



Altered Expression of Autophagy-related Genes in Human Colon Cancer

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Abstract

Background and objectives: Autophagy is a physiologic mechanism, which utilizes the self-digestion of cell organelles to promote cellular homeostasis, such as in the setting of dysfunctional cellular components, cellular stress or energy-deprived states. *In vitro* studies have pointed toward the key role of autophagy in colorectal cancer. However, *in vivo* studies from human colorectal cancer tissues are lacking.

Methods: We collected tissue samples from six patients with colon cancer who received curative surgery at Baylor College of Medicine. We also obtained normal colonic mucosa biopsy from five unrelated polyp-free individuals who were matched to cases individually by age, sex, ethnicity, and colon segment. Total RNA was successfully extracted from fresh frozen tissue biopsies of five tumor tissues and five unrelated normal tissues. We tested the expression levels of 84 genes in a predefined autophagy pathway using the RT² Profiler PCR array. We compared differences using Student's *t*-test. The false-discovery rate was used for multiple testing adjustment. We also used the TCGA dataset to validate our findings.

Results: We observed significant differential expression between colon cancer tissue and normal colon mucosa for 29 genes in the autophagy pathway ($p < 0.05$). After multiple testing adjustment, the expression of 17 genes was significantly down-regulated, with fold-change greater than 2 in colon cancer; these included *ATG4A*, *ATG4C*, *ATG4D*, and *CTSS* ($q < 0.10$). The down-regulation was observed in both early and late stage colon cancer. We observed the same down-regulation of multiple autophagy-related genes using the TCGA data. The *ATG9B* gene was the only statistically non-significantly up-regulated gene after multiple testing adjustment.

Conclusions: This pilot study showed the down-regulation of multiple autophagy pathway genes in human colon cancer, corroborating the increasing clinical relevance of autophagy in human colorectal carcinogenesis. This preliminary finding should be validated in larger studies.

Keywords: Autophagy; Gene expression; Colorectal cancer; ATG4; Cathepsin.

Abbreviations: ATG, autophagy-related genes; CD, Crohn's disease; CRC, colorectal cancer; FC, fold change; FR, fold regulation; FDR, false discovery rate; IHC, immunohistochemical; SNP, single nucleotide polymorphism.

Received: May 18, 2018; Revised: June 25, 2018; Accepted: July 06, 2018

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How to cite this article: Gurwara S, Kourkoumpetis T, Chen L, Chen F, Graham DY, Eissa NT, Ittmann M, El-Serag HB, Jiao L. Altered Expression of Autophagy-related Genes in Human Colon Cancer. *Exploratory Research and Hypothesis in Medicine* 2018;3(3):47–53. doi: 10.14218/ERHM.2018.00007.

Introduction

Colorectal cancer (CRC) is one of the major causes of death worldwide. The carcinogenic processes of CRC involve complex mechanisms. Autophagy is a physiologic mechanism that utilizes self-digestion of cell organelles to promote cellular homeostasis in the setting of dysfunctional cellular components, cellular stress or energy-deprived states. An extensive network of proteins coordinates the process of autophagy.¹

Autophagy can play an instrumental role in the pathogenesis

of CRC.¹ Autophagy reduces carcinogenesis and acts as a tumor suppressor by removing damaged organelles and reducing reactive oxygen species and DNA damage, and by promoting autophagic cell death. However, in established tumors, autophagy promotes cancer growth by providing tumor cells access to nutrients for growth and metabolism as well as increasing drug resistance and ensuring the maintenance of the cancer stem cell.² Autophagy-related genes (ATGs) play an integral role in the complex cellular pathway of autophagy. Thirty-six such genes have been identified to date.³ Early clinical trials have shown the potential benefit of inhibiting parts of the autophagy pathway in many other cancers, including pancreatic cancer, multiple myeloma, and melanoma.³ Novel clinical biomarkers are being discovered via ongoing clinical studies to monitor autophagy in patients.⁴

However, little is known about the expression of autophagy genes in human colon cancer.^{5,6} In this study, we compared the expression levels of ATG between normal and colon cancer tissues using a predefined autophagy pathway array. We hypothesized that the genes involved in autophagy were differentially expressed in colon tumor tissue versus normal control tissues.

Methods

Study participants

The study population and research approaches were described previously.⁷ Our study was conducted at the affiliated hospitals of Baylor College of Medicine (BCM) and involved six patients who were diagnosed with colon cancer and subsequently underwent total curative surgical excision during the years of 2007–2013. None received pre-operative neoadjuvant chemotherapy. The resected tumor tissues and normal adjacent mucosa samples were snap-frozen in liquid nitrogen and stored in -80°C . Additionally, we acquired colon mucosa samples from five unrelated patients who underwent a colonoscopy and were shown to have normal colons endoscopically at the Michael E. DeBakey Veterans Affairs Medical Center (MEDVAMC). These samples served as the control samples when the RNA quality of normal adjacent samples was not adequate for gene expression analysis. The tumor and its unrelated control tissue were matched in relation to colonic segment, race/ethnicity, age (± 5 years), and sex. All patients provided informed consent to participate in a research and use their samples. The Institutional Review Boards of MEDVAMC and BCM approved the study protocol.

RNA extraction and gene expression analysis

In the genomic core lab at the Texas Medical Center Digestive Disease Center, using the NucleoSpin RNA isolation kit (MACH-EREY-NAGEL Inc, Bethlehem, PA, USA), we obtained total RNA from 17 fresh frozen tissue biopsy samples (six biopsies from excised cancerous tissues, six biopsies from adjacent normal colon mucosa, and five from unrelated normal control mucosa). The RNA purity of samples was confirmed with a nucleic acid 260:280 ratio, which was above 2.0 for all. Nonetheless, for one tumor sample and five normal adjacent mucosa samples, the RNA integrity number (RIN) was < 6.0 . These samples were ultimately excluded from the experiment as they were deemed not appropriate for gene expression analysis. Thus, our analysis was restricted to the samples from five mucosal specimens with colon cancer and five biopsies from matched unrelated healthy controls.⁷

We utilized the Qiagen RT² Profiler PCR Array (Valencia, CA, USA) to determine the expression of 84 key genes in the autophagy pathway (primer sequence is available upon request from Qiagen). The array includes genes encoding proteins for autophagic vacuole formation (*AMBRA1* (*NYW1*), *ATG12*, *ATG16L1*, *ATG4A*, *ATG4B*, *ATG4C*, *ATG4D*, *ATG5*, *ATG9A*, *ATG9B*, *BECN1*, *GABARAP*, *GABARAPL1*, *GABARAPL2*, *IRGM*, *MAP1LC3A*, *MAP1LC3B*, *RGS19*, *ULK1*, *WIPI1*), vacuole targeting proteins (*ATG4A*, *ATG4B*, *ATG4C*, *ATG4D*, *GABARAP*), transport proteins (*ATG10*, *ATG16L1*, *ATG16L2*, *ATG3*, *ATG4A*, *ATG4B*, *ATG4C*, *ATG4D*, *ATG7*, *ATG9A*, *GABARAP*, *GABARAPL2*, *RAB24*), autophagosome-lysosome linkage proteins (*DRAM1*, *GABARAP*, *LAMP1*, *NPC1*), ubiquitination proteins (*ATG3*, *ATG7*, *HDAC6*), and proteases (*ATG4A*, *ATG4B*, *ATG4C*, *ATG4D*). At the core facility at BCM, the assays were conducted using the Bio-Rad Lightcycler RealTime PCR system (Bio-Rad, Hercules, CA, USA).

Analysis of mRNA microarray data from TCGA

We also downloaded mRNA expression data of 244 TCGA (The Cancer Genome Atlas) colon adenocarcinoma and rectum adenocarcinoma (COAD/READ) patients on June 14, 2018 (www.firebrowse.org). This dataset included 22 normal samples and 222 CRC tissues. Using these data, we examined mRNA expression levels of *ATG4A*, *ATG4B*, *ATG4C*, *ATG4D*, *GAA*, *GABARAP*, *CTSD*, and *CTSS* genes in the CRC tissues versus the control tissues. The sequencing platform of the mRNA expression data was UNC_IlluminaHiSeq RNASeqV2.

Statistical analysis

The RT² Profiler PCR Array data analysis version 3.5 was utilized for data analysis. The gene expression levels were normalized to *ACTB*, *GAPDH*, and *RPLP0*. The Student's *t*-test was used to compare the expression levels between diverse groups (overall cancer versus control; early- or late-stage cancer versus control) utilizing the normalized gene expression data. Fold-change (FC)/fold-regulation (FR) was calculated using the $\Delta\Delta\text{Ct}$ method (wherein ΔCt was calculated between a gene of interest and an average of reference housekeeping genes, after that the $\Delta\Delta\text{Ct}$ calculation was performed: ΔCt (Test Group) — ΔCt (Control Group)). Subsequently, we calculated FC using the $2^{\Delta\Delta\text{Ct}}$ formula.

To visualize gene expression changes, we used a scatter plot to compare the normalized expression of every gene on the array between the tumor and normal tissues. On the plot, we used the middle line to denote unchanged gene expression. The selected FR threshold was denoted by the dotted lines above or below the middle line. For multiple testing for 84 genes, we adjusted it using the false discovery rate (FDR). An FDR *q* value < 0.10 denoted statistical significance.⁷ For the downloaded TCGA data, we performed the Student's *t*-test comparing CRC tissue with normal tissue.

Results

The five cancer patients with high-quality RNA included in this analysis were all non-Hispanic Caucasian males between 55 and 75 years old, with a mean age of 62 years. Three patients had early-stage tumors, and three patients had late-stage tumors. The characteristics of these study participants have been described.⁷

We observed significant down-regulation of 29 genes in the autophagy pathway ($p < 0.05$) in colon cancer tissues compared to

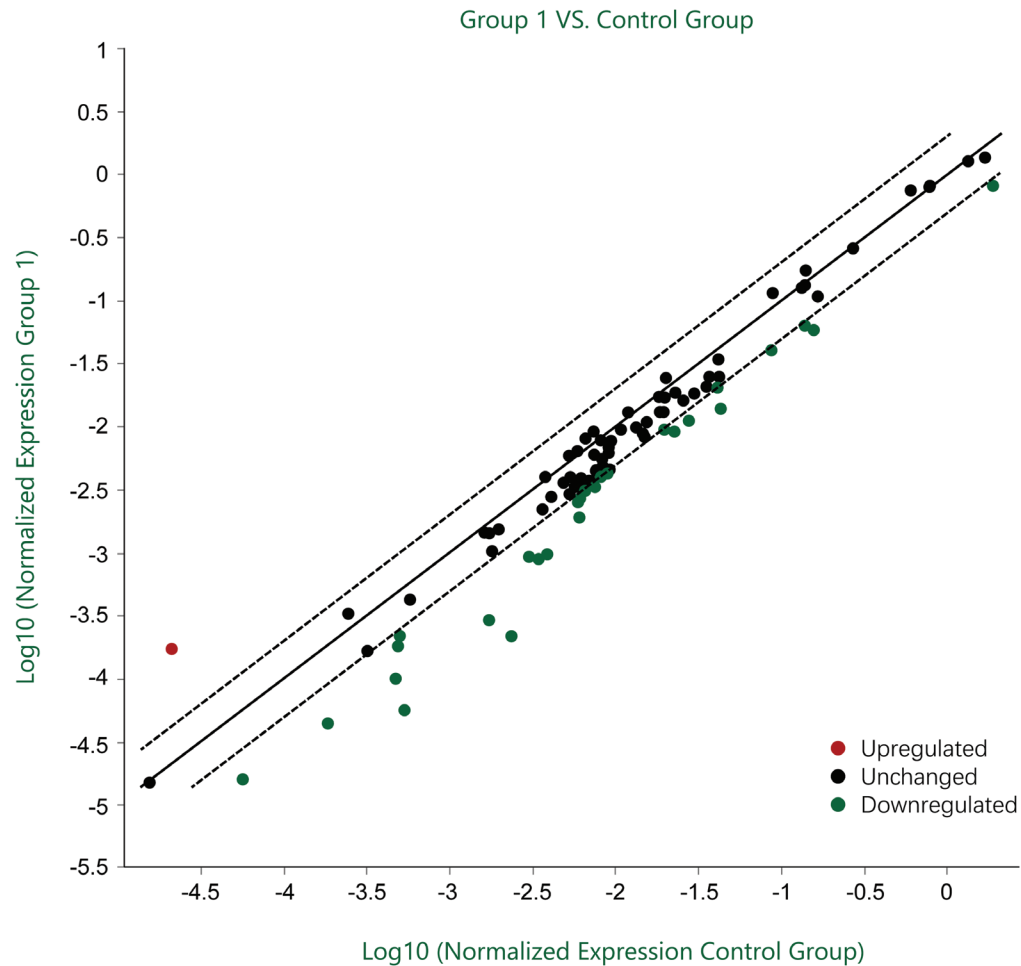


Fig. 1. Scatterplot of autophagy gene expression among colon cancer patients and controls. The dotted lines indicate the selected fold-regulation threshold. Data points beyond the dotted lines in the upper left and lower right sections meet the selected fold-regulation threshold. The figure was generated from the RT² Profiler PCR Array Data Analysis Software, version 3.5.

the normal colonic mucosa (Fig. 1, Supplemental Table S1). The down-regulations were observed for both early- and late-stage tumors. Furthermore, after adjusting for multiple testing, the difference of 17 genes remained statistically significant. The FR was greater than 2 for all 17 genes, including *ATG4A*, *ATG4C*, *ATG4D*, *CTSD*, *CTSS*, *ESR1*, *GAA*, and *GABARAP* (FDR $q < 0.10$; Table 1). *ATG9B* was the only ATG gene that was up-regulated in cancer tissue compared with control tissue (FC=8.39, $p=0.06$). However, the up-regulation was statistically nonsignificant after multiple testing adjustment. The differential expression was consistently observed in early- or late-stage tumors (Table 1).

By comparing CRC tissue with normal tissue using the TCGA data, we also found significant down-regulation of *ATG4A*, *ATG4C*, *ATG4D*, *GAA*, *GABARAP*, *CTSD*, and *CTSS* (Fig. 2).

Discussion

This pilot study suggested differential expression of a number of genes in the autophagy pathway in both early- and late-stage colon cancer compared with normal colonic tissues. Down-regulation of ATG in colon cancer may comprise the host's response in prevent-

ing the accumulation of genetic defects that accompany malignant transformations and therefore promote tumor progression. The analysis based on the TCGA data validated our major findings.

We found that multiple ATGs were down-regulated in colon cancer compared to normal mucosa after accounting for multiple testing, including *ATG4A*, *ATG4C*, and *ATG4D*. *ATG4* genes play multiple roles in autophagy, including autophagic vacuole formation and targeting, and proteases. In yeast, the Atg4-family cysteine proteases play a crucial role in preparing Atg8 for conjugation to lipid membranes and for the deconjugation of Atg8 from the autophagosomes.⁸ There are eight human Atg8 homologues that belong to two subfamilies: LC3 and GABARAP. Atg4a is known to be a potent protease for the GABARAP family but not the LC3 family.⁹ In our study, the *GABARAP* gene was also significantly down-regulated. Contrary to our results, a recent study on gastric cancer reported the higher expression of *ATG4A* in cancer tissues and metastatic lymph nodes than normal tissues. That study also showed that *ATG4A* up-regulated the Notch signaling pathway-induced metastasis in gastric cancer cells.¹⁰

Of all the ATG4 proteases, *ATG4B* has been shown to be the most functionally dominant in autophagy regulation in mammals.¹¹ Liu *et al.*¹² showed that knock-down of the *ATG4B* in CRC

Table 1. Differential expression of selected autophagy genes in human colon cancer tissues and control tissues

	Fold-regulation, All tumor	<i>p</i>	<i>q</i>	Fold-regulation, Early tumor	<i>p</i>	Fold-regulation, Late tumor	<i>p</i>
<i>PIK3CG</i>	-10.58	0.01	0.08	-7.97	0.09	-14.06	0.07
<i>IGF1</i>	-5.84	0.0009	0.08	-2.39	0.09	-14.27	0.04
<i>TNF</i>	-4.59	0.01	0.08	-4.1	0.07	-5.14	0.05
<i>ESR1</i>	-4.05	0.01	0.08	-3.18	0.08	-5.15	0.05
<i>BCL2</i>	-3.89	0.01	0.08	-3.21	0.12	-4.7	0.03
<i>RGS19</i>	-3.19	0.007	0.08	-2.65	0.07	-3.83	0.04
<i>GAA</i>	-3.11	0.01	0.08	-4.39	0.02	-2.21	0.11
<i>TNFSF10</i>	-3.05	0.01	0.08	-2.12	0.12	-4.39	0.04
<i>CTSD</i>	-2.63	0.00085	0.07	-2.08	0.02	-3.31	0.01
<i>PTEN</i>	-2.43	0.01	0.08	-2.67	0.06	-2.2	0.07
<i>ATG4A</i>	-2.25	0.02	0.09	-2.31	0.09	-2.2	0.08
<i>ATG4C</i>	-2.31	0.01	0.10	-2.40	0.07	-2.22	0.06
<i>ATG4D</i>	-2.22	0.01	0.08	-2.14	0.1	-2.3	0.05
<i>CTSS</i>	-2.16	0.01	0.08	-1.78	0.11	-2.64	0.05
<i>GABARAP</i>	-2.12	0.0003	0.07	-2.09	0.03	-2.15	0.04
<i>CASP3</i>	-2.05	0.02	0.08	-1.58	0.17	-2.65	0.04
<i>TGFB1</i>	-2.01	0.01	0.08	-1.92	0.08	-2.1	0.03
<i>ATG9B</i>	8.39	0.06	0.16	18	0.01	3.91	0.22

cell lines induced autophagic flux and reduced cyclin D1 expression to inhibit G1/S phase transition of the cell cycle. However, in our research, the expression of *ATG4B* was statistically non-

significantly down-regulated in tumors, with only a small FC. *ATG4C* gene knock-out in mice has been shown to aid in forming methylcholanthrene-induced fibrosarcomas, suggesting a regu-

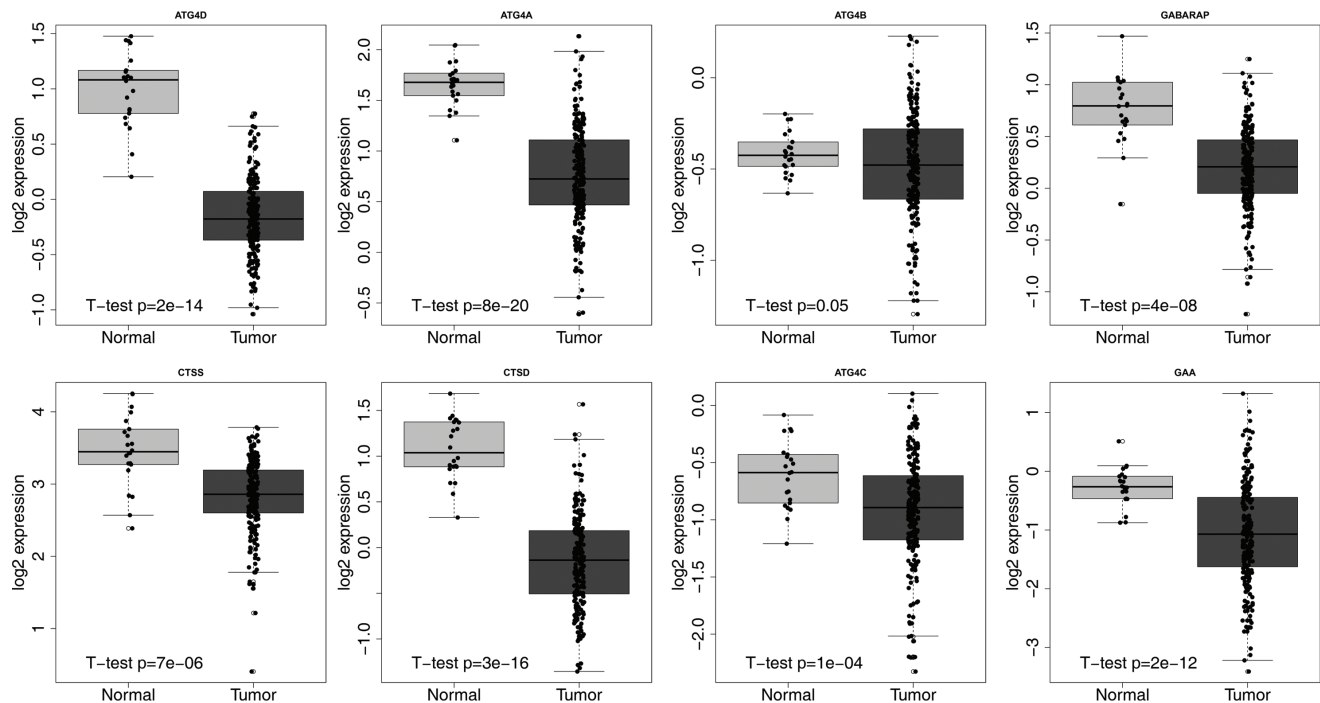


Fig. 2. Box-plot of log2 expression levels of *ATG4* genes, *GABARAP*, *CTSS*, *CTSD* and *GAA* between normal tissue and colorectal cancer tissue, according to the TCGA (The Cancer Genome Atlas) data.

latory impact on tumor cells.¹³ We found the *ATG4C* gene was down-regulated significantly, as was the *ATG4D* gene. In line with our finding, it has been shown that the expression of the *ATG4D* gene was significantly lower in breast cancer tissues than that in the adjacent normal tissues.¹⁴ The identification of diminished *ATG4* gene expression in colon cancer suggested that the formation of autophagosomes may be compromised in colon cancer. In our study, *ATG9B* was the only gene to be highly up-regulated with an FC of 16. However, the difference between colon cancer and normal mucosa was not statistically significant.

Cathepsin is an aspartic endoprotease that is distributed in all lysosomes in the human body.¹⁵ Its primary function is to disassemble proteins and activate cascades in prelysosomal compartments.¹⁶ Cathepsin D (*CTSD*) and cathepsin S (*CTSS*) genes were significantly down-regulated in colon cancer compared to normal mucosa after accounting for multiple testing. Contrary results were reported by Sebzda *et al.*,¹⁷ which showed increased expression of *CTSD* in colon cancer compared to adjacent normal colon mucosa utilizing immunohistochemical (IHC) staining in 68 patients from Poland. Another study also utilizing IHC testing showed that cathepsins B, L, and D activities were higher in a series of malignant tissues compared with adjacent normal colorectal tissues.¹⁸ In our study, *CTSS* was also significantly down-regulated, even after accounting for multiple testing. Our data were incongruous to the prior studies possibly in part because we used mRNA levels to measure gene expression, whereas the prior studies used IHC or enzyme-linked immunosorbent assay. In addition, the function of autophagy genes in cancer development may be tissue-specific.

The research on autophagy and CRC was triggered by a genome-wide association study that identified an association between the single nucleotide polymorphism (SNP) rs2241880 of *ATG16L1* and risk of inflammatory bowel disease.¹⁹ Hampe *et al.*¹⁹ showed a statistically significantly positive association between this SNP and Crohn's disease in a German population. This SNP was also associated with an improved overall survival in CRC.²⁰ However, we did not find the expression of the *ATG16L1* to be associated with colon cancer in our sample.

BECN1 (*Bectin 1*), *LC3B* (*MAP1LC3B*), and *p62* (*SQSTM1*) are three genes that have been widely reported in CRC research. They are expressed in normal colon mucosa as well.²¹ We found *LC3B* and *BECN1* nonsignificantly down-regulated and *p62* up-regulated in cancer tissues. The loss of expression of *BECN1* has been reported for ovarian and esophageal cancers.^{22,23} Contrarily, prior studies showed mRNA and protein expression of *BECN1* or *LC3* were higher in CRC tissue compared with normal adjacent tissue.^{24–26} *p62* is one of five autophagy cargo adaptor molecules that can interact with processed *LC3* at the autophagosome. *p62* accumulation has been observed in liver cancer progression.²⁷ An IHC study also showed that higher *p62* expression was an independent adverse prognostic marker in patients with CRC.²⁸ *p62* and *LC3* also contribute to selective autophagy.^{29,30} For example, mitophagy is a selective mode of autophagy in which mitochondria are targeted for degradation.³¹ Mitophagy affects cell stemness, cell fate determination, inflammation, and DNA damage, and has also been implicated in cancer.³² It is likely that these genes can influence colon tumorigenesis through regulating mitophagy.³³

BECN1 and *PTEN* are important in inducing autophagy in cancer. *PTEN* is a tumor suppressor gene in colon cancer and was down-regulated in our research.³⁴ The down-regulation of autophagy inducers was in line with the overall down-regulation of autophagy pathway genes in colon cancer. A cross-talk exists between apoptosis and autophagy. *BECN1* is also a direct substrate of caspase 3. Several genes related to apoptosis, including *CAPS3*

and *BCL2*, were also down-regulated in colon cancer in our study.

This was a preliminary study with multiple limitations. First, it was based on a small sample size and, thus, false negative and false positive findings could have occurred. The stratified analysis by tumor stage was exploratory given a limited sample size. Furthermore, transcription factor EB (TFEB) is probably one of the most important transcription regulators for autophagy and lysosome-related genes. TFEB could modulate the expression of a broad range of lysosomal genes, including *CTSD* and *CSTS*.³⁵ TFEB over-expression has been seen in a variety of cancers.^{36–38} However, this gene was not included in the predefined panel in the present study. Lastly, one study shows that clinical implications of autophagy biomarkers, such as *LC3* and *p62*, in CRC prognosis may depend on K-ras mutation status.³⁹ As this was a preliminary study, we did not ascertain K-ras mutation status or evaluate protein expression.

In summary, the findings from our study support the tumor suppressor role of autophagy in colon cancer. In particular, the *ATG4* proteases may play an important role in colon cancer development. The precise mechanisms inhibiting or leading to autophagy, and the downstream effectors of autophagy in carcinogenesis need to be fully elucidated in order to identify novel biomarkers for prognosis and target for cancer treatment.

Future research directions

We found that multiple autophagy-related genes were down-regulated in colon cancer tissues. We will validate our findings using larger clinical sample series and by including IHC staining. We will also identify modifiable factors and metabolic stress factors that can contribute to macroautophagy and mitochondria autophagy dysregulation in colon cancer.

Acknowledgments

This research was supported by Gillson Longenbaugh Foundation and Golfers Against Cancer Organization (PI: LJ), Cancer Prevention Research Institute of Texas (RP#140767, PI: LJ), and National Cancer Institute P30 Cancer Center (P30 CA125123) for support of the Human Tissue Acquisition and Pathology Shared Resources. This project was also supported in part by Public Health Service (grant P30DK056338), the expert assistance of Lisa D. White and the Specimen Collection Core E of Texas Medical Center Digestive Diseases Center, and Houston VA HSR&D Center for Innovations in Quality, Effectiveness and Safety (CIN13-413). Finally, we are thankful for Dr. Lang Rao's comments on this manuscript.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study design (LJ), sample acquisition (MI, HBE), data analysis (LC, FC), data interpretation and manuscript writing (SG, TK, DY, MI, NTE, HBE, LJ), critical intellectual input (SG, TK, LC, MI, NTE, HBE, LJ).

Supporting information

Supplementary material for this article is available at <https://doi.org/10.14218/ERHM.2018.00007>.

Supplemental Table S1. Differential expression of all autophagy genes in human colon cancer tissue and normal control tissues.

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