



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 (Tomitsuka 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, hypertrichosis, 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 in tumor	2nd mutation detected in tumor	IHC	Family history of PGL	Cohort
Colon	50	SDHA	Chr 5	223509	C	p.Arg31Ter	Nonsense	NGS capture	>35%	>500x	Yes	rs142441643	No	No	Retained	No	MSKCC
Prostate	48	SDHA	Chr 5	223509	C	p.Arg31Ter	Nonsense	NGS capture	>35%	>500x	Yes	rs142441643	No	No	Retained	No	MSKCC
GIST	21	SDHA	Chr 5	223509	C	p.Arg31Ter	Nonsense	NGS capture	>35%	>500x	Yes	rs142441643	No	Yes	Loss	No	MSKCC
GIST	27	SDHA	Chr 5	223509	C	p.Arg31Ter	Nonsense	NGS capture	>35%	>500x	Yes	rs142441643	LOH	No	Loss	No	MSKCC
Neuroblastoma	3	SDHA	Chr 5	223509	C	p.Arg31Ter	Nonsense	NGS capture	>35%	>500x	Yes	rs142441643	LOH	No	Loss	No	MSKCC
Kidney	55	SDHA	Chr 5	223509	C	p.Arg31Ter	Nonsense	NGS capture	>35%	>500x	Yes	rs142441643	LOH	Yes	Retained	No	MSKCC
Breast	49	SDHA	Chr 5	223509	C	p.Arg31Ter	Nonsense	NGS capture	>35%	>500x	Yes	rs142441643	No	No	Retained	No	MSKCC
Uterus	62	SDHA	Chr 5	233592-235454	n/a	n/a	Intragenic deletion	NGS capture	>35%	>500x	Yes	n/a	No	No	Retained	No	MSKCC
Stomach	34	SDHA	Chr 5	233592-235454	n/a	n/a	Intragenic deletion	NGS capture	>35%	>500x	Yes	n/a	No	No	Retained	No	MSKCC
Bladder	69	SDHA	Chr 5	218356	A	p.Met1Val	missense	NGS capture	>35%	>500x	Yes	rs1061517	No	No	Retained	No	MSKCC
Pheochromocytoma	41	SDHA	Chr 5	223509	C	p.Arg31Ter	Nonsense	Sanger	n/a	n/a	Yes	rs142441643	n/a	No	Loss	No	Utah
Melanoma	35	SDHA	Chr 5	223509	C	p.Arg31Ter	Nonsense	Sanger	n/a	n/a	Yes	rs142441643	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.

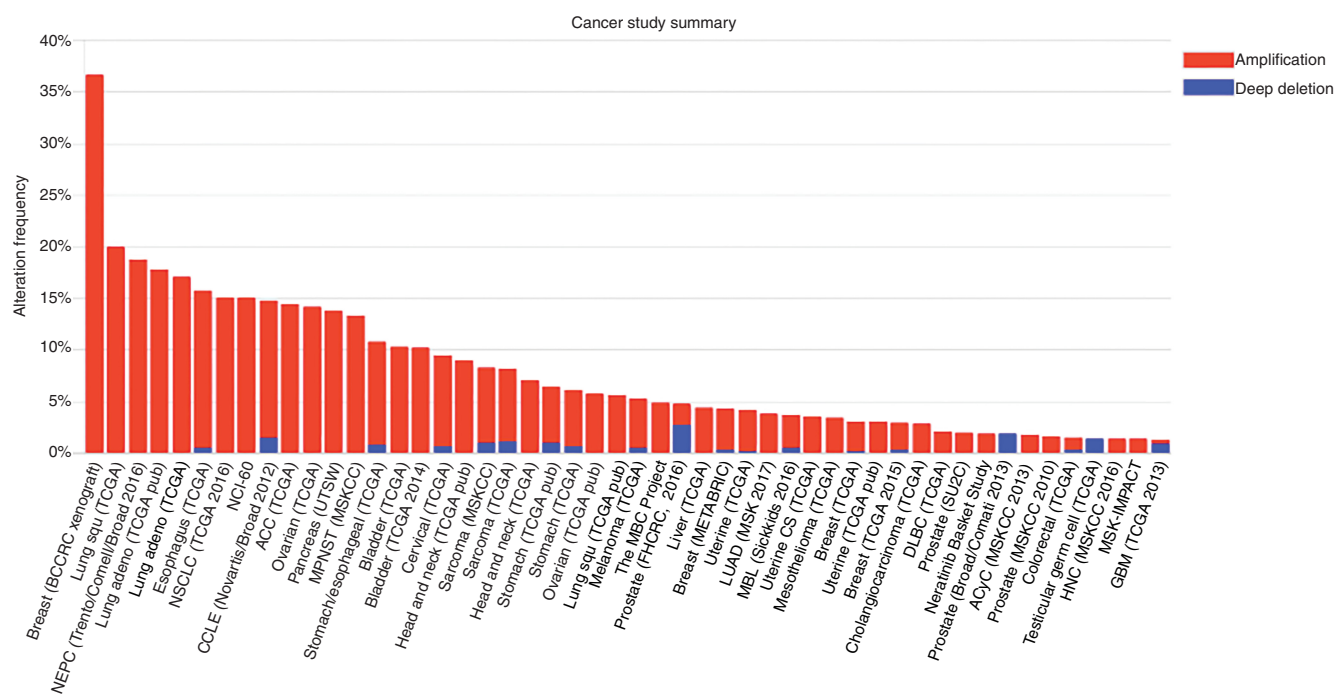


Figure 1. Copy-number alterations of Chromosome 5 shown in 1513/45,870 sequenced cases/patients cataloged in cBioPortal accounting for copy-number loss encompassing SDHA occurs in <1% of cancers studied and cataloged in the cBioPortal. x-axis: specific genomic sequencing study cataloged in cBioPortal; y-axis: alteration frequency of Chromosome 5 in different studies. (Red) amplifications of Chromosome 5 and (blue) deletions of Chromosome 5 in different cancer genomic studies. TCGA, The Cancer Genome Atlas; NEPC, neuroendocrine pancreatic cancer; aden, adenocarcinoma; NSCLC, non-small-cell lung cancer; NCI, National Cancer Institute; ACC, adrenocortical carcinoma; MPNST, malignant peripheral nerve sheath tumor; Stomach/esophageal, stomach/esophageal cancer; Bladder, bladder cancer; Cervical, cervical cancer; Head and neck, head and neck cancer; Sarcoma, sarcoma; Sarc, sarcoma; MSKCC, Memorial Sloan-Kettering Cancer Center; MSKCC pub, Memorial Sloan-Kettering Cancer Center public; Head and neck, head and neck cancer; Stomach, stomach cancer; Stomach (TCGA pub), Stomach (TCGA) public; Ovarian, ovarian cancer; Lung squ, lung squamous cell carcinoma; Lung squ (TCGA pub), Lung squamous cell carcinoma (TCGA) public; Melanoma, melanoma; Melanoma (TCGA pub), Melanoma (TCGA) public; The MBC Project, The Metastatic Breast Cancer Project; Prostate (FHCRC, 2016), Prostate (Fred Hutchinson Cancer Research Center, 2016); Liver, liver cancer; Breast (METABRIC), Breast (Metastatic Breast Cancer Research Consortium); Uterine, uterine cancer; LUAD, lung adenocarcinoma; MBL, medulloblastoma; MBL (MSK 2017), Medulloblastoma (Memorial Sloan-Kettering Cancer Center, 2017); Uterine CS (TCGA), Uterine Cervical Squamous Cell Carcinoma (TCGA); Mesothelioma, mesothelioma; Breast (TCGA), Breast (TCGA); Breast (TCGA pub), Breast (TCGA) public; Breast (TCGA 2015), Breast (TCGA) 2015; Cholangiocarcinoma, cholangiocarcinoma; DLBC, diffuse large B-cell lymphoma; Prostate (TCGA), Prostate (TCGA); Neratinib Basket (SU2C), Neratinib Basket (Stratified Urothelial Cancer Consortium); Prostate (Broad/Comati Study), Prostate (Broad/Comati Study); ACJC (MSKCC 2013), Active Cancer Jointly Cataloged (MSKCC 2013); Prostate (MSKCC 2013), Prostate (MSKCC 2013); Colorectal, colorectal cancer; Testicular germ cell (TCGA), Testicular germ cell (TCGA); HNC (MSKCC 2010), Head and Neck Cancer (MSKCC 2010); HNC (MSKCC 2016), Head and Neck Cancer (MSKCC 2016); MSK-IMPACT, Memorial Sloan-Kettering Cancer Center Institutional Molecular Profiling of Advanced Cancer Tumor; GBM (TCGA 2013), Glioblastoma (TCGA 2013).

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).

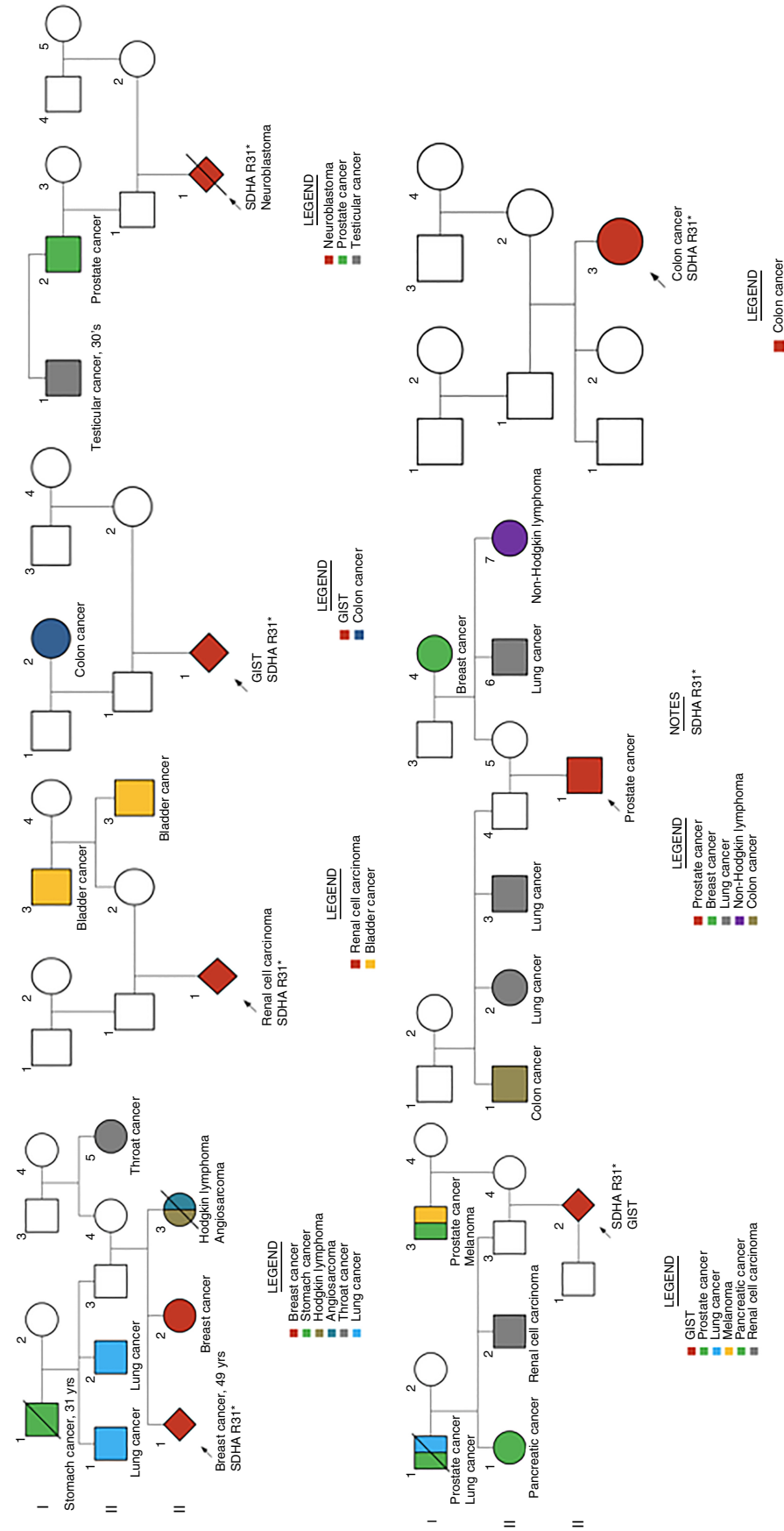


Figure 2. Three-generation pedigrees of families with recurrent *SDHA* c.91C>T (p. Arg31Ter) mutation. The proband cancer diagnoses were breast cancer, renal cancer, gastrointestinal stromal tumor, neuroblastoma, gastrointestinal stromal tumor, prostate cancer, and colon cancer.

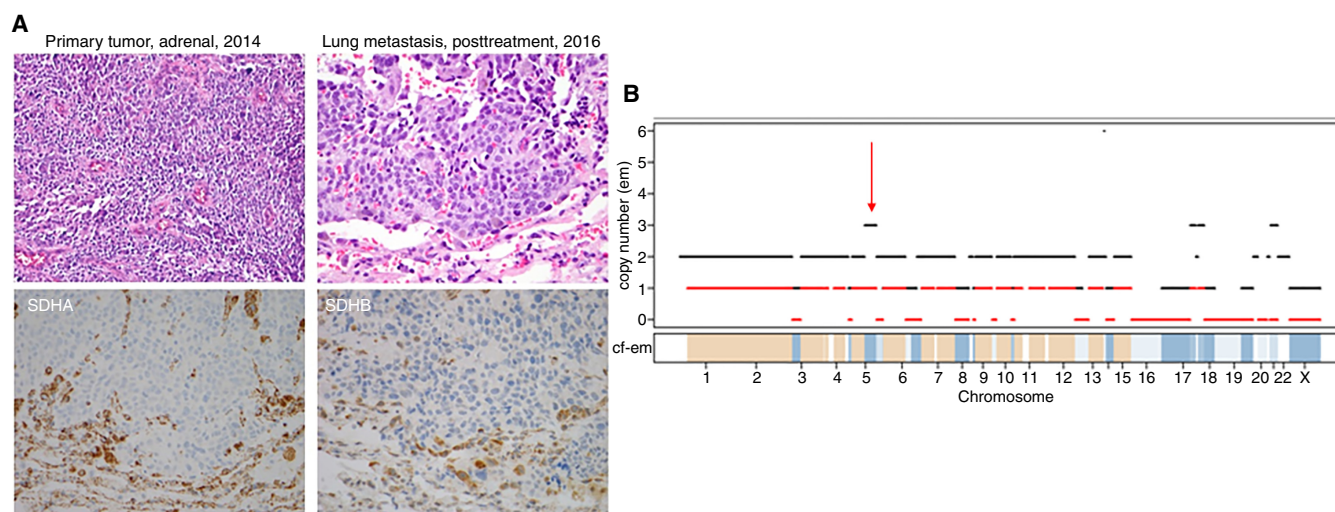


Figure 3. (A) Neuroblastoma primary tumor and lung metastasis with SDHA/SDHB staining loss, (B) schematic illustrating chromosomal copy-number loss in the tumor encompassing the *SDHA* gene locus Chromosome 5p15.33 (red arrow indicates loss of Chromosome 5).

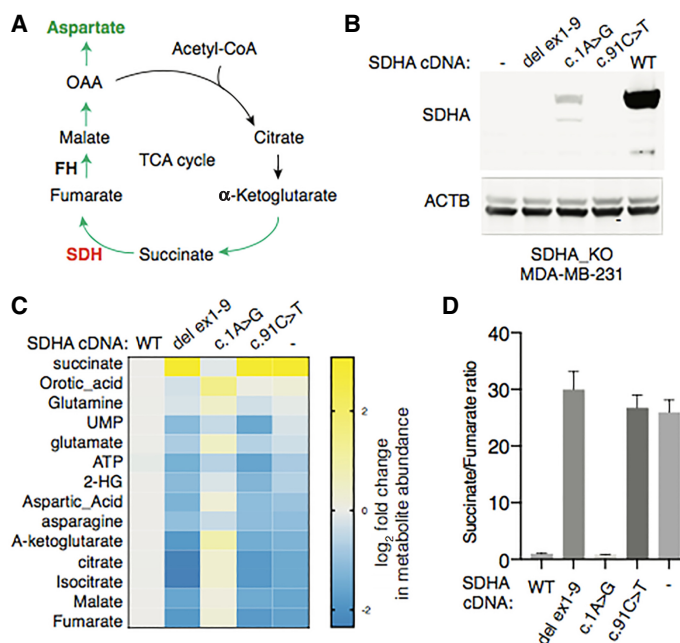


Figure 4. (A) Metabolite intermediates of Krebs cycle. SDH (succinate dehydrogenase; indicated in red). (B) Immunoblot analysis of SDHA knockout MDA-MB-231 breast cancer cell line expressing vector, wild-type (WT), or indicated SDHA cDNA. Actin is used as a loading control. (C) Changes (\log_2) in indicated 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

sgRNAs (caccGCCCATCACCTCGACCCACGG 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- μ m filter to eliminate cells. Target cells in six-well tissue culture plates were infected in media containing 8 μ g/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 μ l 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- d_8 as an internal standard. After a 10-min vortex and centrifugation for 10 min at 4°C at 10,000g, samples were dried under nitrogen gas. Dried samples were stored at –80°C and then resuspended in 100 μ l water; 1 μ l of each sample was injected onto a ZIC-pHILIC 2.1 \times 150 mm (5 μ m 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.

Competing Interest Statement

The authors have declared no competing interest.

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