Punctate LC3B Expression Is a Common Feature of Solid Tumors and Associated with Proliferation, Metastasis, and Poor Outcome

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Abstract

Purpose: Measurement of autophagy in cancer and correlation with histopathologic grading or clinical outcomes has been limited. Accordingly, we investigated LC3B as an autophagosome marker by analyzing nearly 1,400 tumors from 20 types of cancer, focusing on correlations with clinical outcomes in melanoma and breast cancer.

Experimental Design: Staining protocols were developed for automated quantitative analysis (AQUA) using antibodies versus LC3 isoform B (LC3B) and Ki-67. Clinically annotated breast and melanoma tissue microarrays (TMA) and a multitumor array were used. An AQUA program was developed to quantitate LC3B distribution in punctate and diffuse compartments of the cell.

Results: LC3B staining was moderate to high in the large majority of tumors. The percentage of area occupied by punctate LC3B was elevated by 3- to 5-fold at high LC3B intensities. In breast cancer and melanoma TMAs, LC3B and Ki-67 showed strong correlations (P < 0.0001), and in multitumor TMAs, mitotic figures were most often seen in tumors with the highest LC3B expression (P < 0.002). In breast cancer, LC3B expression was elevated in node-positive versus node-negative primaries and associated with increased nuclear grade and shortened survival. In a melanoma TMA with no survival data, LC3B levels were highest in nodal, visceral, and cutaneous metastases.

Conclusions: The results reveal a common expression of LC3B in malignancy and support emerging evidence that autophagy plays a significant role in cancer progression. High LC3B was associated with proliferation, invasion and metastasis, high nuclear grade, and worse outcome. Thus, autophagy presents a key target of therapeutic vulnerability in solid tumors. Clin Cancer Res; 18(2); 370–9. ©2011 AACR.

Introduction

Preclinical studies have established autophagy as a therapeutic target in cancer, and several clinical trials are underway combining autophagy inhibitors with standard cytotoxic and targeted anticancer therapies (1). Autophagy is part of a conserved integrated stress response whereby in times of nutrient deprivation, hypoxia and DNA damage, or endoplasmic reticulum stress, the cell’s own cytoplasmic components—damaged organelles and misfolded proteins—are engulfed into autophagosomes and degraded following fusion of the autophagosomes with lysosomes to form autophagolysosomes. Recycling of degraded cellular components from autophagolysosomes provides a source of amino acids, nucleotides, and lipids for ATP production and macromolecular synthesis (2). Autophagy plays dual roles in cancer. On the one hand, it acts as a suppressor of tumor development (3) and can induce senescence (4). On the other hand, once a tumor is established, autophagy is a cancer cell survival factor (5, 6). For example, autophagy can confer resistance to cytotoxic therapeutics (7, 8). Indeed, malignant cells bearing activating mutations in Ras are autophagy addicted, requiring active autophagy for survival (9). Numerous preclinical studies have shown that genetic or pharmacologic autophagy inhibition can enhance the antitumor efficacy of variety of anticancer therapies (7–14).

While there is growing interest in clinical trials targeting autophagy in cancer therapy, the clinical implications of elevated or suppressed autophagy at different stages and in different malignancies is as yet unknown. A critical shortcoming in the field is the lack of immunohistochemical tools that can be applied to traditional formalin-fixed,
**Translational Relevance**

Preclinical studies indicate that autophagy can be a suppressor mechanism for the initiation of tumorigenesis but a targetable mechanism of cancer cell survival once a tumor becomes established. Translation of these preclinical findings has led to clinical trials involving the autophagy inhibitor hydrox-chloroquine in combination with existing anticancer therapies that may induce autophagy in tumors. To guide the development of autophagy inhibitors for advanced disease and to understand the prognostic significance of high and low levels of autophagy in early disease, robust biomarkers for autophagy need to be developed that can be assessed in commonly available paraffin-embedded tissue. This study reports novel immunohistochemical protocols for LC3 staining that were applied to hundreds of human cancer pathology specimens through the use of clinically annotated tissue microarrays. The results provide comprehensive evidence that autophagy levels are elevated in most human cancers and are associated with aggressive malignancy and poor outcome. High expression of LC3B was found to be prognostic of high nuclear grade, high mitotic rate, metastases, and shortened survival. The staining techniques reported here by light microscopy and quantitative immunofluorescence [automated quantitative analyses (AQUA)] can now be incorporated into prospective and retrospective clinical studies to establish the clinical use of LC3 as a potential prognostic and predictive marker in multiple cancers. These techniques can also be applied in the future for other emerging autophagy markers.

paraffin-embedded (FFPE) tissues. Here, we analyzed autophagy in nearly 1,400 tumor specimens from 20 different types of cancer with a major focus on melanoma and breast cancer. As a marker, we used punctate and diffuse staining of a key autophagosomal modulating protein, LC3 (microtubule-associated protein 1, light chain 3). We used automated quantitative analyses (AQUA) and direct observer scoring to assess LC3 expression, cellular distribution between punctate and diffuse compartments, and associations with tumor progression. AQUA is linked to a fluorescent microscope system that detects the expression of biomarker proteins by measuring the intensity of antibody-conjugated fluorophores within a specified subcellular compartment (typically including the nucleus, cytoplasm, and plasma membrane) within the tumor region of each tissue microarray (TMA) spot. The result is a quantitative score of immunofluorescence intensity for the tumor (15–17). LC3 is first synthesized as pro-LC3 that is cleaved by cysteine protease Atg4B to its cytosolic form, LC3-I. During active autophagy, LC3-I is conjugated to phosphatidylethanolamine, where it resides in the lipid membrane of nascent autophagosomes as LC3-II (1). LC3-II migrates separately from LC3-I on gel electrophoresis (18). LC3-II is localized to the cytosolic and luminal surfaces of immature, double-membranated autophagic vesicles and modulates autophagic vesicle growth and cargo recruitment. Some proteins recruited by LC3-II are themselves cargo adaptor proteins (autophagy receptors) and include p62, Nbr1, and NIX (1). During autophagic flux, autophagosomes fuse with lysosomes to form autolysosomes and the contents are rapidly degraded with lysosomal hydrolases. As this proceeds, cytoplasmic surface LC3-II is delipidated to LC3-I and recycled into the cytosol whereas luminal LC3-II is degraded by hydrolases. Here, we used an antibody that was generated against the N-terminus of full-length human LC3B sequence to investigate LC3 expression and cellular distribution in FFPE specimens of human cancers by immunohistochemistry and AQUA. In mammals, LC3 is expressed as 3 isoforms, A, B, and C. We used the B isoform, LC3B, because of its broad tissue specificities and previous use as an autophagosome marker in cancer (19–21). By a number of approaches, we show herein that LC3B expression is remarkably common in a variety of metastatic human cancers. We chose to investigate the correlation between LC3B staining and lymph node metastases and clinical outcome in melanoma, a cancer reported to have high levels of autophagy (22, 23), and breast cancer, a cancer that is commonly referred to as autophagy deficient because of monoallelic loss of the autophagy gene beclin (24). Here, we find that LC3B is strongly associated with proliferation, metastasis, and at least in breast cancer, with worse patient outcome.

**Materials and Methods**

**Immunohistochemistry**

LC3B (microtubule-associated protein light chain 3, isoform B) was detected using rabbit anti-LC3B polyclonal antibody ab48394 (Abcam, Inc.) generated versus a synthetic peptide corresponding to an N-terminal portion of the human LC3B protein sequence. The LC3B antibody recognizes human LC3BI and LC3BII as confirmed by Western blotting. Ki-67 was detected using mouse anti-human Ki-67 antibody 556026 (BD Biosciences). Sections from FFPE tissues were deparaffinized and rehydrated by standard procedures. For direct observer scoring via light microscopy, slides were stained with anti-LC3B (1:500, without citric acid/antigen retrieval) by standard immunoperoxidase techniques using the brown chromogen generated from diaminobenzidine and counterstained with hematoxylin.

**AQUA**

Fluorescence-conjugated antibodies were used as previously described (15–17). For detection of LC3B, slides were incubated with anti-LC3B (1:5,000, overnight at 4°C) with no citric acid/antigen retrieval. Similar procedures were used to detect Ki-67 through immunofluorescence except that citric acid antigen retrieval was used before staining.
(22). The antibodies were visualized using a species-specific horseradish peroxidase–conjugated secondary antibody (rabbit EnVision, Dako) followed by Cy5 tyramide. The LC3B or Ki-67 signals were read within a tumor mask created using mouse-anti-cytokeratin (1:100 AE1/AE3; Dako) for breast cancer, or a cocktail of anti-S100 (1:100; Biogenex) and anti-HMB45 for melanoma (1:25; Abcam). The tumor mask antibodies were incubated along with the anti-LC3B and visualized with Cy3-goat-anti-mouse IgG (1:100; Jackson ImmunoResearch).

**Light microscope scoring of microarrays**

Multitumor microarray YTMA96 from the Yale Critical Technologies Group was stained by immunohistochemistry for LC3B using the immunoperoxidase reaction coupled with a brown chromogen. Individual sections were evaluated under the light microscope by 2 independent observers (R. Lazova and J.M. Pawelek). Staining intensities were scored and pooled into 2 groups of 0/1 (none to weak) and 2*/3* (moderate to strong).

**Nuclear grade**

Nuclear grade information for the breast carcinoma TMA was obtained from the pathology reports of the individual cases. Nuclear grade information was not available for the melanoma or multitumor TMAs.

**Assessment of LC3B cellular distribution through AQUA**

The relative distribution of LC3B in punctate (granular/vesicular) and diffuse (cytosolic) compartments of the cytoplasm was assessed within antibody masks as described above ($S_{100}$ for melanoma, anti-cytokeratin for breast cancer). The local LC3B immunofluorescence intensity was averaged for each pixel on the basis of the surrounding 15 pixels ($\sim 2\mu m^2$). Pixels with an LC3B expression level above the local mean were designated as "punctate" and the remaining pixels as "diffuse." Oversaturated pixels were excluded from the analysis. The expression of LC3B in each designation was then calculated as was the percentage of pixels assigned to the punctate category. Using small TMAs of melanoma and breast carcinoma with about 40 cases each (Yale Cancer Center Critical Technologies Group), the program was validated through color overlays showing coincidence between the algorithm-generated regions and the actual fluorescence emissions of LC3B.

**Multitumor TMA YTMA96**

A multiple TMA, YTMA96, was provided by the Yale Cancer Center Critical Technologies Group and used for observer evaluation of immunohistochemical staining with the light microscope. The array consists of the following primary tumors: invasive ductal carcinoma of the breast ($n = 20$), invasive colon carcinoma ($n = 20$), clear cell carcinoma of the kidney ($n = 20$), invasive and metastatic malignant melanoma ($n = 20$), papillary thyroid carcinoma ($n = 20$), glioblastoma multiforme ($n = 11$), astrocytoma ($n = 11$), non–small cell carcinoma of the lung ($n = 20$), mixed types of ovarian carcinoma ($n = 20$), hepatocellular carcinoma ($n = 20$), endometrial carcinoma of the uterus ($n = 20$), Hodgkin lymphoma ($n = 10$), mixed non-Hodgkin lymphoma ($n = 10$), mixed sarcomas ($n = 20$), pancreatic adenocarcinoma ($n = 20$), germ cell neoplasms of the testis ($n = 20$), prostatic adenocarcinoma ($n = 20$), gastric adenocarcinoma ($n = 20$), transitional cell carcinoma of the bladder ($n = 20$), and squamous cell carcinoma of the skin ($n = 20$). In addition, unmatched normal specimens from the following normal tissues were included ($n = 10$ of each type): breast, colon, skin, kidney, brain, thyroid, lung, ovary, liver, endometrium, lymph node, muscle, pancreas, testis, prostate, stomach, and bladder. All specimens were taken from deidentified blocks of specimens submitted to Yale University's Department of Pathology. Tumors were of mixed stage and grade.

**Breast carcinoma microarray YTMA49**

A microarray of human primary breast carcinomas, YTMA49, was provided by the Yale Cancer Center Critical Technologies Group for use in the AQUA analyses of fluorescent staining intensities. The microarray consists of 640 cases of node-negative ($n = 321$) and node-positive ($n = 319$) primary tumors. FFPE specimens of breast carcinoma were identified from the archives of the Department of Pathology at Yale University between 1962 and 1980. Complete treatment information was unavailable; however, most of the node-positive patients were treated with local radiation and approximately 15% were given chemotherapy consisting primarily of doxorubicin, cyclophosphamide, and 5-fluorouracil. The node-negative patients were routinely treated with surgery and/or local radiation alone. About one fourth of the patients subsequently received tamoxifen (post-1978). Representative regions of invasive carcinoma were selected for coring by a pathologist (R.L. Camp). All patients were followed until death or for a minimum of 20 years. Patients were censored if they died of causes other than breast cancer or survived more than 20 years after their initial date of diagnosis. The prognostic use of YTMA49 was assessed using standard clinicopathologic variables and is presented in the Supplementary Materials. Correlations between these variables and LC3B expression are also provided.

**Melanoma TMA YTMA20**

Melanoma array YTMA20 was provided by the Yale Cancer Center Critical Technologies Group for use in the AQUA analyses. This array consists of 446 cases of primary ($n = 285$) and unmatched metastases ($n = 161$) from the following sites: lymph node ($n = 86$), skin ($n = 43$), and visceral organs ($n = 32$). This array was constructed from FFPE specimens taken from the archives of the Department of Pathology, Yale University, as available from 1959 to 1994. A dermatopathologist reviewed slides from all blocks to select representative areas of invasive tumor to be cored. No adjudicated survival data exist for this array; however, pathology criteria for the primaries (Breslow depth, Clark levels, tumor-infiltrating lymphocytes, and ulceration) and
metastases (site) are provided in the Supplementary Materials.

**Statistical analyses**

Statistical analyses for AQUA were conducted using Statview 5.0.1 software (SAS Institute Inc.). Prognostic significance was assessed using multivariate Cox proportional hazards model with 30-year follow-up data. Survival curves were calculated using the Kaplan–Meier method, with significance evaluated using the log-rank test. For the distribution of mitotic figures, the P-value was calculated through Pearson χ² test with Yates continuity correction (Supplementary Table S5).

**Results**

**Normal tissues**

While autophagy has been long known to be operational in many disease states in addition to malignancies, less is known about autophagy in healthy tissues and cells. Previous studies in primary cutaneous melanomas from our group revealed that, whereas most if not all invasive melanomas were positive for LC3B, normal epidermal keratinocytes and melanocytes proximal to the tumor as well as dermal fibroblasts and lymphocytes were negative for LC3B staining. On the other hand, melanoma-associated macrophages showed strong expression of LC3B with abundant autophagosomes (22). In the multitumor array YTMA96, there were also a number of normal tissues included that showed a variety of staining LC3B staining patterns (Supplementary Fig. S1A–S1F). Many tissues were negative for LC3B as seen in a section of normal brain tissue (Supplementary Fig. S1A). However, renal (Supplementary Fig. S1B) and pancreatic tissues showed strong LC3B cytoplasmic expression with punctate and diffuse staining in the epithelial lining of tubules and glands. This is consistent with roles for autophagy in the physiology of these organs (25, 26). Stromal fibroblasts and tumor-infiltrating lymphocytes were LC3B negative (Supplementary Fig. S1C–S1E), whereas tumor-associated macrophages were strongly positive with punctate and diffuse staining recently reported (Supplementary Fig. S1e and S1f ref. 22). Thus, at least in some cases, LC3B expression alone cannot be used to distinguish between normal and malignant tissues; however, to avoid this problem, tumor-specific antibody masks were used in the breast carcinoma and melanoma AQUA analyses herein, as described in Materials and Methods.

**Multitumor microarray YTMA96**

The multitumor microarray YTMA96 containing invasive or metastatic specimens from 20 different types of human cancers was stained with the anti-LC3B antibody and scored for staining intensity by 2 independent observers, as described in Materials and Methods. Staining generally, but not always, approached homogeneity in the sections. Examples of different cases with strong and weak LC3B staining are shown (Fig. 1). For all tumors, 84% ± 11% (245 of 292) showed moderate to strong LC3B staining with a range between the different tumor types from 60% to 97% (Supplementary Table S5). The brown LC3B staining was localized predominantly to the cytoplasm, whereas nuclei stained blue from the hematoxylin counterstain. Cases with moderate to strong staining intensities showed prominent punctate patterns, whereas those with weak intensities showed mostly diffuse patterns. Although the tumors were from different tissues of origin, the staining patterns were similar.

**LC3B cytoplasmic distribution**

Because punctate LC3B staining is a marker for autophagosomes and a potential sign of active autophagy (though not a proof in itself), one of us (R.L. Camp) devised an AQUA program to quantitate the amounts of LC3B in the punctate and diffuse compartments of the cytoplasm by combining the parameters of fluorescence-conjugated LC3B emission intensity and percentage of cytoplasmic area, as described in Materials and Methods. We assessed this in melanoma and breast carcinoma small TMAs using the appropriate masks to assure staining of tumor cells, as described in Materials and Methods. For validation of the program, we compared the actual LC3B immunofluorescence patterns with those generated by the algorithm (punctate staining, yellow; diffuse staining, blue; Fig. 2). Figure 2 shows a high LC3B–expressing breast carcinoma (Fig. 2A and B) compared with a low-expressing carcinoma (Fig. 2C and D) with immunofluorescence in the images on the left (2A and 2C) and the algorithm-generated images on the right (2B and 2D). Arrows point to examples of correspondence between the 2 within each tumor. The same correspondence was also seen with the melanoma small TMA (not shown). There was little or no nuclear LC3B staining by the AQUA fluorescence analyses, consistent with the immunohistochemical analyses (not shown).

We thus used the algorithms above to conduct quantitative analyses of LC3B distribution in the same 40-case TMAs of melanoma and breast carcinoma (Fig. 3). For both cancers, LC3B total cellular expression/intensity levels varied widely between different tumors (punctate + diffuse; AQUA score). However, when the tumors were ordered from low to high total LC3B intensity, the percentage of area occupied by the punctate compartment was 3- to 5-fold higher in tumors with the highest versus lowest total expression levels, occupying 25% to 30% of the cytoplasm at the highest and 5% to 10% at the lowest (P < 0.0001 for both cancers; Fig. 3A and B). Interestingly, as the percentage of area occupied by punctate LC3B increased, there was a concomitant reduction in the intensity of LC3B fluorescence intensity in the punctate compartment versus the diffuse compartment (Fig. 3C and D). For melanoma (Fig. 3C), the intensity of LC3B fluorescence in the punctate compartment (but not the percentage of area) was reduced by approximately 60% in tumors with the highest compared with lowest total LC3B expression levels (P < 0.0001). For breast carcinoma (Fig. 3D), the punctate LC3B content was reduced by approximately 20%, although statistical significance for the breast TMA was not reached (P = 0.1218). These results indicate that high total LC3B
expression is associated with increased punctate LC3B area. As discussed below, this is apparently due to a higher total number of autophagosomes per cell but with diminished LC3 concentrations, consistent with active LC3 processing during autophagosome–lysosome fusion.

Breast cancer microarray YTMA49

Using AQUA, we examined LC3B expression in primary breast carcinomas from patients with or without lymph node metastases in microarray YTMA49. Primary tumors with associated lymph node involvement showed higher LC3B expression than did those without ($P < 0.0001$; Fig. 4A). Furthermore, breast cancers with the highest LC3B expression also showed the highest Ki-67 expression, a widely used marker for cell proliferation and tumor aggressiveness ($r = 0.631$; $P < 0.0001$; Fig. 5A; ref. 17). The correlation between LC3B and Ki-67 was significant in all breast cancer subtypes except HER2/neu-positive tumors (Supplementary Table S5). High LC3B expression was also associated with increased nuclear grade ($P < 0.0001$), a predictor of tumor aggressiveness and survival (Fig. 6A). Likewise, Kaplan–Meier analyses of all tumors on YTMA49 revealed a direct correlation between increased LC3B expression and disease-related survival (Mantel–Cox analysis, $P = 0.0016$; Cox univariate analysis, $P = 0.0013$; Fig. 6B). When divided into breast cancer subtypes, luminal A tumors [estrogen receptor (ER) and progesterone receptor (PR) positive and HER2/neu negative, $n = 262$] chiefly accounted for the correlation between LC3B expression and poor outcome ($P = 0.0051$). The other 3 subtypes, luminal B (ER or PR negative and HER2/neu positive, $n = 45$); HER2/neu positive ($n = 46$); and triple negative ($n = 107$), did not reach significance in this regard (Fig. 6C and D; Supplementary Fig. S2). LC3B was significantly higher in larger tumors ($P = 0.0117$) and in tumors...
from women ≤50 versus >50 years ($P = 0.0001$). It was significantly lower in ER- and PR-positive tumors ($P = 0.0009$ and $P = 0.0043$, respectively; Supplementary Table S2). LC3B expression was highest in the HER2/neu-positive subtype, followed by the triple-negative, luminal B, and luminal A subtypes ($P = 0.0021$; Supplementary Table S2).

**Melanoma microarray YTMA20**

Melanoma microarray YTMA20 was designed for AQUA analyses and consisted of primary tumors ($n = 131$) and unmatched lymph node, visceral, and subcutaneous metastases ($n = 250$). LC3B expression was significantly higher in metastases than in primary tumors ($P < 0.0001$; Fig. 4B). This was true for all metastatic locations, and there were no correlations with specific sites (Supplementary Table S4). As with the breast cancer microarray YMTA49, there was a strong correlation between LC3B and Ki-67 expression in melanoma YTMA20 ($r = 0.694$; $P < 0.0001$; Fig. 4B). In primary melanomas, there was a weak but significant correlation of LC3B expression with Breslow depth ($P = 0.0479$) but not with Clark Level ($P = 0.1066$), although Clark level showed a trend in that fashion. LC3B staining intensities were highest in patients aged 60 years or older ($P = 0.0085$). There were no specific associations with the density of tumor-infiltrating lymphocytes, the presence of ulceration, or gender (Supplementary Table S4). There are no data for this TMA about nuclear grade or patient outcome.

**Mitotic figures**

In a survey of 289 tumor sections on microarray YTMA96, 91 contained 1 or more mitotic figures. Of these, we had previously scored 89 of 91 as having moderate to strong LC3B expression levels, whereas only 2 of 91 were scored as LC3B negative to weak. On the basis of random distribution, the expected number of mitotic figures would have been 15 of 91 because 17% of the tumors in the YTMA96 arrays were LC3B weak to negative. Thus, the distribution of mitoses was skewed toward moderate to high LC3B expression ($P < 0.002$; Supplementary Table S6), consistent with the findings on Ki-67 levels in melanoma and breast cancer (Fig. 4). Examples are shown of mitotic figures in close proximity to LC3B-positive punctate structures, presumably autophagosomes, in a breast carcinoma (Fig. 5B) and a melanoma (Fig. 5D). Lower-magnification images of mitotic figures associated with LC3B punctate staining are also seen in Fig. 1.

**Discussion**

From analyses of 1,359 tissue specimens from breast carcinoma, melanoma and, on a smaller scale 18 other cancers, LC3B expression was remarkably common regardless of tissue origin. LC3B expression encompassed both the punctate and diffuse compartments of the cytoplasm but not the nucleus. A novel AQUA-based quantitative imaging technique for LC3B staining determined that tumors with high LC3B expression contain more LC3B-positive vesicles, but within these vesicles, the intensity of LC3B staining was diminished compared with that in tumors which have fewer LC3B-positive vesicles. We suggest that the results are consistent with a model in which higher total (punctate + diffuse) LC3B expression levels reflect increased autophagic flux with...
accelerated autophagic vesicles–lysosome fusion and production of autolysosomes. This would result in decreased LC3B content in the punctate compartment because of increased LC3BII degradation by lysosomal hydrolases as well as delipidation with release into the cytosol. The results do not seem to be consistent with a block in autophagic flux prior to autophagic vesicles–lysosome fusion, as such a block would likely result in increased rather than decreased LC3B in the punctate compartment. However, because we could not measure the dynamics of these processes in the fixed tissues, further studies are necessary to address this model. These results are consistent with emerging evidence that, whereas autophagy can suppress the initiation and development of early tumors, it plays a prosurvival role once cancer cells gain metastatic competence (1, 9, 24). Evidence for autophagy serving as a tumor suppressor mechanism was first seen in a mouse model of monoallelic loss of the autophagy gene beclin-1, in which an increased development of spontaneous tumors and elevated rates of tumor proliferation were observed in vivo (3). In humans, 40% to 75% of carcinomas of the breast, ovary, and prostate showed monoallelic deletions of beclin-1 (24). Thus, these findings and those of others show a role for autophagy as a suppressor of tumor initiation and development. On the other hand, a large volume of literature has emerged showing that once malignancies have become established they rely heavily on autophagy for survival. One explanation for this paradox is that the retained allele of beclin, even in tumors that are haploinsufficient for this gene, is always wild type. Therefore, when necessary, autophagy can be induced to high levels as a response to environmental stress even in tumors with "autophagy-deficient" phenotypes. Support for a survival role for autophagy came early from mouse models of cancer therapy. Experiments involving AKT-driven tumors expressing GFP-LC3 showed that autophagy was critical for survival of cancer cells within the center of growing tumors (27). Preclinical therapeutic models revealed that antitumor therapy, including chemotherapy and targeted therapies, induces autophagy in vivo (7, 10–13) and that blockade of therapy-induced autophagy can augment the efficacy of a variety of cancer therapies. More recently, reports that autophagy promotes survival and aggressiveness of metastatic melanoma (23) and pancreatic cancer (28) have emerged. Many of these early preclinical and clinical investigations have been limited to the use of electron microscopy to measure autophagy in tumor tissue. Whereas electron microscopy provides a wealth of ultrastructural data and with trained investigators can accurately measure autophagy in a semiquantitative manner, it is expensive and time intensive, and without a molecular marker of autophagy included in the analysis the incorrect scoring of nonautophagosomal structures as autophagosomes may skew the results. In addition, reliance on electron microscopy limits autophagy investigations to freshly collected tissue. Reliable, objective methods to measure autophagy in FFPE would allow more thorough investigation of the dozens of genes related to autophagy as potential prognostic or predictive biomarkers for cancer prevention and therapy.

Other groups have previously reported LC3 staining in FFPE. Sato and colleagues showed that 90% of primary colorectal tumors (72 of 80) and 100% of lymph node...
(5 of 5) and liver (6 of 6) metastases were positive for LC3 punctate expression (29). Autophagosomes were confirmed by electron microscopy of surgically removed tumor tissue. Normal mucosal epithelium adjacent to the tumors was negative for punctate LC3 staining. When autophagy was induced in colorectal cancer cell lines by nutrient deprivation, agents that interfered with autophagy markedly increased apoptotic death, indicating that autophagy plays a prosurvival role in colorectal cancer cell. Yoshioka and colleagues showed strong LC3 expression in 53% of esophageal, 58% of gastric, and 63% of colorectal cancers. In early esophageal cancers, this correlated with high Ki-67 expression (30). It was concluded that LC3 expression is advantageous to tumor development. In pancreatic cancers, Fujii and colleagues observed that 87% (62 of 71) were LC3B positive and this correlated with a shorter disease-free period and poor patient outcome (31). In primary invasive melanoma, we previously reported that, whereas normal melanocytes adjacent to the tumor and melanoma cells of early in situ melanomas showed little or no LC3B expression, punctate LC3B staining was widespread in nests of florid in situ tumor cells and in 31 of 31 invasive tumors in the dermis (ref. 22 and Lazova and Pawelek, unpublished data). Autophagosomes were confirmed by electron microscopy showing double-membraned structures filled with melanized melanosomes. While these studies laid the groundwork to explore the importance of LC3B staining as a measure of autophagy in cancer, our findings here included a much larger and broader representation of human malignancy, used objective quantitative immunofluorescence techniques, addressed the issue of LC3B punctate versus total LC3B expression, and established LC3B staining as a potential prognostic marker in multiple malignancies by correlating quantitative staining with survival outcomes.

Conclusions and Significance

Regardless of tissue origin, a large majority of solid tumors contain autophagosomes as assessed by LC3B punctate staining and, in the case of melanoma (22) and colorectal cancer (8), by electron microscopy. Punctate LC3B was associated with tumor cell proliferation, metastasis, high nuclear grade, and worse patient outcome. Taken together, it seems most likely that these results, along with those of

Figure 4. Association of LC3B punctate staining with metastasis. A, breast carcinoma array YTM49-box graphs of LC3B staining intensity in primary tumors comparing lymph node-negative versus lymph node-positive cases (N = 640). B, melanoma array YTM20-box graphs of LC3B staining intensity in primary melanoma compared with that in lymph node, visceral, and cutaneous metastases (N = 342). Mets, metastases.

Figure 5. Associations of LC3B punctate staining with cell proliferation. A, correlation analyses by AQUA of LC3B cytoplasmic staining and Ki-67 nuclear staining in breast carcinoma microarray YTM49. B, mitotic figure (*) and punctate LC3B staining (arrow) in a breast carcinoma section. C, correlation analyses of LC3B cytoplasmic staining and Ki-67 nuclear staining in melanoma microarray YTM20. D, mitotic figure and punctate staining in a melanoma section. IHC, immunohistochemistry.
others, reflect widespread, active autophagy in malignant tumors as a prosurvival mechanism for cancer cells. These findings have several potential therapeutic implications. LC3 staining could be a prognostic marker in many human cancers and could be incorporated into clinical trials of drugs that are known to induce autophagy or inhibit autophagy. The surprisingly common occurrence of autophagosomes in malignancies points to a key role for autophagy in tumor progression and thus presents a common pathway of therapeutic vulnerability in human malignancies.

Disclosure of Potential Conflicts of Interest

R.L. Camp is a stockholder, scientific founder, and consultant to HistoRx, a private corporation to which Yale University has given exclusive rights to produce and distribute the software and technologies embedded in AQUA. Yale University retains patent rights for the AQUA technology. The other authors have disclosed no potential conflicts of interest.

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