. Using targeted sequencing covering the cancer census genes, we identified 10 patients with SDHA germline mutations. Cancer diagnoses for these patients carrying SDHA germline mutations included neuroblastoma (n = 1), breast (n = 1), colon (n = 1), renal (n = 1), melanoma and uterine (n = 1), prostate (n = 1), endometrial (n = 1), bladder (n = 1), and gastrointestinal stromal tumor (GIST) (n = 2). Immunohistochemical staining and assessment of patient tumors for second hits and loss of heterozygosity in SDHA confirmed GIST as an SDHA-associated tumor and suggests SDHA germline mutations may be a driver in neuroblastoma tumorigenesis.

INTRODUCTION

Succinate dehydrogenase (SDH, Complex II) genes play a critical role in mitochondrial respiratory and metabolic function (Henze and Martin 2003). The succinate dehydrogenase enzyme complex is encoded by the genes SDHA, SDHB, SDHC, and SDHD, which localize to the inner mitochondrial membrane and catalyze the oxidation of succinate to fumarate and concomitant reduction of ubiquinone to ubiquinol (Tomitsuaka et al. 2003). Determining the nucleotide sequence, gene function, transcriptional regulation, importance
of 5′ regulatory sequence, and negative regulators of the succinate dehydrogenase energy complex have been accomplished through experimental work in *Bacillus subtilis* and *Escherichia coli* (Magnusson et al. 1985, 1986; Miller et al. 1988; Melin et al. 1990; Xu and Johnson 1995). Investigations of the parasitic bacterium, *Rickettsia prowazekii*, and humans have revealed that SDH gene organization differs between bacteria and *Homo sapiens* (Aliabadi et al. 1993; Hofmeister et al. 1997; Tomitsuka et al. 2003). Recognizing SDH genes’ sequences have diverged between species is noteworthy, as sequence conservation is an evident category for variant classification.

In medical practice, the clinical relevance of the succinate dehydrogenase energy complex has emerged across a number of specialties including medical genetics, endocrinology, and oncology (Cordray et al. 1995; Porquet 1997), and mutations in the succinate dehydrogenase complex are described in cancer-predisposing and nonmalignant-related phenotypes. Germline alterations in the *SDHB*, *SDHC*, and *SDHD* genes and, to a lesser extent, the *SDHA* gene predispose to familial paraganglioma, pheochromocytoma, and GIST tumors (Baysal et al. 2001; Jiang et al. 2015; Renkema et al. 2015; Belinsky et al. 2017; Casey et al. 2017a,b; Rednam et al. 2017).

The classic noncancerous phenotype underpinned by genetic aberrancy in succinate dehydrogenase complex is Leigh syndrome (MIM #256000). Leigh syndrome may occur in autosomal recessive and mitochondrial inheritance patterns depending on the genes that are mutated. Leigh syndrome may manifest with varying clinical features including failure to thrive, ophthalmoplegia and ptosis, optic atrophy, nystagmus, strabismus, pigmentary retinopathy, respiratory failure, hypochondrosis, central nervous system disorders, emotional lability, and lactic acidosis (Van Coster et al. 2003; Burnichon et al. 2010; Korpershoek et al. 2011; McKusick 1986–2016).

As next-generation sequencing (NGS) has evolved and tumor/normal sequencing efforts have been deployed by comprehensive cancer sequencing centers, mutations in the *SDH* complex genes have been detected in patients with non-FPG or GIST (Zhang et al. 2015). Here we describe germline *SDHA* mutations harbored by patients treated at Memorial Sloan Kettering Cancer Center (MSK) who consented to participation in an institutional 12–245 tumor/normal sequencing study. Notably, in our series, the majority of patients shown to have an *SDHA* germline mutation were found to carry the same recurrent genomic alteration: c.91C>T (p. Arg31Ter). Three additional mutations detected in our patients were c.1141C>G, c.1A>G (p. Met1Val), and two patients with an intragenic deletion encompassing exons 1–9. Diagnoses for patients in our cohort with *SDHA* germline mutations included prostate and colon adenocarcinomas, endometrial carcinoma (and a secondary melanoma), urothelial carcinoma, poorly differentiated gastric carcinoma, multifocal gastrointestinal stromal tumors, clear cell renal cell carcinoma, triple negative breast cancer, and neuroblastoma.

## Results

Our study team reviewed a total of 4974 patients undergoing targeted sequencing of approximately 400 cancer genes at 732× depth in tumor and matched normal blood using the MSK-IMPACT platform (Cheng et al. 2015). Within this cohort, 10 patients (0.2%) were found to harbor pathogenic germline *SDHA* mutations. Of these, 2/10 (20%) with a diagnosis of uterine endometrioid carcinoma and poorly differentiated carcinoma of the stomach harbored a deletion of exons 1–9, 1/10 (10%) with a diagnosis of urothelial bladder cancer carried the missense mutations c.1A>G (p. Met1Val), and 7/10 (70%) exhibited a *SDHA* c.91C>T (p. Arg31Ter) nonsense mutation. For those with the recurrent c.91C>T (p. Arg31Ter) mutation, patient diagnoses included gastrointestinal stromal tumor (n = 2), neuroblastoma.
(n = 1), colon cancer (n = 1), prostate cancer (n = 1), renal cell cancer (n = 1), and breast cancer (n = 1). For one of these seven patients, additional evidence of a second somatic missense mutation, SDHA p.S445L, was revealed (patient with a GIST); 2/7 (28.6%) patients had a copy-number loss of the other SDHA allele on Chromosome 5 (neuroblastoma and GIST) (Table 1). As a point of reference, copy-number loss encompassing the SDHA locus occurs in <1% of cancer studies cataloged in the cBioPortal (Fig. 1). To elucidate phenotypes associated with the recurrent SDHA c.91C>T (p. Arg31Ter), we collaborated with The University of Utah and queried their Familial Paraganglioma/Pheochromocytoma Cancer Registry to determine cancer types, associated family histories of patients with the recurrent SDHA c.91C>T (p. Arg31Ter) germline mutation, and if typical tumor types segregated in families (Fig. 2). Family histories of patients with SDHA c.91C>T (p.Arg31Ter) showed lack of paraganglioma and pheochromocytoma tumor types segregating in kindreds, suggesting the SDHA c.91C>T (p.Arg31Ter) mutation is a low-penetrance allele (Fig. 2).

We analyzed population frequencies of the SDHA alterations in our patient cohort compared to control databases. The SDHA c.91C>T (p. Arg31Ter) mutation is found in the Exome Aggregation Consortium (ExAC) database at an allele frequency of 0.000174% (20 allele count/121,408 allele numbers) compared to 0.0018% (7 allele counts/4974 allele number) in the MSK population. This germline SDHA alteration and the others detected in our cohort meet the American College of Medical Genetics and Genomics Standards and Guidelines for pathogenicity based on the nature of the mutations, the rarity of the alterations in population control databases, and functional evidence (Renkema et al. 2015; Richards et al. 2015).

To confirm the pathogenicity of these SDHA mutations in this expanded spectrum of cancers, we examined patient tumors for second mutations in the SDHA gene, loss of heterozygosity or copy-number alterations of the SDHA locus, and retention of SDHA/B proteins determined by immunohistochemistry (IHC) (Table 1). IHC loss for patients harboring germline SDHA mutations has previously been described in patients with GIST and a patient with GIST and secondary renal cell carcinoma (Belinsky et al. 2013, 2017; Jiang et al. 2015; Renkema et al. 2015; Casey et al. 2017a,b; Rednam et al. 2017). Next, 3 of 10 patients in our cohort with SDHA germline mutations had tumors exhibiting, by IHC, loss of SDHA/B proteins (neuroblastoma [n = 1] (Fig. 3) and GIST [n = 2]). IHC staining loss suggests loss of SDH function. In addition to patients tested as part of MSK-IMPACT, two patients were identified with the same recurrent SDHA c.91C>T (p. Arg31Ter) mutation in the University of Utah Familial Cancer Predisposition Registry. Patients in the repository with this germline mutation were diagnosed with pheochromocytoma (SDHA/B, IHC absent) and melanoma (IHC not performed).

As a means to functionally determine the impact of the germline SDHA mutations detected in our patients on cell lines, we performed mutagenesis experiments and measured citric acid cycle metabolite differences compared to a wild-type cell line for SDHA. Functional interrogation of the SDHA alterations detected in our patient population revealed aberrant protein level production, increased accumulation of metabolites of the Krebs cycle with a significant increase in succinate to fumarate ratios in mutagenic cells compared to wild-type (Fig. 4).

**DISCUSSION**

Next-generation sequencing studies have identified germline pathogenic and likely pathogenic mutations in a greater percentage of patients than expected (Pritchard et al. 2016; Mandelker et al. 2017; Walsh et al. 2017). However, there are few examples in which
Table 1. Clinical and molecular characteristics of patients with germline SDHA mutations

| Tumor type         | Age at diagnosis | Gene | Chr | Location | HGVS reference | HGVS protein | Variant type       | Germline platform | Allele frequency normal | Target coverage | Genetic counseling | dbSNP ID | LOH | 2nd mutation detected in tumor | IHC | Family history of PGL | Cohort |
|--------------------|------------------|------|-----|----------|----------------|--------------|--------------------|-------------------|----------------------|----------------|-------------------|----------|-----|-----------------------------|-----|---------------------|--------|
| Colon              | 50               | SDHA | Chr 5 | 223509 | C             | p.Arg31Ter    | Nonsense           | NGS capture       | >35%                 | >500×          | Yes               | rs1424416 43 | No  | No                          | Retained | No                   | MSKCC  |
| Prostate           | 48               | SDHA | Chr 5 | 223509 | C             | p.Arg31Ter    | Nonsense           | NGS capture       | >35%                 | >500×          | Yes               | rs1424416 43 | No  | No                          | Retained | No                   | MSKCC  |
| GIST               | 21               | SDHA | Chr 5 | 223509 | C             | p.Arg31Ter    | Nonsense           | NGS capture       | >35%                 | >500×          | Yes               | rs1424416 43 | No  | Yes                        | Loss    | No                   | MSKCC  |
| GIST               | 27               | SDHA | Chr 5 | 223509 | C             | p.Arg31Ter    | Nonsense           | NGS capture       | >35%                 | >500×          | Yes               | rs1424416 43 | LOH | Loss                       | No       | No                   | MSKCC  |
| Neuroblastoma      | 3                | SDHA | Chr 5 | 223509 | C             | p.Arg31Ter    | Nonsense           | NGS capture       | >35%                 | >500×          | Yes               | rs1424416 43 | LOH | Loss                       | No       | No                   | MSKCC  |
| Kidney             | 55               | SDHA | Chr 5 | 223509 | C             | p.Arg31Ter    | Nonsense           | NGS capture       | >35%                 | >500×          | Yes               | rs1424416 43 | LOH | Yes                        | Retained | No                   | MSKCC  |
| Breast             | 49               | SDHA | Chr 5 | 223509 | C             | p.Arg31Ter    | Nonsense           | NGS capture       | >35%                 | >500×          | Yes               | rs1424416 43 | No  | No                          | Retained | No                   | MSKCC  |
| Uterus             | 62               | SDHA | Chr 5 | 223592-235454 | n/a | n/a | Intragenic deletion | NGS capture       | >35%                 | >500×          | Yes               | n/a        | No  | No                          | Retained | No                   | MSKCC  |
| Stomach            | 34               | SDHA | Chr 5 | 223592-235454 | n/a | n/a | Intragenic deletion | NGS capture       | >35%                 | >500×          | Yes               | n/a        | No  | No                          | Retained | No                   | MSKCC  |
| Bladder            | 69               | SDHA | Chr 5 | 218356 | A             | p.Met1Val     | missense          | NGS capture       | >35%                 | >500×          | Yes               | rs1061517   | No  | No                          | Retained | No                   | MSKCC  |
| Pheochromocytoma   | 41               | SDHA | Chr 5 | 223509 | C             | p.Arg31Ter    | Nonsense           | Sanger             | n/a                  | n/a            | Yes               | rs1424416 43 | n/a | No                          | Loss    | No                   | Utah    |
| Melanoma           | 35               | SDHA | Chr 5 | 223509 | C             | p.Arg31Ter    | Nonsense           | Sanger             | n/a                  | n/a            | Yes               | rs1424416 43 | n/a | No                          | Not available | No       | Utah    |

HGVS, Human Genome Variation Society; LOH, loss of heterozygosity; IHC, immunohistochemistry; PGL, pheochromocytoma or paraganglioma; GIST, gastrointestinal stromal tumors.
unexpected germline mutations are detected in atypical cancers that are accompanied by additional supporting evidence of pathogenicity (Holmfeldt et al. 2013). This study revealed germline SDHA mutations in patients with the following cancer diagnoses: neuroblastoma, pheochromocytoma, melanoma, breast cancer, colon cancer, renal cell carcinoma, prostate cancer, gastric cancer, and endometrial cancer. Supporting biochemical evidence in this study is illustrated for neuroblastoma and GIST. Other cancer types retained SDHA/B staining and are thus less likely to be driven by the SDHA germline alteration. Family history data suggest the SDHA c.91C>T (p.Arg31Ter) allelic variant has a low penetrance and perhaps does not play an oncogenic role in cancers other than neuroblastoma and GIST.

Potential reasons second mutational hits or loss of heterozygosity were not observed in cancer types other than neuroblastoma and GIST include epigenetic alterations and cryptic second hits not detected by our sequencing methodology.

These results have implications in regard to cancer screening and reproductive counseling for patients and family members harboring germline SDHA alterations (Rednam et al. 2017). Our results indicate that neuroblastoma may be a tumor type to consider for screening SDHA mutation carriers if additional evidence surfaces from large scale sequencing studies. Of note, a germline SDHB mutation was reported in a neuroblastoma patient participating in the Pediatric Cancer Genome Project (Zhang et al. 2015).
Figure 2. Three-generation pedigrees of families with recurrent SDHA c.91C>T (p.Arg31Ter) mutation. The proband and cancer diagnoses were breast cancer, renal cancer, gastrointestinal stromal tumor, neuroblastoma, gastrointestinal stromal tumor, prostate cancer, and colon cancer.
Figure 3. (A) Neuroblastoma primary tumor and lung metastasis with SDHA/SDHB staining loss, (B) schematic illustrating chromosomal copy-number loss in the tumor encompassing the SDHA gene locus Chromosome 5:p15.33 (red arrow indicates loss of Chromosome 5).

Figure 4. (A) Metabolite intermediates of Krebs cycle. SDH (succinate dehydrogenase; indicated in red). (B) Immunoblot analysis of SDHA knockout MDA-MB-231 breast cancer cell line expressing vector, wild-type (WT), or indicated SDHA cDNA. Actin is used as a loading control. (C) Changes (log2) in indicated metabolites of SDHA knockout MDA-MB-231 expressing vector, wild-type, and indicated SDHA cDNA. Values were normalized to the average of the wild-type SDHA-expressing controls (n = 3). (D) Ratio of intracellular succinate to fumarate levels of SDHA knockout MDA-MB-231 expressing vector, wild-type, and indicated SDHA cDNA. Values were normalized to the average of the wild-type SDHA-expressing controls (n = 3).
In conclusion, this study reports children and adults with SDHA germline mutations and various cancers. Additional supporting evidence of the SDHA mutations detected in our patient cohort as pathogenic was revealed through biochemical studies for patients with neuroblastoma and GIST. For other patients in our cohort this evidence was lacking. At present, screening recommendations for individuals with SDH complex germline mutations commence at the age of 8 and require routine imaging and biochemical evaluation (Rednam et al. 2017). It is not clear at this time if additional screening is indicated for neuroblastoma, but this should continue to be revisited as more data is generated.

METHODS

We queried patients undergoing MSK-IMPACT tumor/normal sequencing for SDHA germline alterations from January 2014 until October 2017 at MSK and the Family Cancer Clinic at the University of Utah. Patients consented to IRB-approved studies allowing for interrogation of germline alterations and reporting. The patient at MSK consented to participate in a study specifically allowing for germline interrogation and reporting (12–245 Part C). Patients described from the University of Utah enrolled in a Clinical Genetics Study allowing for molecular and pedigree data to be presented. As an additional protection, pedigrees in Figure 2 have been de-identified.

For patients with sequencing data available from NGS, we were able to assess for allele-specific copy-number analysis (ASCN) using our Fraction and Allele-Specific Copy Number Estimates from Tumor Sequencing (FACETS) bioinformatics tool to determine loss of heterozygosity (Shen and Seshan 2016). Of note, copy-number loss encompassing SDHA occurs in <1% of cancers studied and cataloged in the cBioPortal (Fig. 1). Moreover, when possible, the clinical IHC staining was used for interrogating the presence or absence of SDH gene–encoded proteins as performed when assessing for heritable paraganglioma and pheochromocytoma (Fig. 3; Papathomas et al. 2015; Casey et al. 2017a).

We queried our institutional database and accumulated experience from our germline tumor board, taking into account clinical testing and research testing for which patients had consented to return of results. We reviewed PubMed, Web of Science databases, ClinVar, and locus-specific databases taking into account mutations detected in previous germline molecular landscaping efforts. The following keywords and annotations were used: succinate dehydrogenase, germline, SDHA, SDHB, SDHC, SDHD, SDHAF2, pediatric cancer, oncology, supplemental, variant, annotations, mutations, neuroblastoma, gastrointestinal stromal tumor (GIST), and immunohistochemistry (IHC). All references published from inception until 7 October, 2017 were eligible for inclusion in this review. A bibliography was created using EndNote X8.

Generation of the SDHA Knockout Cell Line

gRNAs (caccGCCCATCACCTCGACCACGG and aaacCCGTGGTCGAGGTGATGGGC) were cloned into lentiCRISPR-v1 using Gibson Assembly (NEB). sgRNA expressing vector along with lentiviral packaging vectors Delta-VPR and CMV VSV-G were transfected into HEK-293T cells using the XTremeGene 9 transfection reagent (Roche). For overexpression cell lines, cDNA vectors along with retroviral packaging vectors gag-pol and CMV VSV-G were transfected into HEK-293T cells. Media was changed 24 h after transfection. The virus-containing supernatant was collected 48 and 72 h after transfection and passed through a 0.45-µm filter to eliminate cells. Target cells in six-well tissue culture plates were infected in media containing 8 µg/ml of polybrene, and a spin infection was performed by centrifugation at 2200 rpm for 1 h. Postinfection, virus was removed and cells were selected with puromycin or blasticidin.
For knockout cells, after selection, cells were single-cell-sorted with a flow cytometer into the wells of a 96-well plate containing 200 µl of RPMI supplemented with 20% FBS. Cells were grown for 2 wk, and the resultant colonies were trypsinized and expanded. Clones were validated for loss of the relevant protein via immunoblotting.

**Immunoblotting**

MDA-MB-231 (1.5 million) cells were rinsed twice in ice-cold PBS and harvested in a standard lysis buffer containing 50 mM HEPES (pH 7.4), 40 mM NaCl, 2 mM EDTA, 1.5 mM orthovanadate, 50 mM NaF, 10 mM pyrophosphate, 10 mM glycerophosphate, protease inhibitors (Roche), and 1% Triton-X-100. Proteins from total lysates were resolved by 8%–12% SDS-PAGE and analyzed by immunoblotting as described (Birsoy et al. 2014).

**Metabolite Profiling and Isotope Tracing**

LC/MS analyses were conducted on a Q Exactive benchtop orbitrap mass spectrometer equipped with an Ion Max source and a HESI II probe, which was coupled to a Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific). Polar metabolites were extracted using 1 ml of ice-cold 80% methanol with 10 ng/ml valine-d8 as an internal standard. After a 10-min vortex and centrifugation for 10 min at 4°C at 10,000 g, samples were dried under nitrogen gas. Dried samples were stored at −80°C and then resuspended in 100 µl water; 1 µl of each sample was injected onto a ZIC-pHILIC 2.1 x 150 mm (5 µm particle size) column (EMD Millipore).

**ADDITIONAL INFORMATION**

**Data Deposition and Access**

Variants described in this manuscript were deposited in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) under accession numbers SCV000778379, SCV000778380.1, and SCV000778378.1. Because of lack of patient consent, raw sequencing data was not deposited.

**Ethics Statement**

Patients reported here provided written consent permitting use of molecular and phenotypic description under MSK’s IRB approved studies #06-107 or #12-245 and The University of Utah Cancer Predisposition Familial Cancer Registry. Family histories have been illustrated in a de-identified manner. All patients participating in this study have been provided genetic counseling.

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**Variant Interpretation**

The c.91C>T (p. Arg31Ter) variant in SDHA is entered eight times in ClinVar and annotated as pathogenic/likely pathogenic (Landrum et al. 2016). The variant is linked with the Database for Short Genetic Variations (dbSNP), 142441643, and the National Center for Biotechnology (NCBI), rs142441643. Submission accession numbers for this mutation in ClinVar are SCV000186863.4, SCV000288157.4, SCV000490791.1, SCV000677113.1, SCV000677772.1, SCV000195526.1, SCV000222637.1, and SCV000246126.2.
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