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Prognostic impact of Beclin 1, p62/sequestosome 1 and LC3 protein expression in colon carcinomas from patients receiving 5-fluorouracil as adjuvant chemotherapy

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Autophagy is a cellular degradation process that can be activated in tumor cells to confer stress tolerance. During autophagy initiation and autophagosome formation, Beclin 1 binds microtubule-associated protein-1 light chain 3 (LC3I) that is converted to its membrane-bound form (LC3II) and interacts with the ubiquitin-binding protein p62/sequestosome 1 (SQSTM1). We determined the association of Beclin 1, LC3 and p62 protein expression with clinical outcome in resected stage II and III colon carcinomas (n = 178) from participants in 5-fluororuacil (5-FU)-based adjuvant therapy trials. The immunopercentage for each marker was determined and dichotomized for analysis with overall survival (OS) using Cox models. We found that autophagy markers localized to the tumor cell cytoplasm and showed increased expression relative to normal epithelial cells. Overexpression of Beclin 1, LC3 and p62 proteins were detected in 69, 79 and 85% of tumors, respectively. Expression levels were not significantly associated with clinicopathological variables. In a multivariable analysis adjusting for tumor grade, stage and patient age, Beclin 1 overexpression was independently associated with worse OS [hazard ratio (HR), 1.82; 95% confidence interval (CI), 1.0–3.3; p = 0.042] in patients who received 5-FU-based adjuvant therapy. Neither LC3 nor p62 overexpression was prognostic. In conclusion, Beclin 1 overexpression was associated with reduced survival in colon cancer patients treated with adjuvant 5-FU. These data are consistent with preclinical evidence indicating that autophagy can protect colon cancer cells from 5-FU and support the targeting of autophagy for therapeutic advantage in this malignancy.

Introduction

Colorectal cancer (CRC) is the third most common cancer in the United States and is second only to lung cancer as a cause of cancer-related mortality.[1] 5-fluorouracil (5-FU) remains the most active single agent used in CRC treatment. However, acquired resistance generally develops during therapy that leads to transient responses and eventual disease progression.[2] The role of autophagy in conferring resistance to cellular stress and in maintaining tumor cell survival has recently been recognized.[3] Autophagy is a homeostatic and catabolic process whereby cytoplasmic proteins and organelles are sequestered within autophagosomes and degraded in lysosomes to sustain cellular metabolism.[4] Autophagy is induced in tumor cells to maintain survival in a setting of stress due to increased metabolic demands, a hypoxic microenvironment, or cytotoxic agents.[3,5-9] Studies have shown that inhibition of autophagy in tumor cells can enhance chemotherapy-induced cell death, thus establishing autophagy as a therapeutic target.[3,7,10-13]

Autophagy is regulated by autophagy specific genes (Atg) that include *Beclin 1*, the human homolog of *Atg6* in yeast, which forms a complex with a class III phosphoinositide 3-kinase (PI3K), vacuolar sorting protein 34 (Vps34), that is required for autophagosome formation.[14] *Beclin 1* is also a haplo-insufficient tumor suppressor gene in that *Beclin 1* heterozygous mice are tumor-prone,[15,16] and monoallelic loss of *Beclin 1* has been found in human breast, ovarian and prostate cancers.[6,15,17] Excessive stimulation of autophagy due to Beclin 1 overexpression has been shown to inhibit tumor development.[18] In colon cancer cell lines, we found that knockdown of *Beclin 1* or *LC3* by lentiviral shRNA can enhance apoptosis induction by 5-FU, whereas ectopic expression of *Beclin 1* confered cytoprotection.[7] While Beclin 1 functions in the induction of autophagy, the BH3 domain of Beclin 1 is bound to and inhibited by anti-apoptotic Bcl-2 or Bcl-XL proteins that can thereby disrupt autophagy.[19]

During autophagy initiation, autophagosome formation is associated with the conversion of cytosolic-associated protein

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light chain 3 (LC3-I), also known as Atg8, to the membrane-bound LC3-II form.[20] LC3 then binds to the adaptor protein p62 sequestosome 1 (SQSTM1) (herein referred to as p62) which facilitates the autophagic degradation of ubiquitinated protein aggregates in lysosomes.[21] Defective or impaired autophagy is associated with accumulation of p62.[8] p62 is a multi-domain protein that is degraded by autophagy and is implicated in the activation of the transcription factor NFκB.[22] Both p62 and LC3 are routinely used as biomarkers to monitor the level of autophagy.[23] Evidence suggests that p62 can serve as a link between autophagy and the extrinsic apoptotic pathway in that caspase-8 self-association/activation was shown to be dependent upon p62.[24,25] In a prior study, we found that ectopic *p62* expression can enhance 5-FU-induced apoptosis, whereas *p62* knockdown or functional *p62* mutants was shown to protect colon cancer cells from 5-FU-induced apoptosis and DNA damage.[7]

Given data suggesting that key autophagy regulatory proteins may predict chemoresistance in human cancers, we hypothesized that elevated expression levels of Beclin 1 or LC3 are associated with poor clinical outcome after 5-FU-based adjuvant chemotherapy whereas overexpression of p62 may reflect autophagy inhibition with increased susceptibility to chemotherapy-induced cell death. To address our hypothesis, we analyzed the association of Beclin 1, LC3 and p62 expression with patient survival in surgically resected, primary stage II and III colon carcinomas from participants in randomized clinical trials of 5-FU-based adjuvant chemotherapy.

Results

Study population and tumor characteristics (Table 1). The median age of the patient population was 63.5 y (range, 26.0–81.0) and 99 of 178 (55.6%) colon cancer patients were male. Of the 178 primary colonic adenocarcinomas studied, 32 (18%) were TNM stage II and 146 (82%) were stage III. There were 94 (52.8%) distal and 84 (47.2%) proximal cancers defined relative to the splenic flexure. Histologic grade was categorized as well/moderate differentiation in 119 (66.9%) and poor/undifferentiated in 59 (33.1%) tumors.

Expression of autophagy markers in colon cancer tissues. The expression of Beclin 1, p62 and LC3 proteins was increased in tumor cells relative to normal-appearing and adjacent colonic mucosa in all cases (**Fig. 1**). In carcinomas, the percent tumor cell positivity for each marker was dichotomized into high vs. low groups using an arbitrarily determined cutoff of 50%. A high level of expression Beclin 1 expression (> 50% tumor cell positivity) was detected in 114 of 166 (69%) tumors (**Fig. 2**). For p62 and LC3, high expression was observed in 145 of 171 (85%) and in 119 of 1551 (79%) tumors, respectively (**Fig. 2**). Within tumors, expression of each autophagy marker was primarily localized to the tumor cell cytoplasm (**Fig. 1**). For Beclin1, some perinuclear membrane staining was also observed and infrequent nuclear staining for p62 and LC3 were detected. A statistically significant yet weak correlation was found between Beclin 1 and p62 or LC3. Specifically, the level of Beclin 1 expression was inversely correlated with the level of p62 and positively correlated with LC3 (Spearman correlation coefficients of -0.22 and 0.26, respectively) (both $p < 0.01$). Low levels of Beclin 1 may reflect decreased autophagy with an accumulation of p62 whereas high Beclin 1 levels are consistent with autophagy activation with p62 turnover via autophagy-mediated degradation.[26] The level of p62/ SQSTM 1 expression was not correlated with that of LC3 (correlation coefficient, -0.07; $p = 0.41$).

Table 1. Characteristics of the study population (n = 178)

Study	
NCCTG 89-46-51	53 (29.8%)
NCCTG 91-46-53	125 (70.2%)
Age	
Mean (SD)	62.2 (10.6)
Median and range	63.5 (26.0–81.0)
Gender	
Women	79 (44.4%)
Men	99 (55.6%)
Race	
Caucasian	156 (87.6%)
African American	3 (1.7%)
Native American	2 (1.1%)
Hispanic	1 (0.6%)
Asian	1 (0.6%)
Other	15 (8.4%)
Stage	
Stage II	32 (18%)
Stage III	146 (82%)
Histologic Grade	
Grade ½	119 (66.9%)
Grade ¾	59 (33.1%)
Tumor site	
Distal	94 (52.8%)
Proximal	84 (47.2%)

Autophagy markers and clinical outcome. We determined the association of autophagy marker expression levels with clinicopathological variables and patient survival rates in stage II and III colon cancers from patients treated with 5-FU as adjuvant therapy. The expression levels of Beclin 1, p62 or LC3 were not significantly associated with clinicopathological variables including tumor stage, histologic grade, primary site, or patient age or sex (data not shown). In a univariate analysis, tumor stage and histologic grade were each significantly associated with patient overall survival (OS) (**Table 2**). High vs. low levels of Beclin1 expression in tumors was associated with reduced OS [hazard ratio (HR), 1.78; 95% confidence interval (CI), 0.98–3.24; $p = 0.0540$] (**Table 2**; **Fig. 3A**). Neither p62 nor LC3 expression levels were associated with clinical outcome (**Table 2**; **Fig. 3B and C**). None of the autophagy markers showed a significant interaction with stage for OS (all $p > 0.14$), suggesting that the effects of these markers are similar across stage II and III patients. After adjusting for tumor stage, grade and patient age and stratifying by study

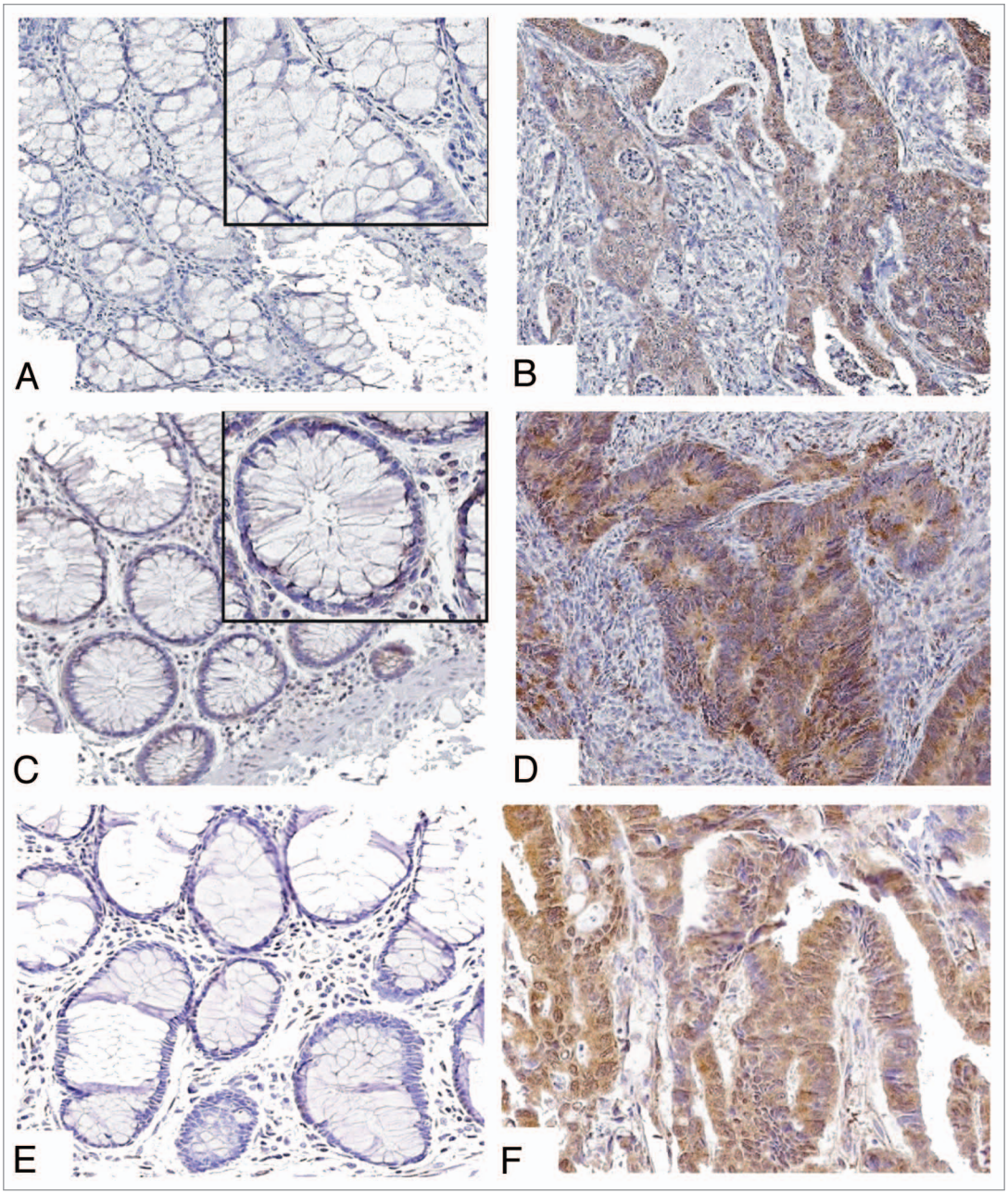

Figure 1. Immunohistochemical staining of Beclin 1, p62/SQSTM 1 and LC3 in human colon carcinomas. (**A and B**) Representative tissue sections show absent Beclin 1 expression in normal colonic crypt epithelial cells (**A**) compared with carcinoma (**B**) where Beclin 1 is localized to the tumor cell cytoplasm (20×). (**C and D**) Expression of p62 can be detected in normal epithelial cells (**C**) and is diffusely expressed in tumor cells (**D**) (20×). (**E and F**) LC3 staining is absent in normal epithelia (**E**), but diffusely expressed in cancer (**F**) (20×).

in a multivariable analysis, we found that a high vs. low level of Beclin1 expression was associated with a statistically significant reduction in OS (HR, 1.82; 95% CI, 0.99–3.32; p = 0.0421) in patients treated with adjuvant 5-FU (**Table 3**).

Discussion

Autophagy confers stress tolerance that enables tumor cells to survive under adverse conditions that include increased metabolic demands, hypoxia, or cytotoxic stimuli.[5,7,27,28] We found that the expression of the autophagy regulatory proteins Beclin 1, p62 and LC3 were increased in the tumor cell cytoplasm relative to normal colonic epithelial cells. A statistically significant association between Beclin 1 and LC3 was found consistent with their known roles in regulating autophagy activation, whereas Beclin 1 and p62 were inversely related given that p62 accumulation is a consequence of autophagy inhibition.[26] Expression of Beclin 1 was detected in at least 50% of tumor cells in 69% of colon cancers. While the mechanisms regulating Beclin 1 expression in colon cancer are unknown, allelic loss of Beclin 1 has

Table 2. Univariate analysis of clinicopathological features and autophagy marker expression in relation to patient overall survival (OS) (n = 178)

Parameter	5-y OS %	Hazard Ratio (95% CI)	p value[§]
Age (1-y increase)	NA	1.02 (0.99, 1.04)	0.1624
Gender			**0.8699**
Female	72%	-	
Male	69%	1.04 (0.64, 1.69)	
TNM Stage			**0.0161**
II	91%	-	
III	66%	2.70 (1.16, 6.25)	
Grade			**0.0074**
I/II	76%	-	
III/IV	59%	1.91 (1.18, 3.10)	
Tumor Site			**0.8402**
Distal	72%	-	
Proximal	68%	0.95 (0.58, 1.55)	
Beclin 1[†]			**0.0540**
Low	79%	-	
High	68%	1.78 (0.98, 3.24)	
p62/SQSTM 1[†]			**0.7679**
Low	69%	-	
High	72%	0.91 (0.47, 1.74)	
LC3[†]			**0.3877**
Low	70%	-	
High	73%	0.74 (0.38, 1.46)	

[§]Score test p value from a Cox regression model after stratifying by study; [†]missing data for Beclin 1 (n = 12); p62 (n = 7); LC3 (n = 43).

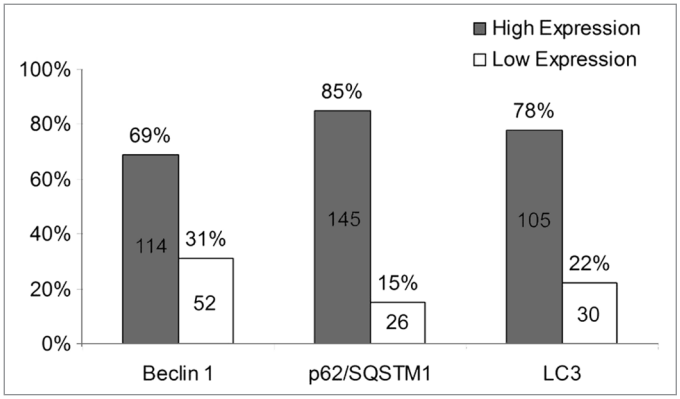

Figure 2. Beclin 1, p62/SQSTM 1 and LC3 expression levels were dichotomized into high and low categories based upon percent tumor cell positivity (≥ 50% high; < 50% low). Missing data for Beclin1 (n = 12), p62 (n = 7) and LC3 (n = 43).

been detected in human breast and genitourinary cancers[15,17] and aberrant DNA methylation of Beclin 1 has been detected in breast cancers.[29] Furthermore, miR-30a was shown to negatively regulate Beclin 1 expression in human cancer cells and to attenuate activation of autophagy by the mTOR inhibitor, rapamycin.[30]

Although data are limited, conflicting reports exist for the association of Beclin 1 expression and prognosis in human cancers.[31-36] Furthermore, data for the prognostic impact of LC3 and p62 are essentially lacking. We found that Beclin 1 overexpression in primary tumors was significantly associated with reduced survival in patients receiving 5-FU as adjuvant therapy after adjustment for covariates. Given that we studied stage II and III tumors, we examined the interactions of the autophagy markers with stage and found that none were statistically significant for OS (all p > 0.14). While another study in colorectal cancers found that high vs. low Beclin-1 expression was associated with poorer survival in patients treated with surgery alone,[36] high vs. low Beclin-1 expression was associated with favorable survival in resected, stage III colon cancers after 5-FU-based adjuvant chemotherapy in a retrospective case series.[35] Studies have also linked Beclin 1 overexpression with upregulation of hypoxia inducible factor 1-α (HIF1-α)[32] and with poor prognosis in nasopharyngeal carcinoma patients receiving chemoradiation.[37] Our study, as well as other reports,[35,38] did not identify an association of Beclin 1 with clinicopathological variables. Accordingly, the prognostic impact of Beclin 1 may be due to increased autophagic capacity with an ability to confer chemoresistance. In this regard, autophagy inhibtion has been shown to sensitize colon cancer cells to 5-FU-induced apoptosis.[39] Furthermore, knockdown of *Beclin 1* enhanced chemotherapy- or radiation-induced apoptosis in human cancer cell lines consistent with a pro-survival role for autophagy.[6,40] Further study is needed, however, to address the role of Beclin 1 as a predictive vs. a prognostic biomarker.

In contrast to Beclin 1, the other key autophagy-related proteins, i.e., p62 and LC3, were not associated with clinical outcome. p62 is a cytosolic adaptor protein and a signaling hub that regulates diverse cellular processes including cell survival and cell death.[22] p62 can facilitate cell death by binding and activating caspase-8,[25] but can also activate the pro-survival transcription factor NF-κB via activation of TRAF6.[41] While upregulation of p62 protein expression in some tumor types was reported to correlate with progression,[42-44] we did not find an association of the level of p62 expression with clinicopathological variables or with patient survival. Similarly, the expression level of LC3 was not associated with clinical features or with patient outcome. Membrane-bound and lipidated LC3-II[45] is commonly used to monitor autophagy.[23] LC3 and Beclin 1 expression were only weakly correlated in colon cancer specimens. Of note, immunohistochemical detection of LC3 does not distinguish between LC3-1 and LC3-II. Furthermore, other mammalian Atg8 homologs besides LC3 [GABARAP and GATE-16 (GABARAPL2)] may have functional redundancy with LC3.[46]

In addition to our data, other evidence in colon cancers also suggests that Beclin 1 may be more closely associated with survival compared with LC3.[47] Beclin 1 is a central regulator of autophagy that interacts with several Beclin 1 binding proteins (UVRAG, Atg14L, Bif-1, Rubicon, Ambra1, survivin).[48] The Beclin 1 network regulates both autophagy and apoptosis whose balance determines the efficacy of anticancer treatment. The BH3 domain of Beclin-1 is bound to and inhibited by anti-apoptotic Bcl-2 or Bcl-XL proteins that can reduce its capacity to induce autophagy.[49,50] Interestingly, caspases can cleave Beclin 1 during

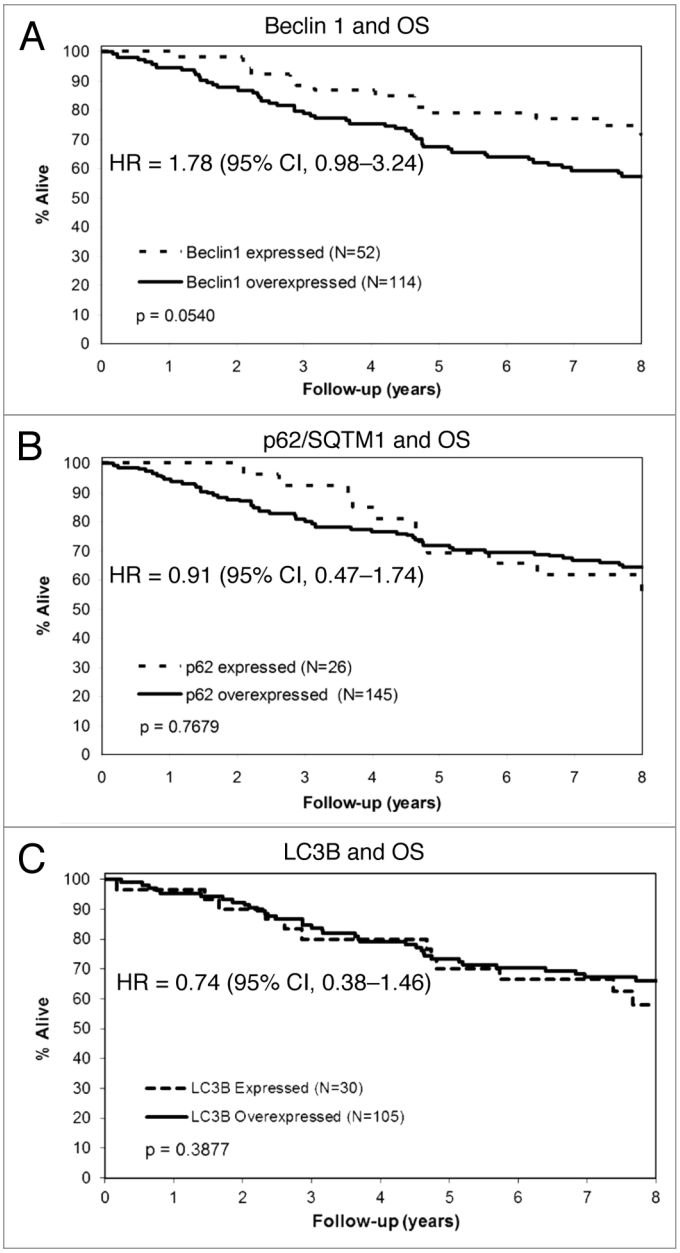

Figure 3. Prognostic impact of autophagy markers in stage II and III colon carcinomas. Overall survival plot compares high vs. low level expression of Beclin 1 (**A**), p62/SQSTM 1 (**B**) and LC3 (**C**) in tumor specimens.

apoptosis, thereby disabling its pro-autophagic activity.[51,52] Beclin 1 exerts an anti-apoptotic role in response to chemotherapy, irradiation and other stimuli by an as yet unclear mechanism.[48] Another Beclin 1 binding partner is HMGB1 (high-mobility group box 1), a nuclear protein and extracellular damage-associated molecule.[53] HMGB1 binds to the receptor for glycosylation end products (RAGE) that sustains autophagy and can promote colon and pancreatic tumor cell resistance to chemotherapeutic agents.[54] Accordingly, Beclin 1 exerts broad regulatory control over cell death processes that appear to contribute to its ability to influence outcome in colon cancer patients.

Table 3. Multivariate analysis of tumor variables and overall survival (n = 166)

Variable	Hazard ratio (95% CI)	p value[§]
Beclin1[†] (overexpressed vs. expressed)	1.82 (0.99, 3.32)	0.0421
Histologic grade (grade III/IV vs. I/II)	1.80 (1.09, 2.99)	0.0253
Stage (III vs. II)	2.72 (1.16, 6.37)	0.0088
Age	1.01 (0.99, 1.04)	0.3759

[§]Likelihood ratio p value after stratifying by study; [†]adjusted for grade, stage and age.

While our data require validation, they suggest that Beclin 1 expression may predict the efficacy of cytotoxic chemotherapy in colon cancer patients. Furthermore, our data support targeting autophagy in vivo to enhance the efficacy of cytotoxic drugs used to treat colorectal cancers. Currently, there are nearly 20 clinical trials registered with the National Cancer Institute exploring autophagy inhibition as a therapeutic strategy against a variety of human cancers. Most of these trials use hydroxychloroquine to inhibit autophagy,[55] although more specific and potent autophagy inhibitors are needed. Study limitations include the fact that current methods to measure autophagy in tissue specimens, including those utilized in this study, represent static measurements of a dynamic process. Furthermore, molecules regulating the autophagy pathway may be influenced by other factors or have their own specific functions that involve processes other than autophagy. In conclusion, we demonstrate that biomarkers of autophagy are upregulated in primary human colon cancers relative to normal-appearing colonic mucosa. Moreover, overexpression of the autophagy initiator Beclin 1 is associated with poor survival in patients with nonmetastatic colon cancers treated with 5-FU-based adjuvant therapy. These data support strategies to target autophagy for therapeutic advantage in this malignancy.

Patients and Methods

Study population. Surgically resected, primary stage II and III colon carcinomas (n = 178) were analyzed from patients who participated in one of two 5-fluorouracil (5-FU)-based adjuvant chemotherapy trials conducted by the North Central Cancer Treatment Group [study numbers: 89-46-51 (n = 53) and 91-46-53 (n = 125)]. We utilized tumors from patients in the 5-FU-based treatment arms to explore the impact of autophagy on patient survival. Our study population represents a subset of the overall clinical trial populations where tumor tissue was available. Details of these completed, randomized trials have been previously reported.[56,57] Study treatments consisted of 5-FU + levamisole vs. 5-FU + levamisole + leucovorin (89-46-51) or 5-FU + leucovorin + standard dose or high-dose levamisole (91-46-53). Patients were followed for a median of 8 y after study randomization for overall survival (OS). The current analysis was in accordance with the original informed consent documents. The

individual adjuvant clinical trials were approved by Institutional Review Boards (IRBs) at the respective study sites and the current analysis was approved by a separate IRB-approved protocol.

Tumor histologic grade was determined as defined by the American Joint Committee on Cancer (AJCC) Prognostic Factors Consensus Conference (grade 1, well differentiated; grade 2, moderately differentiated; grade 3, poorly differentiated; grade 4, undifferentiated).[58] Tumor site was defined relative to the splenic flexure, with tumors located at the splenic flexure included in the distal category.

Immunohistochemical staining. Tissue microarrays (TMAs) had been previously constructed from formalin-fixed, paraffin-embedded (FFPE) tumor blocks that were available from a non-random subset of study participants. TMAs contained three malignant and three normal colon tissue cores per case. Sections of normal liver, tonsil and placenta had been included as controls and navigation markers within the TMAs. Using tissue sections (4–6 μm) cut from TMA blocks, Beclin-1, p62/ SQSTM1 and LC3 protein expression was analyzed by immunohistochemistry (IHC). After deparaffinization, slides were placed in a pre-heated 0.1 mM EDTA (pH 8.0) retrieval buffer in a water bath at 99–100°F for 30 min then cooled and rinsed in distilled water. Endogenous peroxidase activity was blocked and sections were incubated with the primary antibodies against Beclin1 (1:250 dilution of rabbit polyclonal ab79937, ABCAM), p62 (1:500; mouse monoclonal M162-3, MBL) or LC3 (1:500; rabbit polyclonal L7543, Sigma). After rinsing in a TBST wash buffer (DAKO), a secondary incubation was performed using DUAL+/ HRP labeled polymer (K4061, DAKO) for 15 min. All incubation steps were done at room temperature. Slides were placed in 3,3'-diaminobenzidine for 5 min and then counterstained with a modified Schmit's hematoxylin. Negative controls omitted the primary antibody but included all other procedural steps. Each slide contained a unique number that enabled blinding with respect to patient identity and clinical characteristics.

Immunohistochemical scoring. Slides were scanned by a cytotechnologist using a slide scanner (BLISS, Bacus Laboratories, Inc.) that can digitally captures images at 480 × 752 pixel resolution at 40× magnification. Tumors were considered to express a given protein if more than 5% of tumor cells stained positively at light microscopy. Staining extent was defined as the percentage of positive tumor cells and was scored as follows: 0, < 5%; 1, 5–24%; 2, 25–49%; 3, 50–74%; 4, ≥ 75%. Given the limited observations per each category, we collapsed the four categories into two groups for analysis with the immunopercent values categorized as high (≥ 50%) vs. low (< 50%) expression. All specimens were interpreted and scored by a gastrointestinal pathologist (TTW) without knowledge of any clinical information.

Statistical analysis. Our cohort size of 178 patients provided at least 80% power to detect an effect reflected by a hazard ratio (HR) of 1.8 for a two-level factor with a prevalence of 30 vs. 70% (2-sided log rank test, α level = 0.05), assuming exponential survival with 4 y of minimum follow-up on each patient. We regard a HR of 1.8 or greater to be a reasonable threshold for a clinically meaningful impact on outcome. Chi-square or Fisher's exact tests were used to test for an association between categorical variables. The Spearman correlation coefficient was used to determine the association among the autophagy markers. OS, censored at 8 y, was calculated as the number of years from random assignment to the date of death or last contact. The distribution of OS was estimated using Kaplan-Meier methodology. Univariate and multivariate Cox proportional hazards models were used to explore the association of biomarkers or clinical variables with OS. Multivariate models were adjusted for covariates. The score and likelihood ratio test p alues were used to test the significance of each covariate in the univariate and multivariate models, respectively. Statistical tests were two sided, with $p < 0.05$ considered significant. All survival analyses were stratified by study to take into account survival differences between the studies. Statistical analyses were done using Statistical Analysis System software (SAS Institute).

Disclosure of Potential Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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