Germline SDHx variants modify breast and thyroid cancer risks in Cowden and Cowden-like syndrome via FAD/NAD-dependant destablization of p53

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Cowden syndrome (CS), a Mendelian autosomal-dominant disorder, predisposes to breast, thyroid and other cancers. Germline mutations in phosphatase and tensin homolog (PTEN) have been recently reported in 23% of a large series of classic CS. Here, we validated our small (n = 10) pilot study in a large patient series that germline variations in succinate dehydrogenase genes (SDHx) occur in 8% (49/608) of PTEN mutation-negative CS and CS-like (CSL) individuals (SDHvar+). None of these SDHx variants was found in 700 population controls (P < 0.0001). We then found that SDHx variants also occur in 6% (26/444) of PTEN mutation-positive (PTENmut+) CS/CSL individuals (PTENmut+/SDHvar+). Of 22 PTENmut+/SDHvar+ females, 17 had breast cancers compared with 34/105 PTENmut+ (P < 0.001) or 27/47 SDHvar+ patients (P = 0.06). Notably, individuals with SDHvar+ alone had the highest thyroid cancer prevalence (24/47) compared with PTENmut+ patients (27/105, P = 0.002) or PTENmut+/SDHvar carriers (6/22, P = 0.038). Patient-derived SDHvar+ lymphoblastoid cells had elevated cellular reactive oxygen species, highest in PTENmut+/SDHvar+ cells, correlating with apoptosis resistance. SDHvar+ cells showed stabilized and hyperactivated hypoxia inducible factor (HIF)1α signaling. Most interestingly, we also observed the loss of steady-state p53 in the majority of SDHvar+ cells. This loss of p53 was regulated by MDM2-independent NADH quinone oxidoreductase 1-mediated protein degradation, likely due to the imbalance of flavin adenine dinucleotide/nicotinamide adenine dinucleotide in SDHvar+ cells. Our data suggest the potential regulation of HIF1α, p53 and PTEN signaling by mitochondrial metabolism in CS/CSL tumorigenesis. Together, our findings suggest the importance of considering SDHx as candidate predisposing and modifier genes for CS/CSL-related malignancy risks, and a mechanism which suggests ways of therapeutic reversal or prevention.

INTRODUCTION

Cowden syndrome (CS, [MIM 158350]) is autosomal dominant with lifetime risks of 28% for developing female breast cancer, 10% for epithelial thyroid cancer and unknown but finite risks of developing other cancers (1,2). The syndrome is difficult to recognize because of the protean and variable manifestations of the broad phenotype (2) and remains under-diagnosed. The

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incidence of CS was estimated to be 1 in one million (3,4) before gene identification and raised to 1 in 200 000 after (5), which may still be an underestimate. Germline mutations in the phosphatase and tensin homolog deleted on chromosome 10 (PTEN [MIM 601728]) tumor suppressor gene were found in up to 85% of CS cases defined by the strict International Cowden Consortium criteria (6), although in a recent series comprising >3000 probands, only 23% of classic CS carry a germline PTEN mutation (7). This trend in mutation prevalence with time from initial gene discovery is typical for many heritable syndromes as more probands without family histories and without full phenotype are analyzed, thus making molecular diagnosis difficult and predictive testing challenging, often impossible. When individuals have features of CS but do not meet these criteria, they are referred to as CS-like (CSDL) and necessarily represent a heterogeneous series. Only 5% of CSL individuals have germline PTEN mutations (8). Thus, illustrative of many cancer syndromes and relevant to clinical practice, other predisposition genes must exist for PTEN mutation-negative CS/CSDL individuals and families.

Recently, mitochondrial respiratory enzymes have emerged as tumor suppressors, including autosomal genes encoding mitochondrial complex II–succinate dehydrogenase (SDH) (reviewed in 9). Germline homozygous or compound heterozygous mutations in mitochondrial complex genes, including complex II, result in Leigh syndrome, a rare but fatal neurodegenerative disease. Germline heterozygous SDHB/C/D mutations result in hereditary phaeochromocytoma–paraganglioma (PCC/PGL) syndrome (10–12). We subsequently noticed a small subset of individuals with germline SDHB mutations in our population-based PCC registry also developed early onset renal cell carcinoma (RCC) and papillary thyroid carcinoma, reminiscent of two CS component tumors (12,13). Based on these observations, we carried out a small hypothesis-generating pilot study, which found germline SDHB and SDHD variants in a subset of CS/CSDL individuals without PTEN mutations (14). Based on small numbers (n = 10), it appeared that germline SDHx variant carriers may have elevated frequencies of breast, thyroid and RCC compared with those with germline PTEN mutations. It also appeared that the SDH-related thyroid cancers were papillary in contrast to PTEN-related thyroid cancers whereby follicular histology is over-represented. However, the sample size was small. Functionally, CS/CSDL-associated SDHB or SDHD variants showed similar activation of AKT (protein kinase B) and mitogen-activated protein kinase (MAPK) signaling, which are downstream of the PTEN pathway in a variant-dependent manner, indicating the likely cross-talk between the SDH and PTEN signaling pathways (14).

In the present study, we sought to address our hypotheses that germline SDHx variants associate with increased frequencies of component cancers in a large independent series of PTEN mutation-negative CS/CSDL individuals; and that SDHx alleles modify solid tumor risks in individuals with germline PTEN mutations. Because the SDHx alleles are missense variants, it was important to demonstrate functional relevance. So, we then explored mechanisms whereby SDHx variants can lead to tumorigenesis by examining cellular phenotypes such as apoptosis and reactive oxygen species (ROS) status, and candidate dysregulated signaling pathways.

### RESULTS

**SDHB/D germline variants identified in PTEN mutation-negative and PTEN mutation-positive CS/CSDL patients**

Manganese superoxide dismutase (MnSOD) is an indicator of ROS stress and of general mitochondrial dysfunction. Following a similar workflow (Supplementary Material, Fig. S1) as in our pilot paper (14), PTEN mutation-negative CS/CSDL germline samples with elevated MnSOD levels (n = 608, including 10 pediatric patients; Supplementary Material, Table S2) were subjected to SDHB/C/D mutation analysis and their data compared with ancestry-matched controls (n = 700). We found 49 of 608 (8%) PTEN mutation-negative cases carrying non-synonymous germline SDHx mutations/variants (Table 1, upper panel): 17 in SDHB [Ala3Gly (n = 1), Arg27Gly (n = 1), His57Arg (n = 1), Asn120Ser (n = 1) and Ser163Pro (n = 13)], 1 in SDHC [Ala66Val] and 31 in SDHD [Gly12Ser (n = 18), His50Arg (n = 12), His145Asn (n = 1)]. None of these SDHx variants was found in the 700 population controls (P < 0.0001).

In order to determine whether SDHx variation and PTEN mutation are mutually exclusive or can occur together, we examined a nested series of 444 PTEN mutation-positive CS/CSDL patients (Supplementary Material, Table S2) with germline PTEN mutation/variant, including promoter variants and large insertions/deletions, regardless of the MnSOD status. Of the 444, 26 (6%) were also found to carry germline SDHB/D variants, 12 in SDHB [Ala3Gly (n = 1), Gly53Glu (n = 1), Ser163Pro (n = 9), Ala215Thr (n = 1)] and 14 in SDHD [Gly12Ser (n = 3), His50Arg (n = 11)] (Table 1, lower panel).

<table>
<thead>
<tr>
<th>Variation</th>
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<tr>
<td><strong>SDHx mutation-negative subset:</strong> (8%, 49/608)</td>
<td></td>
</tr>
<tr>
<td>SDHB (n = 17)</td>
<td>c.8C&gt;G, p.Ala3Gly 1</td>
</tr>
<tr>
<td></td>
<td>c.9C&gt;G, p.Arg27Gly 1</td>
</tr>
<tr>
<td></td>
<td>c.170A&gt;G, p.His57Arg 1</td>
</tr>
<tr>
<td></td>
<td>c.359A&gt;G, p.Asn120Ser 1</td>
</tr>
<tr>
<td></td>
<td>c.487T&gt;C, p.Ser163Pro 13</td>
</tr>
<tr>
<td>SDHC (n = 1)</td>
<td>c.197C&gt;T, p.Ala66Val 1</td>
</tr>
<tr>
<td>SDHD (n = 31)</td>
<td>c.34G&gt;A, p.Gly12Ser 18</td>
</tr>
<tr>
<td></td>
<td>c.149A&gt;G, p.His50Arg 12</td>
</tr>
<tr>
<td></td>
<td>c.433C&gt;A, p.His145Asn 1</td>
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<tr>
<td><strong>SDHx mutation-positive subset:</strong> (6%, 26/444)</td>
<td></td>
</tr>
<tr>
<td>SDHB (n = 12)</td>
<td>c.8C&gt;G, p.Ala3Gly 1</td>
</tr>
<tr>
<td></td>
<td>c.158G&gt;A, p.Gly53Glu 1</td>
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<tr>
<td></td>
<td>c.487T&gt;C, p.Ser163Pro 9</td>
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<td>SDHC (n = 14)</td>
<td>c.643G&gt;A, p.Ala215Thr 1</td>
</tr>
<tr>
<td></td>
<td>c.34G&gt;A, p.Gly12Ser 3</td>
</tr>
<tr>
<td></td>
<td>c.149A&gt;G, p.His50Arg 11</td>
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Table 1. List of SDHx variants identified in CS/CSDL individuals in PTEN mutation-negative subset and PTEN mutation-positive subset
Increased prevalence of breast and thyroid cancers in 
SDHx variant carriers in PTEN mutation-negative CS/CSL individuals

The snapshot prevalence of breast, epithelial thyroid and RCC were calculated for the 47 adult individuals carrying SDHx variants only (i.e. without PTEN mutations). Of the 47 patients with SDHx variants, 27 (57.4%) had breast cancer, 24 (51.1%) had epithelial thyroid cancers and 3 (6.4%) RCC (Table 2, upper panel). When compared with patients with only PTEN mutations, SDHx variant carriers showed odds ratios (OR) of 2.82 (95% CI 1.39–5.72, P = 0.001) for breast cancer, and 3.01 (1.47–6.19, P = 0.002) for epithelial thyroid carcinoma (Table 2). The prevalence of RCC in SDHx variant carriers is similar to PTEN mutations carriers. Epithelial thyroid cancers seen in the SDHx variant carriers were mostly papillary (22/24, 91%) in histology (majority classic papillary) compared with only one-third among pathogenic PTEN mutation carriers (P < 0.001).

SDHx alleles associated with modified malignancy risks in PTEN mutation carriers

Strikingly, breast cancers developed in 77% (15) of 22 patients carrying both PTEN mutations and SDHx variants (11 invasive ductal carcinomas and 6 ductal carcinoma in situ) when compared with patients with PTEN mutations alone (32.4%) [P < 0.001; OR = 7.10 (95% CI: 2.41–20.86)], or compared with patients with SDHx variants alone (57.4%) [P = 0.06, OR = 2.52, (95% CI: 0.80–7.98)]. There were 6 of 22 (27.2%) carriers of both PTEN mutation and SDHx variant with thyroid cancer compared with 27 of 105 (25.7%) in those with PTEN mutations alone [P = 0.21, OR = 1.08 (95% CI: 0.39–3.05)] in contrast to 24 of 47 (51.1%) with SDHx variants alone [P = 0.038, OR = 0.34 (95% CI: 0.12–1.08)]. While the prevalence of thyroid cancer in patients with both PTEN mutations and SDHx variants is not statistically significantly different from that of PTEN mutation-only patients, the histology of these 6 PTEN/SDHx-related thyroid cancers was exclusively papillary. No RCC were noted in the group with both PTEN mutation and SDHx variant.

Enhanced apoptosis resistance correlated with elevated ROS in SDHx variant carriers

Based on our genotype–phenotype observations above, we hypothesized that the functional consequences of the co-occurrence of PTEN and SDHx alterations would be more marked, beyond those of SDHx alteration alone. To evaluate if SDHx variants have any effect in regulating cell death, patient-derived lymphoblastoid cells from controls, SDHx variant carriers (SDHxvar) and individual samples that have both SDHx variant and PTEN mutation (SDHxvar/PTENmut) were serum starved overnight and allowed to grow under low serum [0.2% fetal bovine serum (FBS)] conditions for 36 h before measuring the percentage of cells in the sub-G1 cell cycle phase by flow cytometry (Fig. 1A). This revealed a pattern of additive cell death resistance with SDHx variants in the presence of PTEN mutations. Although some apoptosis resistance was seen in cells with only SDHx variants, it was not as pronounced as those with both PTEN mutation and SDHx variant. ROS marker microscopy revealed significantly increased intracellular ROS levels in representative samples harboring SDHx variants compared with normal controls (Fig. 1B), with or without PTEN mutation, consistent with our previous finding (14). Quantitative flow cytometry experiments using the same carboxy-H2DCFDA live cell labeling confirmed this observation (Fig. 1C). Interestingly, samples with both PTEN mutations and SDHx variants showed higher ROS compared with samples with SDHx variants alone. Moreover, these observations are inversely correlated with the degree of cell death (Fig. 1A).

Hypoxia inducible factor (HIF)1α stabilized in samples from SDHx variant carriers

In SDH-related PCC/PGL syndromes, pseudohypoxia-induced stabilization of hypoxia inducible factor (HIF)1α was hypothesized (16). In order to investigate if the pseudohypoxia hypothesis is also applicable to CS/CSL in the context of PTEN and SDH cross-talk, we analyzed HIF1α protein expression levels in PTEN or SDHx mutation/variant carriers when compared with normal control samples. SDHx variant-positive samples showed increased levels of HIF1α protein compared with controls (Fig. 2A). This result was

Table 2. Increased cancer frequencies in SDHx variant-only carriers and in PTEN mutation and SDHx variant double-carriers compared with PTEN mutation-only carriers

<table>
<thead>
<tr>
<th></th>
<th>Breast cancer (total number)</th>
<th>Thyroid cancer (total number)</th>
<th>Renal cancer (total number)</th>
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<tbody>
<tr>
<td>PTENmut+</td>
<td>34/105 (32.4%)</td>
<td>27/105 (25.7%)</td>
<td>7/105 (6.7%)</td>
</tr>
<tr>
<td>SDHxvar+</td>
<td>27/47 (57.4%)</td>
<td>24/47 (51.1%)</td>
<td>3/47 (6.4%)</td>
</tr>
<tr>
<td>PTENmut+/SDHxvar+</td>
<td>17/22 (77.2%)</td>
<td>6/22 (27.3%)</td>
<td>0</td>
</tr>
<tr>
<td>SDHxvar+ versus PTENmut+</td>
<td>P-value: 0.001</td>
<td>OR (95% CI): 0.002</td>
<td>0.28</td>
</tr>
<tr>
<td>SDHxvar+ versus SDHxvar+</td>
<td>P-value: 2.82</td>
<td>OR (95% CI): 0.038</td>
<td>0.95 (0.24–3.87)</td>
</tr>
<tr>
<td>SDHxvar+ versus SDHxvar+</td>
<td>P-value: 2.52</td>
<td>OR (95% CI): 0.21</td>
<td></td>
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</table>

P-values derived from Fisher’s two-tailed exact test. P-value < 0.05 is considered statistically significant and is in italics. OR, odds ratio; CI, confident interval.
confirmed by dot blot analyses (Fig. 2B). PTEN nonsense mutation-positive patients showed reduced PTEN protein, while HIF1α protein levels were no different from those of wild-type controls (Fig. 2A). SDHx variant-positive samples showed a lower HIF1α transcriptional expression relative to samples with PTEN mutations (Fig. 2C). In contrast to the protein expression, interestingly, truncating PTEN mutations was associated with moderately decreased HIF1α transcript and virtually no HIF1α protein. As a confirmation of enhanced HIF1α transactivity due to protein stabilization, one of the well-known HIF1α target genes, vascular endothelial growth factor (VEGF) transcriptional expression was examined using quantitative reverse-transcription polymerase chain reaction (RT–PCR). As shown in Figure 2D, relative VEGF mRNA expression was significantly increased in SDHx variant carriers compared with normal controls.

Enhanced baseline p53 ubiquitin-independent degradation in SDHx variant-positive cells

Exposure of cells to high levels of ROS leads to oxidative stress, and should induce a p53-mediated response including apoptosis, whereas we observed the opposite in our cases. Therefore, we decided to investigate if p53 is somehow modified in our patient samples in order to escape the expected apoptotic regulation. Western blots showed reduced p53 protein expression in the majority of samples harboring SDHx variants compared with normal controls (Fig. 3A and B, upper panels). In contrast to p53 protein levels, TP53 mRNA expression was not changed in SDHx variant-positive samples compared with controls (Fig. 3C), suggesting that the loss of p53 protein was not from down-regulated TP53 transcript. Cells treated with proteasome inhibitor MG132
abolished the loss of the p53 protein phenomenon in SDHx variant-positive cells (Fig. 3A and B, lower panels), indicating that the loss of p53 protein is most likely due to enhanced protein degradation. The multiple bands >53 kDa are likely ubiquitinated p53 resulting from proteasomal inhibition.

To further explore the regulation of p53 degradation, we then investigated the main MDM2-related proteasomal degradation pathway (15,17). No corresponding increase in MDM2 or p-MDM2 was observed (Supplementary Material, Fig. S2A). Because NADH quinone oxidoreductase 1 (NQO1) has been shown to bind and stabilize p53 through MDM-independent pathways (18–20), we next checked the NQO1 status in our control and patient cell lines with the hypothesis that either absolute levels of NQO1 could be reduced or the interaction between NQO1 and p53 could be disrupted in the presence of SDHx variation. First, we did not observe obvious decreases of NQO1 expression (Supplementary Material, Fig. S2B) in SDHx variant-positive cells, indicating that decreased p53 protein was not a consequence of decreased NQO1 expression. Secondly, dose-dependent induction of NQO1 by NQO1 inducer dimethyl fumarate (DMF) treatment had no significant impact on either p53 protein expression or cell apoptosis in SDHx variant-positive cells either (Supplementary Material, Figs S2C and S2D), indicating that NQO1 induction cannot rescue the SDHx variant-associated loss of p53. Finally and importantly, co-immunoprecipitation experiments showed decreased binding between NQO1 and p53 in SDHx variant-positive patient cells compared with controls (Fig. 3D), suggesting that the loss of p53 is indeed caused by defective interactions between NQO1 and p53.

Figure 2. HIF1α protein stabilized with accumulation in SDHx variant-positive samples. (A) Western blot analysis of HIF1α, PTEN and β-actin protein expression in PTEN mutation-positive, SDHx variant-positive patients including (patient ID—SDHx variant): 826GV-H50R, 1617LS-G12S, 1902BS-S163P, 2369JB-S163P, positive controls (Pos Ctr, samples stimulated with CoCl2 to mimic hypoxia condition) and normal (Neg Ctr) controls. Relative levels of HIF1α protein were quantitated by densitometry and normalized to β-actin in right panel. Results are fold change in SDHx+ carriers compared with normal control cells. (B) A dot blot analysis of HIF1α protein expression in SDHx+ samples and controls. Relative levels of HIF1α protein were quantitated by densitometry in right panel. Results are fold change in SDHx+ carriers compared with normal control cells. (C) Relative HIF1α mRNA expressions in PTEN mutation-positive and SDHx variant-positive patients compared with normal controls. (D) Relative VEGF mRNA expression is upregulated in SDHx variant-positive samples. Data were presented as (Mean ± SEM). *P < 0.05.

Altered flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NAD) concentrations in SDHx variant carrier samples

We then hypothesized that the impaired binding between NQO1 and p53 may be caused by accumulated flavin adenine dinucleotide (FAD), which is a cofactor for both NQO1 and SDH enzymatic activity. To test this hypothesis, FAD and nicotinamide adenine dinucleotide (NAD)+/
NADH (as product/substrate of NQO1) concentrations were measured directly in control and SDHx variant carrier lymphoblastoid cells. Indeed, we observed elevated FAD level in SDHx variant-positive cells compared with controls (Fig. 4A, left panel). In contrast to higher FAD levels, NAD+ was lower in SDHx variant-positive cells with no change in NADH (Fig. 4A, middle and right panels). When we supplemented normal control cells with an excess (250 μM) of riboflavin (FAD precursor) for 48 h, significant reductions in NAD+ levels (Fig. 4B) were also detected, but not NADH, and were accompanied by increased cellular FAD. This is consistent with our observations in SDHx variant-positive patient cells. More interestingly, FAD-treated control cells showed similar reductions in p53 expression (Fig. 4C), mirroring our observations in non-FAD-treated patients cells carrying SDHx variants (above). FAD treatment of control cells resulted in increased p-AKT and p-MAPK activation, mimicking the inactivation of the PTEN pathway, without changing PTEN protein expression.

DISCUSSION

Individuals with heritable cancer syndromes, such as CS/CSL, who do not carry mutations in the known predisposition genes, bring challenges to molecular diagnosis, predictive testing of family members, genetic counseling and preventive medical management. Identifying additional cancer susceptibility genes for CS/CSL would improve and facilitate the gene-specific personalized medical care. We have recently uncovered an alternative mechanism, germline hypermethylation of the tumor suppressor gene KLLN (encoding KILLIN), accounting for one-third of PTEN mutation-negative CS/CSL (21). Germline KLLN hypermethylation is associated with increased risks of breast and renal cancers over those with PTEN mutations. Our current study not only validates the previous pilot observations that SDHx variants occur in ≈8% of PTEN mutation-negative CS/CSL patients, but also validates the elevated breast and thyroid cancer frequencies over those with PTEN mutations. SDHx, together with PTEN and KLLN, may form a panel of predisposition genes considered for genetic testing for CS/CSL, perhaps prioritized based on the individual patient’s clinical phenotype at presentation and their family history. For example, if a CS/CSL individual has papillary thyroid carcinoma, then SDHx testing should be considered first.

As with all inherited cancer syndromes to date, while we can counsel increased prevalence of specific cancers, we cannot predict which subset of those with PTEN mutations will develop each component cancer. Here we have found that 6% of PTEN mutation/variant-positive CS/CSL patients were also found to have germline SDHx variants, and the presence of SDHx variants appear to further modify PTEN mutation cancer risks over those of PTEN mutation in isolation. Because this is the first observation of SDHx variation modifying PTEN-related breast cancer risk, this will need to be independently validated before translation into the routine clinical armamentarium.

Among all 11 different SDHx variants we detected, there are 4 novel variants not previously reported in either NCBI SNP
database (http://www.ncbi.nlm.nih.gov/snp/) or SDHx mutation database (http://chromium.liacs.nl/lovd_sdh), SDHB Arg27Gly, Asn120Ser, Ala215Thr and SDHC Ala66Val. SDHC Ala66Val detected represents the first SDHC variant in a CSL patient, a 54-year-old patient with invasive breast cancer, follicular thyroid cancer, uterine fibroids and skin hemangioma. Patients with SDHB Arg27Gly, Asn120Ser or Ala215Thr variants all presented with invasive breast carcinoma and either malignant (papillary thyroid cancer) or benign thyroid lesions. The other patient with SDHD His145Asn (rs121908984) variant first reported in our previous pilot study presented with both breast carcinoma and RCC. The fact that carriers of these variants all presented with malignant breast carcinoma suggest physiologic relevance. SDHB Ala3Gly (rs11203289) and His57Arg (rs35962811) were reported in dbSNP but only in African American population, while our samples are derived from white individuals of European ancestry. The most frequent variants SDHB Ser163Pro (rs33927012), SDHD Gly12Ser (rs34677591) and SDHD His50Arg (rs11214077) we seen in our CS/CSL individuals have also been reported in the database. Although these relatively common (1–5% frequency) variants were computationally predicted to be functionally benign (22), our experimental data provide molecular evidence that they could have functional impact in cellular signaling regulation as well. The reason why bioinformatic analysis of prediction fails in SDHx genes is because they are extremely well conserved throughout species (23). With enormous numbers of variations uncovered by whole genome sequencing, it is essential to realize that functional analysis and clinical correlations must be performed to define the true pathogenic effect of DNA variations (24), as we have done in the current study.

Changes in the mitochondrial metabolism have long been linked to cancer, known as the Warburg effect (25). The mechanism(s) of disruption of mitochondrial function leading to neoplasia remain unclear. Succinate, the substrate of SDH, may function as a second messenger between the mitochondria (energy production body) and cytosol. Accumulation of succinate due to SDHx mutations inhibit...
the prolyl-hydroxylase enzyme and contributes to stabilization of HIF1α in turn promoting transcription of genes containing hypoxic response elements believed to promote cancer (16). Our data suggest that the hyperactivation of the HIF pathway is indeed involved in our CS/CSL development. It has been reported that the HIF signaling pathway can also be regulated by AKT and mTOR signaling downstream of PTEN (26). It is notable that HIF1α expression in PTEN mutation-positive samples versus SDHx variant-only samples are different. We observed no obvious accumulation of HIF1α protein with PTEN mutation, consistent with other reports that loss of function of PTEN most likely increases the HIF1α transactivation function by preventing HIF1α binding to HIF inhibitory factor (FIH), instead of stabilizing HIF1α protein expression (27). This contrasts with our observation of SDHx-related hyperactivation of HIF1α. This differential involvement of HIF signaling may partially explain the different levels of predisposition to breast, thyroid and/or renal carcinomas in PTEN mutation positive or SDHx variant positive, versus both.

As ‘energy factories’ through oxidative phosphorylation, mitochondria make endogenous ROS as byproducts of normal respiration. Under normal physiological conditions, complex II is not considered to be a site for ROS generation. However, the structure–function studies of bacterial Sdh and fumarate dehydrogenase indicated ROS production mainly resides at the FAD site of these enzymes (28). The succinate-driven reverse-electron transfer through complex I was shown as another mechanism to yield the highest rates of H2O2 in isolated mitochondria (29). Therefore, accumulated succinate from dysfunctional SDH complex not only could serve as a second messenger activating HIF signaling (as discussed above), but also could drive the intracellular ROS generation. The extra ROS stress has been reported with SDHx mutations (30), and also observed in our SDHx variant carrier cells in general. Certain PTEN mutation-positive cells do show elevated ROS (31), which accounts for the additive increased ROS in samples with both PTEN mutation and SDHx variant. Excessive oxidation of DNA by ROS could be a major cause of DNA damage and genetic instability (32,33), which leads to accumulation of mutations and deletions that eventually contribute to carcinogenesis.

TP53, as a stress-induced tumor suppressor gene, plays important roles in programmed cell death (34). The reduction in basal p53 levels in SDHx variant carriers likely explains the corresponding escape of cell death, which otherwise should be regulated by the p53 pathway under normal cellular responses. A recent study also suggested that mitochondrial respiration deficiency may impair p53 expression and function (35), which is consistent with our observation. We further investigated the underlying mechanism of this p53 impairment caused by SDHx variation. The tightly regulated p53 protein levels are achieved via both ubiquitin-mediated degradation in 26S proteasomes based on interactions between p53 and Mdm2 (36), and an ubiquitin-independent degradation in 20S proteasomes (18–20,37). In the latter, p53 is degraded by 20S proteasomes through direct binding to NQO1. What is intriguing to us is that the function of NQO1 is tightly regulated by mitochondrial redox metabolites. As a key player here, NQO1 is a FAD-containing protein and its activity is highly dependent on intracellular NAD+/NADH, also the product and substrate, respectively, of mitochondrial complex I (38). Our data suggest that in our SDHx variant cells, it is not the loss of absolute NQO1 expression but...
more specifically the loss of functional NQO1 binding to p53 that results in the reduction in p53 protein. The observation that increased FAD and lowered NAD+ concentrations in $SDHx$ variant cells validates the observation of inhibited NQO1 function. Presence of excessive FAD results in a novel mechanism that mitochondrial metabolites regulate cellular signaling, hence mechanistically linking mitochondrial dysfunction to tumorigenesis.

In conclusion, our genetic analyses revealed that germline $SDHx$ variants are associated with elevated cancer risks in CS/CSL individuals, both alone and synergistically with germ-line $PTEN$ mutations. Our functional data suggest that disruption of complex II could lead to mitochondrial metabolite imbalance, and in turn cause the stabilization of HIF1$\alpha$, loss of baseline p53 levels and is at least partially responsible for ROS generation. The cross-talk between SDH and PTEN results in a multi-signaling cascade that contributes to tumorigenesis (Fig. 5). As we are learning more about the heterogeneity of tumor formation, it is not surprising to observe multiple pathways crosstalk, contribute alone or simultaneously to the final outcome of differential organ-specific carcinogenesis. Together, our findings suggest the importance of considering $SDHx$ as candidate predisposing genes and as candidate modifier genes for CS/CSL-related malignancy risks, and may also guide future tailored preventative or therapeutic approaches.

**MATERIALS AND METHODS**

**Subjects and germline DNA extraction**

CS or CSL patients were prospectively enrolled in accordance with our research protocol IRB8458-PTEN, which was approved by the Cleveland Clinic and respective Institutional Review Boards for Human Subjects Protection. All research participants provided written informed consent. To be enrolled in the IRB8458-PTEN, individuals are eligible if he/she meets the full CS diagnostic criteria established by the International Cowden Consortium (Supplementary Material, Table S1) or the relaxed criteria (criteria minus one) according to version 2006 NCCCN Guidelines (39). Patients meeting the relaxed criteria are referred to as individuals with CSL phenotypes or CSL. In other words, CSL was diagnosed when an individual did not fully meet the strict diagnostic criteria but had features with one or two criteria short of the operational diagnostic criteria. Matching the subjects, normal (population) controls are from northern and western European origin and were anonymized prior to storage and analysis.

Germline DNA was extracted from peripheral blood samples from patients and healthy controls by the Genomic Medicine Biorepository (GMB), Genomic Medicine Institute, Cleveland Clinic (protocols are available at GMB website, http://www.lerner.ccf.org/gmi/gmb/methods.php).

**Lightscanner and sequencing for mutation scanning**

Genomic DNA was first analyzed using high-resolution melting LightScanner technology (Idaho Technology Inc., Salt Lake City, UT, USA), which detects nucleic acid sequence variations, by changes in the melting curve. Primers to amplify a total of 20 amplicons spanning the exons, exon–intron junctions and flanking intronic regions were amplified with LCGreen® Plus (Idaho Technology) in a final reaction volume of 10 µl with 20 µl oil overlay. The temperature cycling protocol consisted of an initial denaturation step at 95°C for 2 min, followed by 37 cycles of denaturation at 94°C for 30 s, optimal annealing temperature for each amplicon for 30 s and heteroduplex formation step at 95°C for 30 s and final hold at 25°C. Melting curve analysis was performed on LightScanner with LightScanner software employing three steps, namely, normalization, temperature shift and generating difference plot to cluster samples. Samples with melting curves that clustered differently from reference samples were directly sequenced for $SDHB$, $SDHC$, or $SDHD$, as previously reported by our laboratory (40,41).

**Cell lines and cell cultures**

Human immortalized lymphoblastoid cell lines (LCLs) derived from patients and controls were generated by Genomic Medicine Biorepository, Genomic Medicine Institute, Cleveland Clinic (protocols are available at GMB website, http://www.lerner.ccf.org/gmi/gmb/methods.php). LCLs were cultured in RPMI 1640 supplemented with 20% FBS and 100 units/ml each of penicillin and streptomycin. All cell lines were cultured at 37°C with 5% CO$_2$. NQO1 inducer DMF (Sigma-Aldrich Co., St Louis, MO, USA) was added into cell culture at different doses as described in figure legend.

**RNA extraction and quantitative reverse-transcription–PCR**

Total RNA was extracted from LCLs from controls and patients using RNeasy® Mini Kit (QIAGEN, Inc., Valencia, CA, USA) according to the manufacturer’s protocol, and subsequently treated with DNase I (Invitrogen, Carlsbad, CA, USA). DNase-treated total RNA from each LCL was reverse-transcribed into cDNA using random primers and SuperTranscript™ III Reverse Transcriptase (Invitrogen), as specified by the manufacturer. PCR was performed using SYBR Green PCR Master Mix and run on 7500 Real Time PCR machine (Applied Biosystem, Carlsbad, CA, USA) using the following intron-spanning primers: 5’-GAAAAAGTAAAGTTCTGAACGTCGA-3’ and 5’-CTTTATCAAGATGCGAACTCACA-3’ for HIF1α; 5’-CATTCCCCACATGCCAAGTG-3’ and 5’-TATTCTCCCTCTTCT-3’ for VEGF; 5’-CTGTCCTCCTTCCACAAACCT-3’ and 5’-GGAGTACGTCGCAAGTGAA-3’ for P53; 5’-GGGCTGCTTIDTTAATCTTCTGTA-3’ and 5’-TGGGTGGAATCATATTGGAAC-3’ for GAPDH.

**Protein analysis**

Whole-cell lysates were prepared as described before (42) with M-PER Mammalian Protein Extraction Reagent (ThermoFisher Scientific, Waltham, MA, USA) supplemented
with protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma-Aldrich). Lysates were either separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose or applied to nitrocellulose with a dot blot apparatus (BioRad, Hercules, CA, USA). The resulting blots were subjected to western blot analysis for PTEN (6H2.1, Cascade Bioscience, Portland, OR, USA), MnSOD (Upstate Biotechnology, Waltham, MA, USA), HIF1α (BD Biosciences, San Jose, CA, USA), p53, Actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and α-tubulin (Sigma-Aldrich) protein levels.

ROS measurement

The measurement of ROS was performed using carboxy-dichlorodihydrofluorescein diacetate (Carboxy-H2DCFDA), a reliable fluorogenic marker for ROS in live cells (Molecular Probes, Invitrogen). The cells were washed with HBSS/Cal/Mg buffer, centrifuged, resuspended in HBSS/Cal/Mg and incubated with 25 μM carboxy-H2DCFDA for 30 min at 37°C. Hoechst 33342 was added at a final concentration of 1 μM to the carboxy-H2DCFDA staining solution during the last 5 min of the incubation. Fluorescence imaging was taken immediately after washing and mounting the samples. For flow cytometry measurement, cells were washed and resuspended in HBSS/Cal/Mg buffer after incubation and count with FACSscan (Becton-Dickinson) immediately.

Cell cycle analysis by FACS flow cytometry

LCLs were serum starved overnight and allowed to grow under 0.2% FBS condition for 36 h before 70% ethanol fixation for cell cycle analysis using FACSscan flow cytometer (Becton-Dickinson).

FAD, NAD+/NADH quantification

FAD concentration was measured using FAD assay kit #357-100 (BioVision, Mountain View, CA, USA) following product protocol. In brief, cells were homogenized and deproteinized. Samples and FAD standard were incubated with FAD enzyme mix, OxidRed Probe and assay buffer for 15–60 min in duplicates before measured by the colorimetric method (λ = 570 nm).

NAD and NADH concentrations were measured using NAD+/NADH quantification kit #337-100 (BioVision) following product protocol. In brief, cells were extracted by freeze/thaw two cycles (20 min on dry ice, then 10 min at room temperature). Extracted samples were filtered through 10 kDa molecular weight cut off filters (BioVision #1997-25) to remove enzymes consuming NADH before performing the assay. To detect total NADt (NADH and NAD), the samples and NADH standard were incubated directly with NAD cycling mix (cycling buffer and enzyme mix). To detect NADH, samples were heated to 60°C for 30 min to decompose NAD before incubating with NAD cycling mix. Duplicated samples were then mixed with NADH developer and incubated at room temperature for 1–4 h before colorimetric reading at OD 450 nm. The amount of NAD in samples was calculated by subtracting NADH from NADt.

Statistical analysis

The frequency of each of the carcinomas in SDHx variant-positive individuals were compared with that in a cohort of 105 PTEN mutation-positive individuals with the CS/CSL phenotype. Fisher’s two-tailed exact test was applied with the significance at P < 0.05.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

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REFERENCES


