Germline Mutations and Variants in the Succinate Dehydrogenase Genes in Cowden and Cowden-like Syndromes

Ying Ni,[1,2,3,7] Kevin M. Zbuk,[1,2,7] Tammy Sadler,[1,2] Attila Patocs,[1,2] Glenn Lobo,[1,2] Emily Edelman,[1,2,4] Petra Platzer,[1,2] Mohammed S. Orloff,[1,2,4] Kristin A. Waite,[1,2,4] and Charis Eng[1,2,3,4,5,6,*]

Individuals with *PTEN* mutations have Cowden syndrome (CS), associated with breast, thyroid, and endometrial neoplasias. Many more patients with features of CS, not meeting diagnostic criteria (termed CS-like), are evaluated by clinicians for CS-related cancer risk. Germline mutations in succinate dehydrogenase subunits SD*HB-D* cause pheochromocytoma-paraganglioma syndrome. One to five percent of SD*HB*/SD*HD* mutation carriers have renal cell or papillary thyroid carcinomas, which are also CS-related features. SD*HB-D* may be candidate susceptibility genes for some *PTEN* mutation-negative individuals with CS-like cancers. To address this hypothesis, germline SD*HB-D* mutation analysis in 375 *PTEN* mutation-negative CS/CS-like individuals was performed, followed by functional analysis of identified SD*H* mutations/variants. Of 375 *PTEN* mutation-negative CS/CS-like individuals, 74 (20%) had increased manganese superoxide dismutase (MnSOD) expression, a manifestation of mitochondrial dysfunction. Among these, 10 (13.5%) had germline mutations/variants in SD*HB* ($n = 3$) or SD*HD* (7), not found in 700 controls ($p < 0.001$). Compared to *PTEN* mutation-positive CS/CS-like individuals, those with SD*H* mutations/variants were enriched for carcinomas of the female breast (6/9 SD*H* versus 30/107 *PTEN*, $p < 0.001$), thyroid (5/10 versus 15/106, $p < 0.001$), and kidney (2/10 versus 4/230, $p = 0.026$). In the absence of PTEN alteration, CS/CS-like-related SD*H* mutations/variants show increased phosphorylation of AKT and/or MAPK, downstream manifestations of PTEN dysfunction. Germline SD*H* mutations/variants occur in a subset of *PTEN* mutation-negative CS/CS-like individuals and are associated with increased frequencies of breast, thyroid, and renal cancers beyond those conferred by germline *PTEN* mutations. SD*H* testing should be considered for germline *PTEN* mutation-negative CS/CS-like individuals, especially in the setting of breast, thyroid, and/or renal cancers.

Introduction

Phosphatase and tensin homolog deleted on chromosome ten (*PTEN* [MIM 601728]) is a ubiquitous tumor suppressor that plays a role in both heritable and sporadic neoplasias.[1] Cowden syndrome (CS [MIM 158350]) is a difficult-to-recognize autosomal-dominant inherited cancer syndrome characterized by benign and malignant breast, thyroid, and endometrial neoplasias in addition to cutaneous findings and macrocephaly.[2] Germline *PTEN* mutations have been found in 85% of those with classic CS although 15% remain mutation negative despite extensive analyses including the promoter and looking for large deletions and rearrangements.[3,4] Many more patients with features reminiscent of CS, not meeting diagnostic criteria (National Comprehensive Cancer Center Practice Guidelines [NCCN]; Table S1 available online) and referred to as CS-like, are evaluated by clinicians for CS and cancer risk.

CS is believed to be without genetic heterogeneity:[5] to date, only *PTEN* has been implicated in this syndrome. However, there must exist other susceptibility genes for CS and CS-like phenotypes, especially in the latter, which appears to be a heterogeneous disease. We often obtain clues to disease etiology by examining whether certain CS/CS-like clinical features resemble those in other syndromes, by examining downstream signaling, and/or by looking at phenotype in murine models. In this situation, the murine model only vaguely resembles human CS.[1] One prominent feature in the mouse model is pheochromocytoma, a neoplasia of the adrenal medulla, and its closely related neural crest-derived paraganglioma (PGL).[6] Pheochromocytoma and PGL are not known component features of CS (NCCN; Table S1).

Succinate dehydrogenase (SDH) belongs to mitochondrial complex II, which participates in both the electron transport chain and the Kreb's cycle (reviewed by C.E.[7]). SDH comprises four subunits, SDHA, B, C, and D, each of which is encoded by autosomal genes on three different chromosomes. Whereas homozygous/compound heterozygous mutations in SD*HA* (MIM 600857) cause severe neurological dysfunction and cardiomyopathy, heterozygous germline mutations in SD*HB-D* (MIM 185470, 602413, 602690) cause a pheochromocytoma-PGL syndrome.[8] Approximately 1%–5% of carriers of SD*HB* or SD*HD* mutations have been found to have renal cell carcinoma or papillary thyroid cancer,[9,10] which are also features of CS. Fumarate hydratase (FH) is the enzyme immediately downstream of SDH. Homozygous germline mutations cause severe neurological dysfunction and death whereas heterozygous mutations are associated with hereditary leiomyomatosis and renal cell carcinoma (HLRCC).[11] In vitro evidence also suggests that

[1]Genomic Medicine Institute, [2]Lerner Research Institute, [3]Department of Molecular Medicine, Cleveland Clinic Lerner College of Medicine, [4]Taussig Cancer Institute, Cleveland Clinic Foundation, Cleveland, OH 44195, USA; [5]Department of Genetics, [6]CASE Comprehensive Cancer Center, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA
[7]These authors contributed equally to this work
*Correspondence: engc@ccf.org
DOI 10.1016/j.ajhg.2008.07.011.

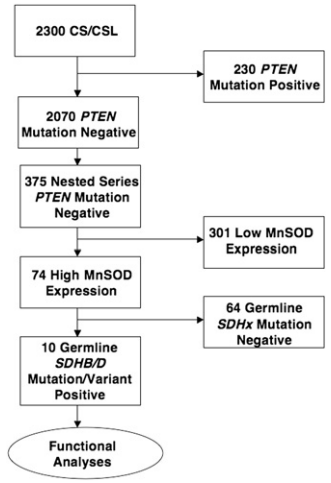

Figure 1. Experimental Design for SD*H* Mutation Testing and Functional Analysis

Note *PTEN* gene testing encompasses intragenic PCR-based mutation analysis, promoter, and large deletion analysis. From the 2270 *PTEN* mutation-negative CS/CS-like individuals, the most proximal (i.e., most recent) consecutive 375 *PTEN* mutation-negative subjects were selected to proceed to MnSOD expression analysis. It is these 375 subjects that represent the series for this SD*H* study.

mitochondrial caspases and HIF1 are downstream molecules of the PTEN pathway.[12,13] Therefore, we hypothesized that SD*Hx* might represent susceptibility genes, other than *PTEN*, for CS/CS-like syndromes.

Material and Methods

Patients

We utilized peripheral blood samples accrued from 375 CS and CS-like individuals who were germline *PTEN* mutation negative after comprehensive mutation analysis that includes all nine coding exons, flanking intronic regions, and minimal promoter region of *PTEN* and examination for large deletions and rearrangements (Figure 1). Classic CS was diagnosed when the operational diagnostic criteria of the International Cowden Consortium were met (Table S1).[14] The diagnosis of CS-like was made when an individual did not meet any of the strict diagnostic criteria but had features that were one or two criteria short of the operational diagnostic criteria (Table S1). We utilized peripheral blood samples from 700 normal white populational controls of northern and western European origin, which were anonymized prior to storage and analysis. Informed consent was obtained for all subjects (CS/CS-like individuals and controls) in accordance with procedures and protocols approved by the respective Human Subjects Protection Committee of each participating institution. All subjects, whether CS/CS-like or controls, participated on a voluntary basis.

Mutation Analysis

Genomic DNA was extracted from peripheral leukocytes, and PCR amplification and direct sequencing (ABI3730xl) of *PTEN, SDHB, SDHC*, and SD*HD* were performed as previously reported by our laboratory.[15,16] It is important to note that all 700 controls had the entire sequence of SD*HB*, SD*HC*, and SD*HD* sequenced and no variants identified.

Cell Lines and Cell Culture

Human immortalized lymphoblast cell lines obtained from patients and controls were cultured in RPMI 1640 supplemented with 20% fetal bovine serum (FBS). All cell lines were cultured at 37°C with 5% CO_2.

Protein Analysis

Whole-cell lysates were prepared with Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) supplemented with protease inhibitor cocktail (Sigma). Lysates were either separated by SDS-PAGE and transferred to nitrocellulose or applied to nitrocellulose with a dotblot apparatus (BioRad). The resulting blots were then subjected to western blot analysis[17] for either: SDHB (AbCam, USA), MnSOD (Upstate Biotechnology, Waltham, MA), PTEN[17] (Cascade Biosciences, Portland, OR) P-MAPK, MAPK, AKT, P-AKT, or actin (Cell Signaling Co., Beverly, MA). For the phosphorylation of MAPK, we utilized an antibody that recognizes the activation phosphorylation of residues Thr187 and Thr189 of the p44-MAPK and the equivalent phosphorylation in p42-MAPK. For Akt phosphorylation, we utilized an antibody that recognizes the activation phosphorylation of Ser473. Both of these antibodies are traditionally utilized to monitor phosphorylation, and thus activation, of these enzymes. Proteins were detected with ECL substrate (Amersham Biosciences, Inc., Chicago, IL) and autoradiography.

Confocal Microscopy

Images were collected with a Leica TCS SP2 AOBS confocal microscope (Leica Micro-Systems, Heidelberg, GmbH) with a HCX Plan Apo 63x/1.4 NA oil immersion lens. The cells were excited with 488 nm light from an Argon laser and emitted light was collected between 500 and 550 nm. Collection parameters remained constant for all samples. Quantitation of ROS was performed by standard FACS (HFE), with controls normalized to 1.

Statistical Analysis

The frequency of each of the established CS-specific component carcinomas (breast and epithelial thyroid) and two of the strongly suspected component carcinomas (renal cell and endometrial) in SD*Hx* mutation-positive individuals were compared to that in a cohort of 230 *PTEN* mutation-positive individuals with CS/CS-like phenotypes. Both groups were ascertained by identical clinical criteria, as noted in the first section of Material and Methods. Fisher's 2-tailed exact test was applied with significance at $p < 0.05$.

Results

To address our hypothesis, we screened protein lysates from 375 *PTEN* mutation-negative CS/CS-like individuals for increased expression of manganese superoxide dismutase (MnSOD) because the latter is a good indicator and first screen for general (complex I–VI, especially complex II or V) mitochondrial dysfunction[8,16,17] (Figure 1). Dot blot analysis of these patients' protein lysates and 18 population controls identified 74 (20%) *PTEN* mutation-negative patients with elevated MnSOD protein levels (Figures 1 and 2A). These 74 were subjected to SD*Hx* mutation analysis (Figure 1). Those that do not have elevated MnSOD levels were not included because a pilot study of 40 CS/CS-like

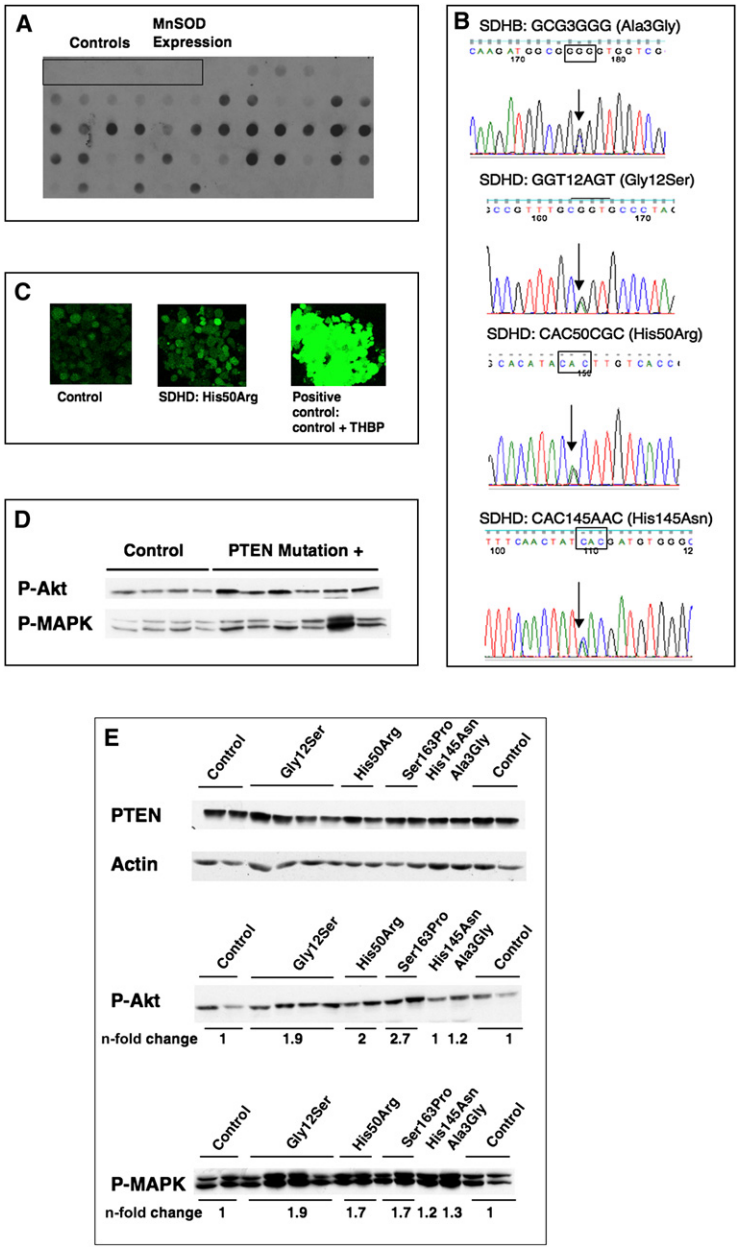

Figure 2. Genetic and Biochemical Analyses of CS/CS-like Patients without Germline *PTEN* Mutations Reveal a Subset with Germline SD*H* Mutations Resulting in Biochemical Dysfunction

(A) Dot blots to screen for increased MnSOD protein levels. Boxed dots represent controls with low MnSOD levels.

(B) Illustrative sequencing chromatograms of germline heterozygous mutations of SD*H* genes identified in patients with CS/CS-like phenotypes (mutations as noted above each chromatogram). The germline mutations/variants are heterozygous manifested by overlapping peaks (arrows).

(C) Increased ROS in peripheral lymphoblasts from an individual with germline SD*HD* His50Arg. Increased ROS levels are measured by increased carboxy-H2DCFDA staining as seen in cultured lymphoblast cells from the patient with SD*HD* His50Arg mutation denoting 1.5-fold higher ROS levels (middle) compared to a lymphoblast cell line derived from a normal control individual (left; $p < 0.001$, Student's t test, 3 replicates). Finally, a control lymphoblast cell line treated with tert-butyl hydroperoxide for 90 min was used as a superpositive control and suprainduced ROS expression is noted by markedly increased carboxy-H2DCFDA staining (right).

(D) Protein expression of P-AKT and P-MAPK (P-ERK44/42) in germline heterozygous *PTEN* mutation-positive individuals. Note different mutations result in varying activation of P-Akt and/or P-MAPK.

(E) Germline protein expression of PTEN, actin (loading control), P-Akt, and P-MAPK (as labeled, from top to bottom). Fold change values beneath the P-Akt and P-MAPK blots represent the mean of normalized densitometrically obtained expressional levels of patient sample(s) relative to controls. In other words, (Patient P-Akt or P-MAPK intensity/corresponding patient actin intensity)/(Control P-Akt or P-MAPK intensity/corresponding control actin intensity). The ratio of control P-Akt or P-MAPK intensity to control actin intensity was normalized to 1.0. We have chosen to use this type of quantitation (taking the ratio of the ratios) because it results in the most conservative (i.e., underestimated) fold changes.

PTEN mutation-negative levels without elevated MnSOD were shown not to harbor any SD*Hx* mutations (A.P., K.M.Z., and C.E., unpublished data). Of the 74 with germline elevations of MnSOD, 10 (13.5%; 95% confidence interval [CI] 7.3%–23.3%) were found to have germline mutations/variants in SD*HB* (n = 3) or SD*HD* (n = 7): Ala3Gly and Ser163Pro (n = 2) in SD*HB* and His145Asn (n = 1), His50Arg (n = 2), and Gly12Ser (n = 4) in SD*HD* (Figure 2; Table 1). None of these SD*H* mutations were found in 700 normal controls ($p < 0.001$, Fisher's 2-tailed exact test). All three genes, SD*HB/C/D*, were sequenced in the controls and no variants uncovered.

We then subjected the five different SD*H* mutations/variants, identified in the 10 CS/CS-like individuals, for functional analysis (Figure 2; Table 2). First, the increased MnSOD protein levels noted on dot blot were confirmed by western blot (Table 2). Because it is known that SDH dysfunction can result in increased production of reactive oxygen species (ROS),[18,19] we also tested the pathogenicity of these different SD*H* mutations by direct measurements of increased ROS levels by using carboxy-H2DCFDA and confocal microscopy (Figure 1C; Table 2). SD*HB* Ser163Pro, SD*HD* Gly12Ser, and His50Arg resulted in increased ROS levels (Table 2). SD*HB* Ala3Gly and SD*HD* His145Asn, in contrast, had normal ROS levels (Table 2).

PTEN is a tumor suppressor that downregulates the anti-apoptotic/proproliferative AKT (protein kinase B)[20] and mitogen-activated kinase (MAPK) pathways.[17] Therefore, PTEN dysfunction is associated with activation of these pathways, whose downstream readouts include phosphorylated AKT and MAPK (p42/44ERKs) (Figure 2D). All 10 CS/CS-like patients with the germline SD*HB/D* mutations

Table 1. Genotype and Clinical Phenotype for *PTEN* Mutation-Negative Patients with Germline SD*HB* or SD*HD* Mutations/Variants

	SD*H* Genotype		Patient's Clinical Features				
Age/Sex	Gene	Mutation	Breast	Thyroid	Renal	Uterus	Family History
41F	SD*HB*	Ala3Gly	C			L	endometrial Cancer
29F	SD*HB*	Ser163Pro		C		B	breast cancer, PTC
54F	SD*HB*	Ser163Pro		C		B	breast cancer, PTC
69F	SD*HD*	Gly12Ser	C			C	breast cancer, endometrial
62F	SD*HD*	Gly12Ser	B	B	C	L	none
46F	SD*HD*	Gly12Ser	C	C		L	none
42F	SD*HD*	Gly12Ser	C			L	none
56F	SD*HD*	His50Arg	C	C			breast cancer
55M	SD*HD*	His50Arg		C			breast cancer, PTC
53F	SD*HD*	His145Asn	C	B	C		none

Abbreviations: C, carcinoma; B, benign pathology; L, uterine leiomyomas; PTC, family history of papillary thyroid carcinoma.

showed activation of AKT and MAPK manifested by increased phosphorylated AKT and MAPK in their germline, when compared to normal controls (Figure 2D). Interestingly, the SD*HD* His145Asn mutation and the SD*HB* Ala3Gly mutation, which did not affect ROS, showed activation of the MAPK pathway and no or only mild activation of the AKT pathway (Table 2; Figure 2E).

Renal cell carcinoma was present in 2/10 (20%; 95% CI 5%–52%) CS/CS-like individuals with germline SD*H* mutations/variants (Table 1) compared to 4/230 (1.2%; 95% CI 0.5%–4.5%) with germline *PTEN* mutations ($p = 0.03$, Fisher's 2-tailed exact test). Epithelial thyroid carcinoma was found in 5 of 10 (50%; 95% CI 25%–76%) SD*H* mutation-positive individuals (Table 1) compared to 15/206 (7.2%; 95% CI 4%–12%) with germline *PTEN* mutations ($p < 0.001$). Interestingly, the histology of all the SD*H*-related thyroid cancers was papillary thyroid carcinoma compared to only one of the 15 thyroid cancers in *PTEN* mutation carriers ($p < 0.001$). Female breast cancer was found in 6 of 9 (66.7%; 95% CI 36%–88%) SD*Hx* mutation-positive women (Table 1) compared to 28% (95% CI 22%–34%) of women with germline *PTEN* mutations ($p < 0.001$). It is important to note that the frequencies of uterine endometrial carcinomas and uterine leiomyomas in our women with SD*Hx* mutations were similar to those in women with *PTEN* mutations ($p > 0.05$). One individual, the 55-year-old man with germline SD*HD* His50Arg, was incidentally found to have a unilateral pheochromocytoma.

Discussion

Our observations suggest that a subset of CS or CS-like individuals, without germline *PTEN* mutations, may be accounted for by germline mutations or variants in either the SD*HB* or SD*HD*, but not SD*HC*, genes. SD*HB* and SD*HD* are the susceptibility genes for familial pheochromocytoma-PGL syndrome.[7] At least one of the five different mutations found in the 10 CS/CS-like individuals, SD*HD* His145Asn, has never been described before in individuals and families with pheochromocytoma and/or PGL (SD*Hx* Mutation Database). Because these mutations are neither in dbSNP nor in our 700 control individuals, this almost certainly is a pathogenic germline mutation. His145 is also highly conserved through mouse, sheep, and cow, arguing for the biological importance of this amino acid residue. Functional analyses corroborate the pathogenicity of this missense mutation. This mutation

Table 2. Identified Germline SD*HB* or SD*HD* Mutations/Variants in *PTEN* Mutation-Negative CS/CS-like Individuals and Their Functional Consequences

Mutation/Variant	MnSOD[a]	ROS	P-Akt Fold Change	P-MAPK Fold Change
SD*HB* Ala3Gly	increased	normal	1.2	1.3
SD*HB* Ser163Pro	increased	increased	2.7	1.7
SD*HD* Gly12Ser	increased	increased	1.9	1.9
SD*HD* His50Arg	increased	increased	2.0	1.7
SD*HD* His145Asn	increased	normal	1.0	1.2

Fold change values represent the mean of normalized densitometrically obtained expressional levels of patient sample(s) relative to controls (where P-Akt/actin or P-MAPK/actin is set to 1.0), i.e., a ratio of ratios.

ROS measurements were quantitated and normalized against controls (latter set at 1). The three mutations resulting in increased ROS had 1.5-fold over controls.

See legend to Figure 2 for further details.

[a] Note that patients chosen for SD*H* analysis were selected for increased MnSOD protein expression.

shows activation of the MAPK, but not AKT, pathway (Table 2; Figures 2D and 2E), mimicking PTEN dysfunction via the latter's nuclear role and/or protein phosphatase activity.[21] Thus, taken together, these genetic and functional data represent strong evidence that SD*HD* His145Asn mutation lends susceptibility to *PTEN* mutation-negative CS/CS-like disorders.

The SD*HB* Ala3Gly variant is shown in dbSNP from the Human Genome sequencing project, but no frequency is noted. The latter usually means that it is an extremely rare variant or it may be found in a nonwhite population. Nonetheless, our germline Ala3Gly variant occurred in a CS/CS-like individual who is white of Northern/Western European ancestry, and this variant is absent in 700 normal chromosomes originating from 700 white controls of the same ancestral background. More importantly, Ala3Gly results in obvious activation of the MAPK pathway and mild activation of the AKT pathway (Figure 2E; Table 1). It would be important to note that dot blot analysis of >700 normal controls showed that none of these control samples had increased MnSOD, increased P-MAPK, or increased P-AKT (K.A.W., T.S., and C.E., unpublished data). Thus, the functional data strongly suggest that Ala3Gly is pathogenic and may function in a low-penetrance fashion in the CS/CS-like setting.

There are human genetic reports that both support and refute SD*HD* Gly12Ser and His50Arg as pathogenic.[22–24] These two variants have been reported to occur in 1.1%–3% of Spanish population controls. His50Arg has been described in 2%–3% of a French Canadian cohort as well. SD*HB* Ser163Pro has been described in African-Americans at a 2% frequency. However, none of our 700 control chromosomes, originating from whites of Northern and Western European ancestry, were found to harbor these three variants ($p < 0.001$). None of our CS/CS-like patients nor any of our controls are of Spanish, French Canadian, or African ancestry. More importantly, we have shown that these three variants do result in increased increased ROS levels (Table 2; Figure 2). Of significance to this report, moreover, Ser163Pro, His50Arg, and Gly12Ser all result in activated signaling down the AKT and MAPK pathways (Figure 2E; Table 2), mimicking PTEN dysfunction although none of these samples had PTEN alterations. Our genetic and functional data, together with recent evidence showing that most rare missense variants are deleterious,[25] therefore, suggest that these three variants are pathogenic at least in the context of our CS/CS-like individuals, and might either be a lower penetrance allele or also signal down other unknown pathways. Although we have provided genetic and strong functional evidence to show that these three variants are pathogenic in the CS/CS-like context, how do we explain the 1.1%–3% prevalence of Gly12Ser and His50Arg in the Spanish or French-Canadian populational controls and the 2% prevalence of Ser163Pro in the black population? One hypothesis is that because these are populational controls (in contrast to healthy controls), these might be individuals who have CS/CS-like phenotypes and have not been recognized given that these syndromes are extremely difficult to diagnose, and more plausibly, these are individuals with partial phenotypes (i.e., *formes frustes*), the latter of which are quite common in the general population.

DNA from family members is not currently available for testing segregation of the mutations with clinical phenotype within families, noting that four individuals do not have any family history. However, maternal imprinting of SD*HD*[11] and decreased penetrance of SD*HB*[11] mutations, even in classic familial pheochromocytoma/paraganglioma syndromes, may make this type of family analysis, especially in this present setting, much less informative. Importantly, we suggest that not only do these mutations likely cause some sort of mitochondrial dysfunction as evidenced by increased expression of MnSOD and/or increased ROS, but they also show increased signaling down the PI3K-AKT and/or MAPK pathways, the latter of which can occur with pathogenic *PTEN* mutations as well. The reason why not all individuals found to have increased MnSOD also have germline SD*Hx* mutations is because MnSOD levels are a broad and general (and not necessarily specific) indication of mitochondrial complex I-VI (electron transport/respiratory chain) function.

We did not have access to the tissues or tumors for the patients with germline SD*H* variants and mutations. However, because of the continuing lack of understanding of SD*H*-related carcinogenesis, it may not be helpful to look for loss of the remaining wild-type allele in tumors of these current cases. In classic SD*H*-related pheochromocytoma/paraganglioma syndromes, sometimes there is somatic loss of the remaining wild-type allele accompanying the germline mutation, but retention of the wild-type allele is also observed.[26,27] Even more puzzling is the well-documented maternally imprinted SD*HD*-related tumors. With maternal imprinting, one would not expect to see loss of the remaining allele but monoallelic expression of the mutant (paternally transmitted) SD*HD* allele. In contrast, paraganglioma from germline SD*HD* mutation-positive individuals still show clear biallelic SD*HD* expression.[26]

The precise mechanism leading to neoplastic transformation in patients with mutations of mitochondrial tumor suppressors is not fully understood. One hypothesis suggests that succinate, the substrate of SDH, functions as a second messenger between the mitochondria and cytosol and inhibits the prolyl-hydroxylase enzymes, thus stabilizing HIF1.[28] This inhibition could contribute to stabilization of HIF and promote transcription of genes containing hypoxic response elements.[29] An alternative hypothesis is that mutations in SD*H* result in increased ROS.[18,19] This metabolic stress results in genomic instability and accumulation of HIF1.[18] Additionally, increased ROS levels can inactivate proteins, including PTEN, via protein oxidation.[30] Because of these reports and our finding germline SD*HB/D* mutations/variants in CS/CS-like individuals, we hypothesized that at least a subset of germline *PTEN* mutations

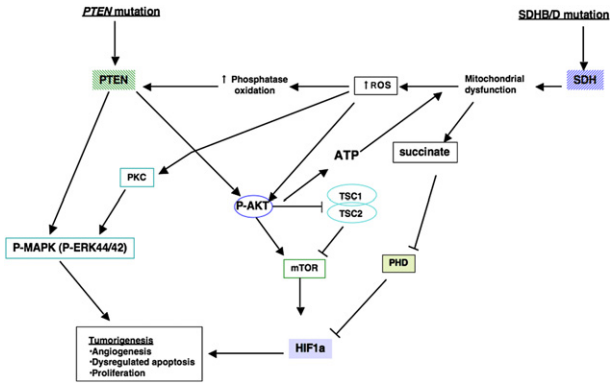

Figure 3. Proposed Model for the Final Common Pathway of Putative Mitochondrial Dysfunction Resulting from Either *PTEN* or SD*Hx* Mutation in Cowden and Cowden-like Syndromes

A simplified version of the signaling pathways involved in tumorigenesis in the setting of dysfunctional PTEN or SDH (represented by hatched colors). These pathways crosstalk, leading to the final common outcome of tumor angiogenesis, cell proliferation, and inhibition of apoptosis. Note that one of the functions of SDH is the conversion of succinate to fumarate as part of the Kreb's tricarboxylic acid cycle. SDH dysfunction will therefore lead to an accumulation of succinate, which inhibits prolyl hydroxylases (PHD) and subsequently leads to the stabilization of HIF-1a. The stabilization of the latter also occurs a number of steps downstream of dysfunctional PTEN signaling. It is interesting to note that activated Akt (P-Akt) can increase ATP levels that result in increased ROS, presumably via mitochondrial dysfunctional signaling. This is postulated to set up a double feedback loop linking both the PTEN and SDH pathways.

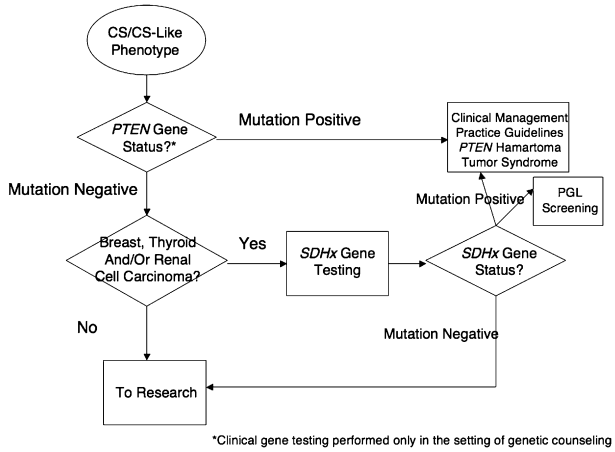

Figure 4. Suggested Algorithm for Clinical *PTEN* and SD*H* Testing for CS/CS-like Individuals

See text for details.

may also lead to mitochondrial dysfunction. As proof of principle, we found that 5 of 11 (45%) patients with germline *PTEN* mutations actually had overexpression of MnSOD protein in the absence of SD*H* mutation (A.P. and C.E., unpublished data). Although our observations and the existing literature suggest interesting signaling crosstalk between the PTEN and mitochondrial signaling pathways, it is also entirely possible that SD*H*-related CS/CS-like phenotypes might be unrelated to the PTEN-deficient mechanism and may represent a previously undescribed syndrome.

In summary, we have shown that a subset of patients with CS or CS-like phenotypes likely has mitochondrial dysfunction irrespective of *PTEN* mutation status, and that this dysfunction can occur by different molecular mechanisms (Figure 3). CS-associated SD*HB* or SD*HD* mutations may be associated with activation of similar antiapoptotic pathways as observed with germline *PTEN* mutations, and that degree of mitochondrial dysfunction might differentially affect the AKT and MAPK pathways. Thus, failure of apoptosis regulation in patients, mediated by either germline *PTEN* or SD*H* mutations, resulting in mitochondrial dysfunction, could be a unifying explanation for tumorigenesis in these patients (Figure 3). Germline SD*H* mutation carriers have significantly higher frequencies of breast, thyroid, and renal cell carcinomas compared to those with germline *PTEN* mutations (Table S1). It would be important to note that germline SD*H* mutation carriers have significantly higher frequencies of breast, thyroid, and renal cell carcinomas compared to those without germline *PTEN* and without SD*H* mutations as well (C.E., unpublished data). In this study, all SD*H*-related thyroid cancers are papillary in contrast to *PTEN*-related epithelial thyroid cancers where all but one are follicular histologies. The frequencies of benign and malignant uterine disease were virtually identical between those with germline *PTEN* mutations and those with SD*H* mutations.

Our data have important implications for both patient care and genetic counseling. Since 1997, the only susceptibility gene for CS and individuals with some neoplasias mimicking CS (CS-like) has been *PTEN*. Now, we suggest SD*H* as a susceptibility gene for a subset of *PTEN* mutation-negative patients with tumors reminiscent of those component to CS. Because this study has analyzed in detail only 10 SD*H* mutation-positive individuals with CS/CS-like features, these data should be validated. Until then, however, it would appear that SD*H* mutation-positive CS/CS-like patients and their families may have significantly increased risks of carcinomas of the breast, thyroid (especially papillary thyroid carcinoma), and kidney beyond those of *PTEN*-related CS. Germline *PTEN* mutation-negative CS/CS-like individuals should be offered SD*H* testing, especially in the setting of breast, papillary thyroid, and/or renal carcinomas (Figure 4). Clinicians may wish to consider annual renal ultrasounds and PGL-type surveillance, beyond the NCCN practice guidelines for *PTEN* hamartoma tumor syndrome, should an individual with tumors similar to those found in CS carry a germline SD*Hx* mutation or variant (Figure 4).

Supplemental Data

Supplemental data include one table and are available at http://www.ajhg.org/.

Acknowledgments

The authors have no conflicts of interest to declare. We thank Jen Stein, MS, CGC, then at the Genomic Medicine Institute, Cleveland Clinic, for acting as genetic counselor-coordinator of the PTEN study at an early stage of the study, and Frank Mularo, MS, and Jin-Liang Chen, MS, both of the Eng Laboratory, Genomic Medicine Institute, Cleveland Clinic, for technical assistance. This study was funded, in part, by the Breast Cancer Research Foundation, the William Randolph Hearst Foundation, and the National Cancer Institute, Bethesda, MD (1P01CA124570-01A1) (all to C.E.). K.M.Z. was a Cleveland Clinic Crile Fellow; A.P. was an International Scholar of The Endocrine Society; and Y.N. is a Howard Hughes Medical Institute Predoctoral Fellow in Molecular Medicine at the Cleveland Clinic Lerner College of Medicine. C.E. is a recipient of the Doris Duke Distinguished Clinical Scientist Award and is the Sondra J. and Stephen R. Hardis Endowed Chair of Cancer Genomic Medicine at the Cleveland Clinic.

Received: June 7, 2008
Revised: July 7, 2008
Accepted: July 10, 2008
Published online: August 7, 2008

Web Resources

The URLs for data presented herein are as follows:

dbSNP, http://www.ncbi.nih.gov/SNP
National Comprehensive Cancer Network, http://www.nccn.org
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/
SD*Hx* mutation database, http://chromium.liacs.nl/lovd_sdh/

References

1. Zbuk, K.M., and Eng, C. (2007). Cancer phenomics: RET and PTEN as illustrative models. Nat. Rev. Cancer *7*, 35–45.
2. Eng, C. (2003). *PTEN*: One gene, many syndromes. Hum. Mutat. *22*, 183–198.
3. Marsh, D.J., Coulon, V., Lunetta, K.L., Rocca-Serra, P., Dahia, P.L.M., Zheng, Z., Liaw, D., Caron, S., Duboué, B., Lin, A.Y., et al. (1998). Mutation spectrum and genotype-phenotype analyses in Cowden disease and Bannayan-Zonana syndrome, two hamartoma syndromes with germline *PTEN* mutation. Hum. Mol. Genet. *7*, 507–515.
4. Zhou, X.P., Waite, K.A., Pilarski, R., Hampel, H., Fernandez, M.J., Bos, C., Dasouki, M., Feldman, G.L., Matloff, E., Ivanovich, J., et al. (2003). Germline *PTEN* promoter mutations and deletions in Cowden/Bannayan-Riley-Ruvalcaba syndrome result in aberrant PTEN protein and dysregulation of the phosphoinositol-3-kinase/Akt pathway. Am. J. Hum. Genet. *73*, 404–411.
5. Nelen, M.R., Padberg, G.W., Peeters, E.A., Lin, A.Y., van den Helm, B., Frants, R.R., Coulon, V., Goldstein, A.M., van Reen, M.M., Easton, D.F., et al. (1996). Localization of the gene for Cowden disease to chromosome 10q22–23. Nat. Genet. *13*, 114–116.
6. Stambolic, V., Tsao, M.S., Macpherson, D., Suzuki, A., Chapman, W.B., and Mak, T.W. (2000). High incidence of breast and endometrial neoplasia resembling human Cowden syndrome in *pten*$^{+/-}$ mice. Cancer Res. *60*, 3605–3611.
7. Eng, C., Kiuru, M., Fernandez, M.J., and Aaltonen, L.A. (2003). A role for mitochondrial enzymes in inherited neoplasia and beyond. Nat. Rev. Cancer *3*, 193–202.
8. Neumann, H.P.H., Brauch, B., McWhinney, S.R., Bender, B.U., Gimm, O., Franke, G., Schipper, J., Klisch, J., Altehöher, C., Zerres, K., et al. (2002). Germ-line mutations in nonsyndromic pheochromocytoma. N. Engl. J. Med. *346*, 1459–1466.
9. Vanharanta, S., Buchta, M., McWhinney, S.R., Virta, S.K., Peczkowska, M., Morrison, C.D., Lehtonen, R., Januszewicz, A., Jarvinen, H., Juhola, M., et al. (2004). Early-onset renal cell carcinoma as a novel extraparaganglial component of SDHB-associated heritable paraganglioma. Am. J. Hum. Genet. *74*, 153–159.
10. Benn, D.E., Gimenez-Roqueplo, A.P., Reilly, J.R., Bertherat, J., Burgess, J., Byth, K., Croxson, M., Dahia, P.L., Elston, M., Gimm, O., et al. (2006). Clinical presentation and penetrance of pheochromocytoma/paraganglioma syndromes. J. Clin. Endocrinol. Metab. *91*, 827–836.
11. Launonen, V., Vierimaa, O., Kiuru, M., Isola, J., Roth, S., Pukkala, E., Sistonen, P., Herva, R., and Aaltonen, L.A. (2001). Inherited susceptibility to uterine leiomyomas and renal cell cancer. Proc. Natl. Acad. Sci. USA *98*, 3387–3392.
12. Zundel, W., Schindler, C., Haas-Kogan, D., Koong, A., Kaper, F., Chen, E., Gottschalk, A.R., Ryan, H.E., Johnson, R.S., Jefferson, A.B., et al. (2000). Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev. *14*, 391–396.
13. Tang, Y., and Eng, C. (2006). p53 downregulates phosphatase and tensin homologue deleted on chromosome ten (PTEN) protein stability partially through caspase-mediated degradation in cells with proteosome dysfunction. Cancer Res. *66*, 736–742.
14. Liaw, D., Marsh, D.J., Li, J., Dahia, P.L., Wang, S.I., Zheng, Z., Bose, S., Call, K.M., Tsou, H.C., Peacocke, M., et al. (1997). Germline mutations of the *PTEN* gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat. Genet. *16*, 64–67.
15. McWhinney, S.R., Pilarski, R.T., Forrester, S.R., Schneider, M.C., Sarquis, M.M., Dias, E.P., and Eng, C. (2004). Large germline deletions of mitochondrial complex II subunits SDHB and SDHD in hereditary paraganglioma. J. Clin. Endocrinol. Metab. *89*, 5694–5699.
16. Mutter, G.L., Lin, M.C., Fitzgerald, J.T., Kum, J.B., Baak, J.P., Lees, J.A., Weng, L.P., and Eng, C. (2000). Altered PTEN expression as a diagnostic marker for the earliest endometrial precancers. J. Natl. Cancer Inst. *92*, 924–930.
17. Weng, L.P., Brown, J.L., Baker, K.M., Ostrowski, M.C., and Eng, C. (2002). PTEN blocks insulin-mediated ETS-2 phosphorylation through MAP kinase, independently of the phosphoinositide 3-kinase pathway. Hum. Mol. Genet. *11*, 1687–1696.
18. Ishii, T., Yasuda, K., Akatsuka, A., Hino, O., Hartman, P.S., and Ishii, N. (2005). A mutation in the SDHC gene of complex II increases oxidative stress, resulting in apoptosis and tumorigenesis. Cancer Res. *65*, 203–209.
19. Slane, B.G., Aykin-Burns, N., Smith, B.J., Kalen, A.L., Goswami, P.C., Domann, F.E., and Spitz, D.R. (2006). Mutation of succinate dehydrogenase subunit C results in increased O2.-, oxidative stress, and genomic instability. Cancer Res. *66*, 7615–7620.
20. Stambolic, V., Suzuki, A., de la Pompa, J.L., Brothers, G.M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J.M., Siderovski, D.P., and Mak, T.W. (1998). Negative regulation of

PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell *95*, 29–39.

21. Chung, J.H., and Eng, C. (2005). Nuclear-cytoplasmic partitioning of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) differentially regulates the cell cycle and apoptosis. Cancer Res. *65*, 8096–8100.
22. Kytola, S., Nord, B., Elder, E.E., Carling, T., Kjellman, M., Cedermark, B., Juhlin, C., Hoog, A., Isola, J., and Larsson, C. (2002). Alterations of the SDHD gene locus in midgut carcinoids, Merkel cell carcinomas, pheochromocytomas, and abdominal paragangliomas. Genes Chromosomes Cancer *34*, 325–332.
23. Perren, A., Barghorn, A., Schmid, S., Saremaslani, P., Roth, J., Heitz, P.U., and Komminoth, P. (2002). Absence of somatic SDHD mutations in sporadic neuroendocrine tumors and detection of two germline variants in paraganglioma patients. Oncogene *21*, 7605–7608.
24. Cascon, A., Ruiz-Llorente, S., Cebrian, A., Leton, R., Telleria, D., Benitez, J., and Robledo, M. (2003). G12S and H50R variations are polymorphisms in the SDHD gene. Genes Chromosomes Cancer *37*, 220–221.
25. Kryukov, G.V., Pennacchio, L.A., and Sunyaev, S.R. (2007). Most rare missense alleles are deleterious in humans: implications for complex disease and association studies. Am. J. Hum. Genet. *80*, 727–739.
26. Baysal, B.E., Ferrell, R.E., Willett-Brozick, J.E., Lawrence, E.C., Myssiorek, D., Bosch, A., van der Mey, A., Taschner, P.E., Rubinstein, W.S., Myers, E.N., et al. (2000). Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma. Science *287*, 848–851.
27. McWhinney, S.R., Pilarski, R., Forrester, S., Schneider, M., Sarquis, M.M., Dias, E.P., and Eng, C. (2004). Large germline deletions of mitochondrial complex II subunits SDHB and SDHD in hereditary paraganglioma. J. Clin. Endocrinol. Metab. *89*, 5694–5699.
28. Koivunen, P., Hirsila, M., Remes, A.M., Hassinen, I.E., Kivirokko, K.I., and Myllyharju, J. (2007). Inhibition of hypoxia-inducible factor (HIF) hydroxylases by citric acid cycle intermediates: Possible links between cell metabolism and stabilization of HIF. J. Biol. Chem. *282*, 4524–4532.
29. Selak, M.A., Armour, S.M., MacKenzie, E.D., Boulahbel, H., Watson, D.G., Mansfield, K.D., Pan, Y., Simon, M.C., Thompson, C.B., and Gottlieb, E. (2005). Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. Cancer Cell *7*, 77–85.
30. Lee, S.R., Yang, K.S., Kwon, J., Lee, C., Jeong, W., and Rhee, S.G. (2002). Reversible inactivation of the tumor suppressor PTEN by H2O2. J. Biol. Chem. *277*, 20336–20342.