Abstract

Purpose: Cowden syndrome (CS), a Mendelian autosomal-dominant disorder, predisposes to breast, thyroid, and other cancers. Germline variations in succinate dehydrogenase genes (SDHx) occur in approximately 10% PTEN mutation-negative CS and CS-like (CSL) individuals (SDHxmut+). We previously showed that SDHx variants result in elevated reactive oxygen species (ROS), disruption of nicotinamide adenine dinucleotide (NAD) equilibrium, and destabilization of p53 hence apoptosis resistance in CS/CSL patient-derived lymphoblastoid cells. In the present study, we sought to address the tumorigenic impacts of increased ROS and the potential of protecting SDHxmut+ cells with antioxidants.

Experimental Design: We measured the lipid peroxidation levels in patient-derived SDHxmut+ lymphoblastoid cells and sequenced 74 controls or SDHxmut+ germline DNA samples for mitochondrial hypervariable region II (HVRII) polymorphisms. SDHxmut+ lymphoblastoid cells were treated with various antioxidants to check p53 expression and sub-G1 cell population with cell-cycle analysis.

Results: We showed that elevated ROS results in higher lipid peroxidation in SDHxmut+ cells. Accumulation of polymorphisms in mitochondrial HVRII was observed in SDHxmut+ samples. Interestingly, α-tocopherol (vitamin E) treatment, but not other antioxidants, rescued SDHxmut+ cells from apoptosis resistance and protected SDHxmut+ cells from oxidative damage such as decreased lipid peroxidation as well as partially recovered p53 expression and NAD/NADH levels.

Conclusions: We conclude that disruption of complex II because of SDHx variants leads to increased ROS generation, specifically accompanied by lipid peroxidation. The lipid soluble antioxidant α-tocopherol can selectively protect SDHxmut+ cells from oxidative damage, apoptosis resistance, and rebalance redox metabolites NAD/NADH. Clin Cancer Res; 18(18); 4954–61. ©2012 AACR.

Introduction

Cowden syndrome [CS, (MIM 158350)] is an autosomal dominant disorder with lifetime risks of up to 85% for developing female breast cancer, 35% for epithelial thyroid cancer, and increased risks of developing other cancers (1). Germline mutations in the phosphatase and tensin homolog deleted on chromosome 10 tumor suppressor gene [PTEN (MIM 601728)] are found in 25% of patients with classic CS accrued from the community (2). When individuals have features of CS but do not meet these criteria, they are referred to as CS-like (CSL) and necessarily represent a heterogeneous series. Only up to 5% of CSL individuals have germline PTEN mutations (2, 3). Other than PTEN, we have recently uncovered alternative mechanisms, germline hypermethylation of the tumor suppressor gene KLLN (encoding KILLIN) and germline variants in succinate dehydrogenase (SDH) genes SDHB-D, accounting for approximately 35% and approximately 10%, respectively, of PTEN mutation negative CS/CSL (4, 5). Germline KLLN hypermethylation is associated with increased prevalence of breast and renal cancers, whereas SDHB-D variants show increased prevalence of breast and thyroid cancers, over those with PTEN mutations.

Mitochondrial respiratory enzyme SDH (or complex II) is involved in both electron transport and the Krebs tricarboxylic-acid cycle, catalyzing FAD-dependent oxidation of succinate to fumarate. Germline homozygous or compound heterozygous mutations in mitochondrial complex
Genes, including SDH, result in Leigh syndrome, a rare but fatal neurodegenerative disease. Germline heterozygous SDHB/C/D mutations result in hereditary pheochromocytoma–paraganglioma (PCC/PGL) syndrome (6–9). Functionally, we discovered that SDHx variants leads to increased ROS generation, specifically accompanied by lipid peroxidation. The lipid-soluble antioxidant \( \alpha \)-tocopherol functions as a lipid peroxyl radical scavenger protecting \( SDHx \) cells from oxidative damage, apoptosis resistance, and rebalance redox metabolites NAD/NADH. Our study supports the notion that \( \alpha \)-tocopherol may be useful as a therapeutic adjunct or preventative agent, especially for individuals with germline \( SDHx \) variants/mutations or cancers with somatic mutations in complex II.

**Translational Relevance**

Whereas the involvement of germline \( SDHx \) mutations is well known in hereditary pheochromocytomas/paragangliomas, the role of impaired SDH in other tumors is still unclear. We reported that \( SDHx \) variants could be important risk factors for breast cancer or thyroid cancer in CS/CSL. Our data suggest that disruption of complex II because of \( SDHx \) variants leads to increased ROS generation, specifically accompanied by lipid peroxidation. The lipid-soluble antioxidant \( \alpha \)-tocopherol functions as a lipid peroxyl radical scavenger protecting \( SDHx \) cells from oxidative damage, apoptosis resistance, and rebalance redox metabolites NAD/NADH. Our study supports the notion that \( \alpha \)-tocopherol may be useful as a therapeutic adjunct or preventative agent, especially for individuals with germline \( SDHx \) variants/mutations or cancers with somatic mutations in complex II.

**Materials and Methods**

**Research participants**

Patients with CS or CSL 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 (Supplemental Table S1) or the relaxed criteria (criteria minus 1) according to version 2006 NCCN Guidelines (17). 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 1 or 2 criteria short of the operational diagnostic criteria. Matching the subjects, normal (population) controls are from Northern and Western European origin and were anonymized before storage and analysis.

Germline \( SDHx \) variants were detected in both PTEN mutation-negative and PTEN mutation-positive CS/CSL individuals as we reported previously (5). The updated \( SDHx \) variant lists in both patient subsets are summarized in Supplemental Table S2.

**Mitochondrial mutation 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). PCR amplification and direct sequencing (ABI3730xl) of mitochondrial hypervariable region II were carried out with primer L163405'-AGCCATTACGCTACATGCA-3' and H4085'-TGTTAAAAGTGCATAACCGCA-3'. Revised Cambridge Reference Sequence (NC_012920.1) was used as reference mitochondrial sequence.

**Cell lines and cell cultures**

Human immortalized lymphoblastoid cell lines (LCL) derived from patients and normal healthy 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% fetal bovine serum (FBS) and 100 units/mL each of Penicillin and Streptomycin. All cell lines were cultured at 37°C with 5% CO2. NQO1 inducer dimethyl fumarate (DMF), dimethyl sulfoxide (DMSO), N-acetyl-cysteine (NAC), ascorbic acid, and \( \alpha \)-tocopherol (Sigma-Aldrich Co) were added into cell culture at different doses as described in figure legend.

**Protein analysis**

Whole-cell lysates were prepared as described previously (18) with M-PER Mammalian Protein Extraction Reagent (ThermoFisher Scientific) supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma-Aldrich). Lysates were either separated by SDS-PAGE and transferred to nitrocellulose (BioRad). The resulting blots were subjected to Western blot analysis for PTEN (6H2.1, Cascade Bioscience), NQO1, p53 (Santa Cruz Biotechnology), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cell Signaling), and \( \alpha \)-tubulin (Sigma-Aldrich) protein levels.
ROS and lipid peroxidation measurement

The measurement of ROS was conducted using carboxy-dichlorodihydrofluorescein diacetate (Carboxy-H2DCFDA), a reliable fluorogenic marker for ROS in live cells (Molecular Probes, Invitrogen). The cells were washed with HBSS/Ga/Mg buffer, centrifuged, resuspended in HBSS/Ga/Mg and incubated with 25 μmol/L carboxy-H2DCFDA for 30 minutes at 37°C. Hoechst 33342 was added at a final concentration of 1 μmol/L to the carboxy-H2DCFDA staining solution during the last 5 minutes of the incubation.

For flow cytometry measurement, cells were washed and resuspended in HBSS/Ga/Mg buffer after incubation and counted with FACScans (Becton-Dickinson) immediately.

The measurement of lipid peroxidation was conducted using the Lipid Peroxidation Microplate Assay Kit (Oxford Biomedical Research) according to the manufacturer’s protocol to measure malondialdehyde (MDA) and 4-hydroxyalkenals (HAE), the products upon decomposition of polyunsaturated fatty acid (PUFA) peroxides. In brief, cells were washed and lysed with ice-cold 20 mmol/L PBS with 5 mmol/L butylated hydroxytoluene (BHT) to prevent sample oxidation during preparation. Lysed protein were incubated with Reagent R1 and 37% HCl (for MDA only) or R2 (for MDA + HAE) for 60 minutes at 45°C. The incubated products were then read at 586 nm wavelength to calculate the concentration based on the standard curve and normalized to the protein concentration measured using separate protein aliquots.

Cell-cycle analysis by FACS flow cytometry

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

NAD/H/NADH quantification

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 2 cycles (20 minutes on dry ice, then 10 minutes at room temperature). Extracted samples were filtered through 10 Kd molecular weight cut off filters (BioVison #1997-25) to remove enzymes consuming NADH before conducting 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 minutes to decompose NAD before incubating with NAD cycling mix. Duplicated samples were then mixed with NADH developer and incubate at room temperature for 1 to 4 hours before colorimetric reading at OD450 nm. The amount of NAD in samples was calculated by subtracting NADH from NADt.

Statistical analysis

The results are presented as means ± Standard Errors of the Mean (S.E.M.) of the indicated number of samples in each mutation/variant group. Significant differences (P < 0.05) were evaluated with 2-tailed Student t test.

Results

Increased lipid peroxidation in SDHx variant–positive cells

We have previously reported that the cellular ROS is significantly increased in patient with CS/CSL samples harboring SDHx variants compared with normal controls. To investigate if the elevated ROS generated can cause damage to lipids such as lipid peroxidation, we measured the byproducts of PUFA peroxides upon decomposition—MDA and HAE, in lymphoblastoid cells derived from normal control and patients with SDHx variant carrier. Compared with controls, CS/CSL samples with SDHx variants showed 2.1-fold increase in lipid peroxidation, whereas patients with both PTEN mutations and SDHx variants had the highest level, 2.6-fold increase, which correlates with overall ROS increase patterns in these 3 groups as we noted previously (5). Interestingly, CS/CSL patients with PTEN pathogenic truncation mutations (R130X or R335X) also had 3.7-fold increased lipid peroxidation compared with controls. As positive controls, paraganglioma patients with SDHx pathogenic mutations were included and had the highest levels of lipid peroxidation among all 5 groups, a mean 6.3-fold increase (Fig. 1).
Accumulated mitochondrial polymorphisms in SDHx variant–positive cells

Exposure of cells to high levels of ROS leads to oxidative stress and may cause DNA damage, especially to the fragile mitochondrial genome that lacks DNA repair machinery and has greatest proximity to the mitochondrial electron transport chain (19). To explore if SDHx variants may accumulate more mitochondrial polymorphisms, 10 control genomic DNA samples and 64 SDHx variant–positive genomic DNA samples (39 with SDHx variant alone, 25 with both PTEN and SDHx variant) were sequenced for mitochondrial hypervariable region II (HVRII), which is the most polymorphic region in the human mitochondrial genome. In total, 38 different alterations were found in both controls and patients screened, 30 of which were unique in cases not seen in controls (Supplemental Table S3). Out of these 38 polymorphisms, 34 are reported in Mitomap database (http://www.mitomap.org/bin/view.pl/MITOMAP/PolymorphismsControl), whereas 4 are novel and only presented in patients with SDHx variant positive. Compared with controls (median = 3, range 2–5), we observed significantly more genetic polymorphisms in both SDHx variant carriers (median = 5 polymorphisms/patient, range 2–8; \(P = 0.004\)) and PTEN/SDHx double variant carriers (median = 4, range 2–7; \(P = 0.011\); Fig. 2).

\(\alpha\)-Tocopherol, but not other antioxidants, rescues p53 loss and apoptosis resistance in SDHx variant–positive cells

We previously showed the loss of steady state p53 expression in SDHx variants previously (5), because of reduced NQO1 and p53 interaction, most likely caused by disrupted mitochondrial metabolism. To test if ROS relief by antioxidant treatment could reverse the apoptosis-resistance phenotype, we treated both control and SDHx variant–positive cells with various antioxidants and measured p53 protein expression and percentage of cells in the sub-G1 phase of the cell cycle. Treating cells with the most direct water-soluble antioxidant NAC induced dose-dependent increase of NQO1 expression, but not p53 in both control and SDHx variant (Fig. 3A). Cell-cycle analysis did not reveal significant changes in cell number in the sub-G1 phase after treatment (Fig. 3B). When we tested water-soluble vitamin C (ascorbic acid) and lipid-soluble vitamin E (\(\alpha\)-tocopherol) exposures, interestingly, neither of these 2 vitamins had any effect on NQO1 expression, unlike NAC or DMF. Only lipid-soluble \(\alpha\)-tocopherol, but not vitamin C, treatment recovered p53 expression in SDHx variant–positive cells (Fig. 3C and D). Correlating with the relative levels of p53 expression, only \(\alpha\)-tocopherol completely rescued SDHx variant–positive cells from apoptosis resistance to the level of control cells (Fig. 3E).

\(\alpha\)-Tocopherol protects SDHx variant–positive cells from redox imbalance

Because \(\alpha\)-tocopherol is lipid-soluble, which is the most distinguishable characteristic compared with the other antioxidants we tested, we hypothesized that \(\alpha\)-tocopherol may directly protect cells from ROS damage, specifically lipid peroxidation. As shown in Fig. 4A, \(\alpha\)-tocopherol treatment significantly reduced overall ROS levels in SDHx variant cells. As we expected, lipid peroxidation in CS/CSL patients’ cells with SDHx variation was also reduced, as well as in 2 positive control groups, namely CS/CSL patients’ cells with PTEN pathogenic mutations and PGL patients’ cells with SDHx pathogenic mutations (Fig. 4B). To further investigate whether \(\alpha\)-tocopherol is involved in NAD metabolic regulation, we measured NAD/NADH levels after \(\alpha\)-tocopherol...
treatment. As shown in Fig. 5A, α-tocopherol treatment had no additional effect on the NAD/NADH ratio in control cells. In contrast, α-tocopherol treatment led to complete recovery of this NAD/NADH reduction back to normal levels. This was true despite DMSO treatment being associated with a significant decrease of the NAD/NADH ratio in SDHx variant-positive cells. Western blot showed slight increases of p53 expression in SDHx variant–positive samples compared with controls after treatment, which was accompanied by decreased pAKT and Phosphorylation of mitogen-activated protein kinases (pMAPK; Fig. 5B).

Discussion

Mitochondrial dysfunction has long been observed in cancer cells, known as the Warburg effect (20). Decades of research implicated the important roles of mitochondrial abnormalities contributing to tumorigenesis, including mitochondrial DNA mutations, oxidative stress, loss of p53, and aberrant expression of metabolic enzymes. Many studies have shown that cancer cells tend to have elevated levels of ROS, compared with normal cells (21). Even though the exact mechanisms of the ROS generation in cancer cells are not clear, we and others proposed mitochondrial dysfunction, such as mitochondrial mutations or the imbalance of redox system, as one of the plausible reasons (5, 10, 22, 23).

Among all the targets ROS could impact, lipid peroxidation is particularly harmful because it facilitates the propagation of free radical reactions. Lipid peroxidation has been reported in numerous human cancers (24–26). We previously presented data that lymphoblastoid cells derived from CS/CSL patient with SDHx variants showed elevated ROS compared with the controls (5, 10). In this study, we further proved that as a consequence of ROS on PUFA, lipid peroxidation was also elevated accordingly in these SDHx variant–positive cells. ROS is not a prominent cellular phenotype with PTEN mutations alone (Ni and Eng, unpublished data). However, germline mutations within the ATP-binding motifs of PTEN showed enhanced ROS production especially with cellular senescence (11), suggesting that only certain PTEN mutations may have similar cellular phenotypes as those with SDH variant which may explain the observed increased lipid peroxidation in the PTEN mutation–positive group. Thus, we believe that the increased oxidative stress is not a universal cellular phenotype in PTEN mutation carriers, as we observed in SDH mutation/variant carrier cells. The fact that the PCL patients with SDHx pathogenic mutations presented with...
extremely high lipid peroxidation suggests that complex II abnormalities had a more severe impact on membrane redox homeostasis, notably mitochondrial membranes. The most abundant aldehydes identified as products of PUFA decomposition upon lipid peroxidation are 4-HNE and MDA. Other than being used as a measure of lipid peroxidation status, both of them form adducts with DNA and are mutagens (27, 28). As the most direct target of mitochondrial free radicals, the mitochondrial genome is prone to DNA damage, as we observed in SDHx variants cells that accumulated more mitochondrial DNA mutations than controls.

We previously showed the NQO1 inducer DMF increased NQO1 protein expression in a dose-dependent manner, without any impact on p53 expression and cell apoptosis resistance (5). These data are consistent with our previous report that the p53 degradation is enhanced by loss of interaction with NQO1 but not loss of absolute NQO1 expression. The most interesting, at first puzzling, observation was that only vitamin E treatment, specifically \( \alpha \)-tocopherol, rescued the cell apoptosis resistance phenotype, but others such as vitamin C and NAC had no effect.

Given that \( \alpha \)-tocopherol is lipid-soluble compared with the other 2, which are water-soluble, we suspect that protection of cells from lipid peroxidation could play a critical role. Vitamin E is a well-known antioxidant which functions as a peroxyl radical scavenger, and has been reported to be colocalized to the mitochondrial membrane (29). Indeed, our data showed that \( \alpha \)-tocopherol treatment not only inhibited overall ROS generation but also reduced lipid peroxidation in SDHx variant–positive cells, thus protecting cells from oxidative damages. Redox-silent analog of vitamin E such as \( \alpha \)-tocopheryl succinate (\( \alpha \)-TOS) has been reported to specifically target ubiquinone-binding sites in the SDH complex (30, 31) and causes rapid production of ROS in cancer cells triggering apoptosis. However, in our case, the existing complex II abnormality may silence or even saturate the response to \( \alpha \)-TOS, whereas antioxidant \( \alpha \)-tocopherol works by a completely distinct mechanism from \( \alpha \)-TOS. We and others reported that altered metabolic intermediates such as FAD and NAD/NADH could inactivate PTEN/PI3K pathways with elevated phosphorylation.
of AKT and MAPK (5, 32, 33). Recovered NAD/NADH ratio upon α-tocopherol treatment in SDHx variants cells suggest that the protection from α-tocopherol may function even at the redox metabolite level. The inhibition of AKT/MAPK activation corroborates that the intervention of α-tocopherol on PTEN/P13K signaling. Therefore, we think α-tocopherol may have intriguing clinical implications such as utility as a potential preventive adjunct for CS/CSL individuals with SDHx variants. In addition to all the preclinical and epidemiology evidence of its antican-


cer property, supplemental vitamin E for prostate cancer prevention has also been investigated by several clinical trials, such as the α-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) and Selenium and Vitamin E Cancer Prevention Trial (SELECT). Compared with a 32% prostate cancer risk reduction by α-tocopherol in the ATBC study, SELECT did not show any preventive effectiveness but paradoxically had higher risks of prostate cancer with vitamin E supplementation (34–36). However, the dose (400 IU/d) used in the SELECT trial is 8-fold higher than that in ATBC, with the latter the dosage we chose to use in this current study, which speaks to dosage effect. Indeed, treating SDHx variant–positive cells with 800 μmol/L (8-fold higher dose) α-tocopherol showed the opposite effect, that is, further reduction of p53 expression and even slight activation of AKT and MAPK pathways (Sup-


plementary Fig. S1).


In summary, we report that disruption of complex II because of SDHx variants leads to increased ROS generation, specifically accompanied by lipid peroxidation. The lipid soluble antioxidant α-tocopherol can protect SDHx variant–positive cells from oxidative damage, apoptosis resistance, and rebalance redox metabolites NAD/NADH. The protective effect from α-tocopherol most likely is because of its lipid peroxyl radical scav-

ger property preventing ROS damage and PTEN inactivation. Our study supports the notion that α-tocopherol may be useful as a therapeutic adjunct or preventative agent, especially for individuals with germ-


l ine SDHx variants/mutations or cancers with somatic mutations in complex II.


Disclosure of Potential Conflicts of Interest

C. Eng is co-PI of a sponsored research agreement from IntegraGen for autism genomic markers, receives royalties from Quest Diagnostics for test to differentiate benign from malignant thyroid neoplasia, is an unpaid member of the External Scientific Advisory Boards of Ecoros.com and of GenomOncology, and is an unpaid member of the Genomic Medicine Advisory Board of Complete Genomics, Inc. No potential conflicts of interest were disclosed by the other author.


Authors’ Contributions

Conception and design: Y. Ni, C. Eng

Development of methodology: Y. Ni

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Ni, C. Eng

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Ni, C. Eng

Writing, review, and/or revision of the manuscript: Y. Ni, C. Eng

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Eng

Study supervision: C. Eng

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References


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