Ethylmalonic Encephalopathy 1 Protein Is Increased in Colorectal Adenocarcinoma

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Abstract. *Background/Aim:* Ethylmalonic encephalopathy 1 protein (ETHE1) plays an important role in sulfide catabolism and polysulfide formation. As sulfides and polysulfides have recently been identified as playing important roles in cancer, we hypothesized that ETHE1 expression would be increased in colon cancer. *Materials and Methods:* We used tissue microarray analysis to compare ETHE1 expression in benign colonic epithelium compared to colonic adenocarcinoma. In total, 26 benign colonic epithelial samples were compared to 122 cases of colonic adenocarcinomas. *Results:* Compared to benign colonic epithelium, ETHE1 expression was significantly increased (~two-fold) in colonic adenocarcinoma. Additionally, this expression increased with increasing colon cancer tumor grades. *Conclusion:* ETHE1 expression is increased in colon cancer compared to benign colonic epithelium. These data, combined with previous studies, suggest that ETHE1 may contribute to colon carcinogenesis by promoting tumor cell bioenergetics and polysulfide formation.

Ethylmalonic encephalopathy 1 protein (ETHE1), also known as sulfur dioxygenase, is located on chromosome 19q13 and encodes a metallo β -lactamase family enzyme containing a mononuclear iron center (1-4). Loss-of-function ETHE1 mutations are associated with ethylmalonic encephalopathy,

a rare infantile autosomal recessive metabolic disorder affecting the brain, gastrointestinal tract, and peripheral vessels, characterized by psychomotor regression with generalized hypotonia, evolving into tetraparesis, dystonia, and subsequent global neurological failure and death in the first decade (3-5). Patients with ethylmalonic encephalopathy exhibit lactic acidemia, elevated urine ethylmalonic and methylsuccinic acids, and low skeletal muscle mitochondrial respiratory complex IV activity (3-5).

ETHE1 functions in the mitochondrial matrix oxidizing sulfides to sulfate or sulfate esters, and taurine, through the sequential actions of sulfide: quinone oxidoreductase, ETHE1, and rhodanese (6). Specifically, ETHE1 detoxifies/oxidizes hydrogen sulfide (H₂S) and glutathione polysulfides (glutathione-persulfide, -trisulfide, and -tetrasulfide), but not cysteine- or homocysteine- persulfides/polysulfides (6-8). Interestingly, ETHE1 also exhibits cysteine protein polysulfidation activities. ETHE1 persulfide dioxygenase and polysulfidation activities are dependent on Cys247 polysulfidation and replacement of the Cys247 with serine ablates both enzymatic activities (8). The enzymes that synthesize H₂S, cystathionine β -synthase (CBS), cystathionine γ -lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST), also function in polysulfide synthesis; suggesting that these enzymes and ETHE1 function together to regulate cellular polysulfide levels (8-10).

Recently, one or more of the enzymes that synthesize H₂S have been found to be increased in many different human malignancies, with one study of oral squamous cell carcinoma also showing significantly increased tumor H₂S levels (11-13). Additionally, there is recent evidence that polysulfides also play a role in cancer promotion (12). Based on this, we hypothesized that ETHE1 may be increased in cancer. Herein we used tissue microarray analysis (TMA) to examine ETHE1 expression in benign colonic epithelium compared to colonic adenocarcinoma.

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Materials and Methods

Tissue microarray (TMA). The TMA CO1921 was purchased from US Biomax, Inc. (Rockville, MD, USA). The TMA contained 32 benign colon biopsies and 159 cases of colonic adenocarcinoma. The TMA was interrogated by an ETHE1 antibody. All tissue samples in the TMAs were 1.0 mm in diameter.

ETHE1 immunohistochemistry (IHC). The concentration of the primary ETHE1 antibody was optimized to normal kidney as a positive control tissue. The negative control was the same tissue interrogated without the primary ETHE1 antibody. The staining of the TMA was performed at the Tissue Core Histology Lab Facility at the Moffitt Cancer Center. The microarray slides were stained using a Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ, USA) as per the manufacturer's protocol with proprietary reagents. Briefly, the slide was deparaffinized on the automated system with EZ Prep solution (Ventana Medical Systems). The heat-induced antigen retrieval method was used in Cell Conditioning (Ventana Medical Systems). Rabbit monoclonal antibody to human ETHE1 (rabbit anti-ETHE1: EPR11697 ab174302, Abcam, Cambridge, MA) was used at a 1:1,000 concentration in Dako antibody diluent (Dako, Carpinteria, CA, USA) and incubated for 60 min. Ventana anti-rabbit secondary antibodies were used for 16 min. The detection system used was the Ventana OmniMap kit. Slides were then dehydrated and coverslipped per standard protocol.

Evaluation of staining. Relative ETHE1 protein expression was determined as average sample immunostain intensity scored on a 0 to 3 scale as follows: no staining: 0, light staining: 1, moderate staining: 2, and heavy staining: 3. The percentage of cells stained was measured, with no detectable staining as 0, 1-33% as 1, 34-66% as 2, and 67-100% as 3. The final IHC score was the product of the percentage of cells stained multiplied by the intensity score, allowing for a minimal score of 0 and a maximal score of 9. Cytoplasmic ETHE1 staining was seen in all tissue samples examined, although at low to moderate levels in benign colonic epithelium. The cytoplasmic staining showed a punctate pattern, suggestive of a mitochondrial distribution. Based on this, ETHE1 staining in the cytoplasmic compartment was measured and quantified.

Statistical analysis of TMA results. The standard error of the mean (SEM) IHC score was calculated by using the standard deviation for the staining scores of each tumor type and dividing this number by the square root of the sample size (14).

Results

Following IHC processing, 6 of the 32 samples of benign colonic epithelium were lost. In the colonic adenocarcinoma samples, 37 were not counted, leaving 122 cases. Of those not counted, some were lost in processing, while cases of signet ring cell or mucinous adenocarcinomas, or colonic adenocarcinomas graded with more than one grade were also excluded from the study. Of the remaining colonic adenocarcinomas 19 were grade I, 89 were grade II, and 14 were grade III. The number of cases examined for ETHE1 staining, the quantified IHC results, and the SEM of each

Table I. *Relative ethylmalonic encephalopathy 1 protein immunohistochemical staining in benign colonic epithelium and colonic adenocarcinoma. ETHE1, Ethylmalonic encephalopathy 1 protein; IHC, immunohistochemistry; SD, standard deviation; SEM, standard error to the mean.*

Tissue	Number of cases	ETHE1 staining	SD	SEM
Benign colon	26	2.96	0.19	0.039
All colonic adenocarcinomas	122	5.67	2.02	0.17
Grade I	19	5.02	2.35	0.53
Grade II	89	5.49	1.80	0.39
Grade III	14	6.39	2.03	0.22

data set are given in Table I. Examples of ETHE1 IHC are shown in Figure 1.

ETHE1 exhibited strong apical localization in benign colonic epithelium, with lower expression deeper in the colonic epithelia. Staining in the colonic adenocarcinoma was uniform and moderately increased compared to benign colonic adenocarcinoma, as previously found (15). Overall, 38.40% of the colonic adenocarcinoma samples scored 3 by the analysis employed here, 53.90% scored 6, and 17.73% scored 9. When ETHE1 expression in grades I-III adenocarcinoma was compared, a slight to moderate increase in expression was seen with increasing tumor grades (Table I and Figure 1).

Discussion

ETHE1 plays a significant role in H₂S metabolism and protein polysulfide formation, suggesting that it might play a role in cancer (8-15). Previously, ETHE1 was found to be increased in the sera of early lung cancer patients by protein serologic array analyses of 352 lung cancer cases, compared to 93 healthy samples, and 101 patients with benign pulmonary lesions (16). Similarly, ETHE1 expression was increased in 6 of 6 colorectal cancer cell lines compared to a nonmalignant colonic cell line, and in 5 of 7 resected colorectal adenocarcinoma cases, compared to adjacent benign colonic epithelium (15). Interestingly, in this study ETHE1 expression in benign epithelium showed strong apical (luminal) localization, with lower expression deeper in the lamina, while in colonic adenocarcinoma the expression was higher and uniform (15). Our work herein replicates these findings. Additionally, ETHE1 was over-expressed, roughly 27-fold, in phenotypically normal colonic epithelial cells from individuals with familial adenomatous polyposis, compared to normal colonic epithelial cells (17). Elevated ETHE1 expression in two colon cancer cell lines also increased aerobic glycolysis (the Warburg effect), oxidative phosphorylation, and mitochondrial biogenesis. Interestingly, higher ETHE1 expression lowered

Figure 1. Representative ethylmalonic encephalopathy 1 protein immunostaining of benign colonic epithelium (A), Grade I adenocarcinoma (B), seminoma (C), Grade II adenocarcinoma (D), and (E). Grade III adenocarcinoma. Original magnification $\times 100$.

cellular H₂S concentrations (measured by lower glutathione persulfide levels), resulting in increased phosphodiesterase activity (PDE), and adenosine monophosphate-activated protein kinase (AMPKp), sirtuin1 (SIRT1), and peroxisome proliferation-activated receptor γ -coactivator-1 α activation (PGC1 α , 18). In a murine xenograft model, high ETHE1 expression also accelerated colon cancer cell line growth, with increased Ki-67 and CD31 expression, reflecting increased cell growth with concomitant intensified tumor angiogenesis (18).

Conversely, a comparison of twenty colonic adenocarcinomas to patient-matched benign tissues revealed lower ETHE1 mRNA and protein expression in malignant tissues compared to benign (19). Additionally, a meta-analysis

revealed suppressed colonic adenocarcinoma ETHE1 expression compared to benign colonic epithelium. This event was hypothesized to allow the malignant cells to more efficiently produce energy under hypoxic conditions (20).

The reason for these discrepancies may lie in several factors. First, TMA studies do not usually compare patient-matched tissues, while studies that do may show more exact resolution of benign/malignant expression differences. Also, different analytical techniques and sample sizes may give different results. Herein we analyzed a relatively large number of colonic adenocarcinoma samples and found moderate ETHE1 induction compared to benign colonic epithelium. Interestingly, in 38% of the colon adenocarcinomas examined

ETHE1 IHC was roughly the same as that seen in benign tissue, with 54% showing moderately increased expression, and only 18% showing high ETHE1 expression. Additionally, Libiad *et al.* (15), found increased ETHE1 expression in 5 of 7 matched benign colon epithelium and matched colonic adenocarcinoma cases, with two cases showing high and equal ETHE1 expression between benign and malignant colonic tissues. Taken together, these data indicate that ETHE1 expression is on average moderately induced in colon adenocarcinoma, with the degree of induction showing some variation. Further support for ETHE1 being increased in colonic malignancy comes for our observation that ETHE1 expression increases with increasing tumor grades.

Polysulfide formation has been implicated in several malignancies. For example, polysulfides are higher in high-grade gliomas compared to glioma-free areas of the brain, while brain hemispheres with glioblastoma show higher polysulfides than the contra-lateral hemispheres without glioblastoma (21, 22). Similarly, surface-enhanced Raman spectroscopy of 186 stage III or IV ovarian cancers indicated that higher CSE expression correlated with increased tumor polysulfides, tumor cisplatin resistance, and a poor prognosis. Cisplatin resistance was found to be related to polysulfide synthesis (23).

Herein we found that ETHE1 expression is increased in colonic adenocarcinomas. Previously ETHE1 was found to catabolize cellular H₂S while synthesizing polysulfides, and also stimulate aerobic glycolysis, mitochondrial biogenesis, and PDE, AMPK α , and PGC1 α activities, while potentiating tumor growth and angiogenesis (15, 18). These data suggest that ETHE1 contributes to colon carcinogenesis, in part, by modulating tumor cell bioenergetics via the conversion of H₂S into polysulfides, contributing to colon cancer carcinogenesis and growth. Further studies on the roles of polysulfides in cancer and cancer cell biogenetics will be needed to clarify this hypothesis. Lastly, the finding that ETHE1 expression shows a strong apical localization and lower expression in underlying benign colonic epithelium, while colon adenocarcinoma shows strong and uniform ETHE1 expression, suggests that these expression differences may be used in IHC analyses to identify colon cancer in conjunction with histologic analysis and other IHC stains (15).

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

All Authors contributed to the writing of the manuscript. Drs. Shackelford and Kevil also performed the literature searches. Dr. Ozluk also took the photographs.

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