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Elevated Plasma Succinate Among *PTEN, SDHB* and *SDHD* Mutation Positive Individuals

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Abstract

Purpose—Cowden Syndrome (CS) results from germline mutations in phosphatase and tensin homologue deleted on chromosome ten (*PTEN*) and from variants in succinate dehydrogenase (SDH) B and D subunits. We hypothesized that succinate accumulation may be common among individuals with *SDH* variants/mutations and those with *PTEN* mutations.

Methods—Urine and blood were collected from individuals meeting full or partial CS diagnostic criteria, those with paraganglioma or a known susceptibility PGL-associated gene mutation and succinate was measured. *PTEN*, *SDHB*, *SDHC*, and *SDHD*-genes were sequenced from genomic DNA.

Results—Elevated plasma succinate was observed in 13/21 (62%) individuals with germline *PTEN*, *SDHB* or *SDHD* mutations compared to 5/32 (16%) controls ($p<0.001$); 10/15 (67%) individuals with pathogenic *PTEN* mutations, but in <20% of mutation negative individuals meeting identical criteria; and among individuals with mutations in *SDHB* (1/1, 100%) and *SDHD* (2/5, 40%).

Conclusions—Our data suggest that mutations in *PTEN, SDHB and SDHD* reduce catalytic activity of SDH and result in succinate accumulation and identify a common biochemical alteration between these two patient populations. Plasma organic acid analysis may provide an effective and inexpensive screening method to determine when more expensive gene sequencing of *PTEN* and *SDH*-genes is warranted.

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Keywords

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INTRODUCTION

Cowden Syndrome (CS, OMIM #158350) is an under-diagnosed, difficult-to-recognise (affecting 1 in 200,000 individuals) autosomal dominant cancer syndrome with high penetrance. It is primarily associated with an increased risk of breast, follicular thyroid and endometrial cancers; however, papillary thyroid cancer and renal cell carcinoma have also been reported.[1–3] Clinical diagnosis of CS is made when an individual meets diagnostic criteria, a combination of pathognomonic major and/or minor criteria, established by the International Cowden Consortium.[1] Many individuals meet partial diagnostic criteria for CS (defined full criteria minus one), and are referred to as CS-like. Germline mutations or deletions in phosphatase and tensin homologue deleted on chromosome ten (*PTEN*, OMIM +601728), a ubiquitously expressed tumour suppressor, have been identified in approximately 25% of individuals with CS, and somatic *PTEN* mutations have been variably observed in a large number of sporadic malignancies.[2, 4]

Germline heterozygous mutations in *SDHB* and *SDHD* have been identified in approximately 10% of *PTEN* mutation negative individuals with CS.[5] *SDHB* and *SDHD* encode the B and D subunits of succinate dehydrogenase (SDH), a Krebs cycle enzyme that catalyzes oxidation of succinate to fumarate and also participates in the electron transport chain (complex II, succinate-ubiquinone oxidoredictase).[5–7]

Similar to Cowden syndrome, female breast cancer, papillary thyroid cancer and renal cell carcinoma have been variably associated with individuals with germline heterozygous mutations in *SDHB* and *SDHD*. [5–7] Like *PTEN*, *SDHx* genes also function as tumour supressors and mutations in these genes result in mitochondrial dysfunction and tumourigenesis via upregulation of angiogenic and hypoxic pathways.[7, 8] Mutations in *SDHA*, *SDHB*, *SDHC*, *SDHD* and succinate dehyrogenase complex assembly factor 2 (*SDHAF2*) underlie most cases of familial paraganglioma (PGL) giving rise to paraganglioma syndromes type 4 (PGL-4, *SDHB*, OMIM 115310), type 3 (PGL-3, *SDHC*, OMIM 605373), type 1 (PGL-1, *SDHD*, OMIM 168000), and type 2 (PGL-2, *SDHAF2*, OMIM 601650) respectively.[9–11] Although paragangliomas and pheochromocytomas both arise from paraganglial cells, paragangliomas are confined to the head and neck and pheochromocytomas are localized to adrenal glands, extraadrenal abdominal and thoracic locations.[6] This is how we have defined these terms, however, we recognize that these definitions may vary depending on group or country. In addition to *SDHA*, *SDHB*, *SDHC*, *SDHD* and *SDHAF2*, four additional genes are associated with the development of hereditary paraganglioma and/or pheochromocytoma, including *RET* (multiple endocrine neoplasia type 2, OMIM 164761), *VHL* (von Hippel-Lindau disease, OMIM 193300), *TMEM127* (transmembrane spanning protein associated with Golgi, endosomes and lysosomes, hypothesized to play a role in protein trafficking and reported to negatively

regulate the TOR signaling pathway, OMIM 171300), and rarely NF1 (neurofibromatosis type 1, OMIM162200).[7, 12]

In addition to their recent association with paraganglioma in its heterozygous state, germline homozygous or compound heterozygous *SDHA* mutations have more commonly been associated with Leigh syndrome (OMIM 256000), a rare neurometabolic disorder. Succinate accumulation has been observed in SDHA mutant fibroblasts and in SDHB mutant tumor tissues[13, 14] and elevated urinary succinate has been associated with, but is not specific to, mitochondrial disorders, hypoxia and seizures. Therefore, our current study was designed to ascertain whether or not common biochemical alterations could be identified in individuals with CS, CS-like and individuals with *SDHx* associated pheochromocytomas and paragangliomas. We hypothesized that elevated succinate could be measured in urine and plasma from patients with *SDHx* mutations and also in individuals with *PTEN* mutations meeting full or partial CS diagnostic criteria. A PubMed literature search failed to identify other studies that evaluated urine and plasma succinate in patients with tumors suggesting that ours is the first such report.

MATERIALS AND METHODS

Participants

Between October 2007 and February 2011, patients identified in the Center for Personalized Genetic Healthcare of the Genomic Medicine Institute at the Cleveland Clinic were recruited for study. Inclusion criteria included patients who met full operational diagnostic criteria for Cowden syndrome, partial criteria (full criteria minus one) for Cowden syndrome (termed Cowden syndrome-like or CSL), or a personal or family history of pheochromocytoma or paraganglioma. Written informed consent was obtained from all participants. The study received ethical approval by the Cleveland Clinic Institutional Review Board for Human Subjects' Protection.

Procedures

Genomic DNA was isolated and *PTEN*, *SDHB*, *SDHC*, and *SDHD* sequencing were performed based on patient phenotype using Light Scanner technology. Multiplex ligation dependent probe amplification (MLPA) was performed to identify *PTEN*, *SDHB*, *SDHC*, and *SDHD* gene duplications or deletions in select mutation negative individuals.

Random urine and/or blood samples were obtained during routine patient visits and de-identified. Urine and plasma were aliquoted and frozen within 1 hour of collection and were stored at −80°C until organic acid analyses were performed. Organic acid concentrations were determined using Gas Chromatography-Mass Spectrometry in the Biochemical Genetics Laboratory of ARUP, Salt Lake City, Utah. This lab was blinded to the mutation status and clinical diagnosis associated with the plasma and urine samples. Measured organic acids in urine included, but were not limited to: lactic acid, pyruvic acid, succinic acid, fumaric acid, 2-ketoglutaric acid, methylmalonic acid, 3-hydroxybutyric acid, acetoacetic acid, 2-keto-3-methylvaleric acid, 2-ketoisocaproic acid, 2-ketoisovaleric acid, ethylmalonic acid, adipic acid, suberic acid, sebacic acid, 4-hydroxyphenylacetic acid, 4-

hydroxyphenyllactic acid, 4-hydroxy-phenylpyruvic acid and succinylacetone. Measured organic acids in plasma included, but were not limited to: lactic acid, pyruvic acid, succinic acid, 3-hydroxybutyric acid, acetoacetic acid, 2-keto-3-methylvaleric acid, 2-ketoisocaproic acid, 2-ketoisovaleric acid, and citric acid. Reference ranges for urine and plasma organic acids were established in an age-matched population by the Biochemical Genetics Laboratory at ARUP. Urinary organic acids were reported as mmol of acid/mole of creatinine and plasma values as μmol/L.

Statistical Analysis

Comparison of frequencies was performed with the Fisher's 2-tailed exact test with $p<0.05$ considered statistically significant.

RESULTS

A total of 66 patients (55 plasma samples; 65 urine samples) were enrolled in the study. All individuals presented to our cancer genetics clinic for an initial visit or follow-up care. Study participants whose phenotypes and family history were consistent with CS or CSL were screened for germline *PTEN*, *SDHB*, *SDHC* or *SDHD* mutations. The majority of *PTEN* mutation negative CS and CSL individuals were also assessed for *PTEN* duplications and deletions. We identified 15 *PTEN* mutation positive individuals who met CS diagnostic criteria (*PTEN* mutation positive), 15 *PTEN* mutation negative individuals who met CS diagnostic criteria (*PTEN* mutation negative, CS), 4 CSL individuals (*PTEN* mutation negative, CSL) and 3 individuals with *PTEN* variants of unknown significance (*PTEN* VUS) (Table 1).

Individuals who presented with paraganglioma, or a family history of a known *SDH* mutation, were screened for germline *SDHB*, *SDHC*, *SDHD* and *PTEN* mutations. Deletions and duplications in these genes were assessed in select mutation negative individuals. In total, we enrolled 1 *SDHB* mutation positive individual, 5 *SDHD* mutation positive individuals, 10 individuals with paraganglioma with no identifiable mutations, duplications or deletions in *SDHB*, *SDHC* or *SDHD* (*SDH* mutation negative, PGL), 1 individual with a known *VHL* mutation, and 1 individual with a known *TMEM127* SNP (Table 1).

Organic acid analyses revealed elevated plasma succinate in 13/21 (62%) individuals with germline mutations in any examined gene compared to 5/32 (16%) mutation negative controls ($p<0.001$). The majority of *PTEN* mutation positive individuals (10/15; 67%) had elevated plasma succinate, this finding was not observed in *PTEN* mutation negative CS individuals (3/15; 20%) or the *PTEN* mutation negative, CSL group (1/4; 25%) or in individuals with *PTEN* VUS (1/3; 33%) (Table 1).

Elevated plasma succinate was recorded in individuals with *SDHB* (1/1; 100%) and *SDHD* mutations (2/5; 40%), and in one individual harboring a *TMEM127* SNP (1/1; 100%). Elevated plasma succinate was not found in *SDH* mutation negative individuals with PGL (0/10; 0%), or in one individual with a mutation in *VHL* (0/1; 0%) (Table 1). Elevated urine succinate was observed in some (6/19, 32%), but not all, individuals with elevated plasma

succinate (Supplemental Digital Content Table 1). No other organic acids in plasma or urine were consistently elevated or decreased for any patient group (Data not shown).

DISCUSSION

This report demonstrates that elevated plasma succinate is a common finding among individuals with known pathogenic mutations in *PTEN*, *SDHB*, *SDHD* and *TMEM127*. Previous studies have reported elevated succinate levels in tumor derived tissue from patients with *SDHB* mutations and in *SDHA* mutant fibroblasts,[13, 14] but to the best of our knowledge this is the first report demonstrating an elevation of succinate in plasma from patients with germline mutations in *SDHB*, *SDHD*, *TMEM127* and *PTEN*. Although elevated plasma succinate levels might be expected for individuals with *SDHx* mutations, it was unexpected for *PTEN* mutation positive individuals and implies that PTEN mutations somehow reduce the catalytic activity of the SDH protein complex.

Consistent with previous studies assessing succinate levels in tumor-derived tissue,[14] more than half of our *SDHD* mutation positive individuals (3/5, 60%) did not demonstrate elevated plasma succinate. Similarly, 33% of *PTEN* mutation positive individuals did not exhibit elevated plasma succinate. Although the reason for this finding is unclear, our data suggest that elevated plasma succinate does not correlate with a specific mutation(s) or phenotype(s) (Supplemental Digital Content Table 1). The conversion of succinate to fumarate, along with several other Krebs cycle enzymatic reactions, is reversible. It is possible some mutations and/or variants impair and/or enhance the reversibility of these reactions thereby reducing succinate to normal levels. This is one possible explanation for the *SDHD* and *PTEN* mutation positive individuals who do not exhibit elevated plasma succinate.

Elevated urine succinate was observed in some, but not all, individuals with elevated plasma succinate. The reason for this inconsistency is not entirely clear, however it likely stems from the fact that the succinate elevations we observed in plasma samples were not severe, but were mild to moderate in nature.

One of three patients harboring *PTEN* polymorphisms exhibited elevated succinate in both plasma and urine. This patient, unlike the other two, met full CS diagnostic criteria. It is conceivable that this intronic variant c.210 −7del5, although currently classified as a polymorphism, may actually be a pathogenic mutation leading to splicing defects.

One plausible explanation for the link between *PTEN* mutations and elevated plasma succinate is PTEN-induced kinase 1 (*PINK1*), a mitochondrial localized serine-threonine kinase, transcriptionally activated by PTEN.[15] Studies of *PINK1* knockout mice showed impaired mitochondrial respiration in striatum, specifically; a significant decrease was seen in the state III activities for complex I and complex II.[15] Because the oxidation of succinate to fumarate is coupled with the reduction of ubiquinone to ubiquinol we expect that a reduction in complex II activities would silmultaneously be associated with a reduction in the activity of succinate dehydrogenase in the Krebs cycle. Therefore, mutations that affect

the stability or activity of PTEN likely affect *PINK1* transcription and downstream function of mitochondrial complex II.

Currently, individuals presenting to genetics clinics who meet CS diagnostic criteria or who present with familial paraganglioma are offered the option of gene sequencing to establish the underlying cause of disease and to provide disease management. Estimated cost for PTEN sequencing, deletion and duplication analysis is approximately US$2,000 per sample.[16] Likewise, cost for *SDHB*, *SDHC*, and *SDHD* mutation analyses are approximately $1,000, $1,300 and $700, respectively. Clinical MLPA analysis for *SDHB*, *SDHC*, and *SDHD* is approximately $550. In contrast, plasma organic acid analysis is a relatively low-cost assay. The cost of plasma organic acid analysis is approximately $230.00 per sample.[16] It is likely that the cost under contract to a medical institute would be even less. Therefore, based upon our finding that a large proportion of individuals with pathogenic *PTEN*, *SDHB* and *SDHD* mutations exhibit elevated plasma succinate, we suggest that plasma organic acid analysis may be a useful and cost-effective preliminary screening tool to identify individuals for which more costly gene sequencing is warranted.

In conclusion, we have demonstrated that elevated plasma succinate is a common biochemical disturbance in the majority of *PTEN*, *SDHB* and *SDHD* mutation positive individuals and provides a plausible biochemical link for the shared phenotypic findings across these groups. Furthermore, screening for elevated succinate provides a rapid, inexpensive analytical tool to identify individuals who are likely to harbor a germline mutation in *PTEN*, *SDHB* or *SDHD*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CSL	Cowden syndrome-like
PTEN	phosphatase and tensin homologue deleted on chromosome ten
SNP	single nucleotide polymorphism
SDHB	succinate dehydrogenase B subunit
SDHC	succinate dehydrogenase C subunit
SDHD	succinate dehydrogenase D subunit
SNP	single nucleotide polymorphism

TMEM127	transmembrane protein 127
VHL	von Hippel-Lindau

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Table 1

Summary of elevated urine and plasma succinate data

Classification	Patient IDs	Patients (plasma), n	Elevated plasma succinate, n (%)	Patients (urine), n	Elevated urine succinate, n (%)
***PTEN* mutation positive**	21–44	15	10 (67)	24	4(17)
***PTEN* mutation negative, CS**	1–16	15	3 (20)	16	3 (19)
***PTEN* mutation negative, CSL**	17–20	4	1 (25)	4	0 (0)
***PTEN* VUS**	45–47	3	1 (33)	2	1 (50)
***SDH* mutation negative, PGL**	48–58	10	0 (0)	11	2 (18)
***SDHB* mutation positive**	59	1	1 (100)	1	0 (0)
***SDHD* mutation positive**	60–64	5	2 (40)	5	1 (20)
***TMEM127* SNP**	65	1	1 (100)	1	0 (0)
***VHL* mutation positive**	66	1	0 (0)	1	0 (0)
Total number study participants		55		65	