LC3A-Positive "Stone-Like" Structures Predict an Adverse Prognosis of Gastric Cancer

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ABSTRACT

Microtubule-associated protein light chain 3 (LC3A) is a reliable marker of autophagy that displays three distinct patterns of immunohistochemical staining in solid tumors: diffuse cytoplasmic staining, juxtanuclear staining, and staining of "stone-like" structures. These three patterns have a different prognostic significance in many solid tumors, but little is known about their influence in gastric cancer (GC). This study was a retrospective analysis of 188 GC patients from stages I to IV. The pattern of LC3A expression was examined in tumor and nontumor tissues by immunohistochemistry. Then, the association between the pattern of LC3A expression in GC and the prognosis was investigated by Kaplan-Meier analysis and the Cox proportional hazards model. Two distinct patterns of LC3A immunostaining (diffuse cytoplasmic expression and "stone-like" structures) were observed in GC tissues. LC3A-positive "stone-like" structures were found only in the tumors, and the number of such structures was correlated with both the tumor type and tumor stage. In addition, a high number of LC3A-positive "stone-like" structures was closely associated with an increased risk of recurrence after radical resection of stages I–III cancer ($P < 0.001$; HR = 0.205) and was associated with a lower overall survival rate for stage IV cancer ($P < 0.001$; HR = 0.364). Taken together, our data demonstrate that LC3A-positive "stone-like" structures can be used as an independent biomarker for an adverse prognosis of GC, suggesting that "stone-like" structures are correlated with the malignancy of this disease. Anat Rec, 297:653–662, 2014. © 2014 The Authors The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology Published by Wiley Periodicals, Inc.

Key words: gastric cancer; autophagy; LC3A; stone-like structure; prognosis

Autophagy is an auto-degradation process that involves the breakdown of cellular constituents in lysosomes and plays a crucial role in maintaining cellular homeostasis and genomic integrity (Mizushima et al., 2002; Eskelinen and Saftig, 2009). Because autophagy has a pivotal role in cellular functions, considerable attention has been focused on the relationship between autophagy and cancer. Autophagy seems to be a two-edged sword (Kimmelman, 2011), because it can prevent the malignant transformation of normal cells, but it may also confer a survival advantage on cancer cells subjected to various stresses. For instance, cancer cells often suffer from lack of nutrients as a result of their high proliferation rate and insufficient blood supply. Under these conditions, autophagy could be activated by tumor cells to degrade cellular contents and provide substrate for energy production. There is considerable evidence that abnormal autophagy promotes the progression of many solid tumors and is negatively correlated with the prognosis (Yang et al., 2011; Liu and Ryan, 2012). Abnormal autophagy may also make a contribution to the acquisition of drug resistance (Chen and Karantza, 2011; Ding et al., 2011). Hence, blocking the process of autophagy could be a novel strategy for cancer therapy.

Microtubule-associated protein light chain 3 (LC3), a mammalian homolog of yeast protein ATG8, is a component of autophagosomes that has been used as a biomarker of autophagy (Fujii et al., 2008). LC3 expression is markedly upregulated in some malignancies, such as gastrointestinal cancer (Yoshioka et al., 2008) and pancreatic cancer (Yang and Kimmelman, 2011). Increased expression of this autophagic protein is closely associated with aggressive tumor behavior and a poor outcome in patients with pancreatic cancer. So far, three isoforms of LC3 have been identified (LC3A, LC3B, and LC3C), which are localized in autophagosomal membranes (He et al., 2003; Marino and Lopez-Otin, 2004). Recent light-microscopic studies have demonstrated three distinct staining patterns for LC3A in several cancers—diffuse cytoplasmic staining, juxtanuclear staining, and staining of stone-like structures (SLS) (Sivridis et al., 2010a). It has been suggested that the diffuse cytoplasmic and juxtanuclear staining patterns reflect basal autophagic activity, while LC3A-positive SLS are thought to represent excessive autophagic activity associated with tumor progression and a poor prognosis in patients with breast cancer (Sivridis et al., 2010b), ovarian cancer (Spowart et al., in press), colorectal cancer (Giatromanolaki et al., 2010), and lung cancer (Karpathiou et al., 2011). However, the influence of LC3A staining patterns in gastric cancer (GC) is still unknown.

We hypothesized that upregulation of LC3A expression in GC would be correlated with an adverse prognosis. In this study, we examined 188 GC specimens and 62 specimens of adjacent noncancerous tissue by immunohistochemistry using an LC3A antibody. We investigated the patterns of LC3A staining in GC and analyzed the clinicopathologic significance of each pattern. We also assessed the relationship of LC3A expression to disease-free survival and overall survival in order to determine whether LC3A staining patterns could predict the clinical outcome.

MATERIALS AND METHODS
Patients and Specimens

This study was approved by the Nanfang Hospital Ethical Review Board. Formalin-fixed (10% formaldehyde), paraffin-embedded pathological specimens obtained from 188 GC patients between 2000 and 2010 were accessed from the archives of the Department of Pathology, Nanfang Hospital (Guangzhou, China). All of the patients were diagnosed as having GC at this hospital and tumor staging was performed according to the UICC/AJCC cancer staging manual (2010). None of the patients received chemotherapy or other anticancer treatment before surgery. Because more than 95% of the tissue specimens that were collected were classified as gastric adenocarcinomas, other histopathological types were not included in the study. In addition, according to the Lauren classification (Lauren, 1965), 93 of the cases were of the intestinal type GC, 67 of the diffuse type and 28 of the mixed type. There were 148 patients in stages I–III who underwent radical resection (25 in stage I, 47 in stage II, and 76 in stage III), whereas the 40 stage IV patients underwent palliative surgery. The median follow-up period was 23.1 months (range, 0.5–61.0 months).

An unbiased sampling method was used. The tissue was cut into 1.5 cm × 1.0 cm × 0.2 cm, and then embedded in paraffin oil. Previous to our experiment, the paraffin blocks from 188 patients had been confirmed to be cancerous tissues by pathologists from our hospital (the first five slices were discarded, and the 6–9th slices were used for diagnosis). On the basis of diagnostic quality control, we cut one section (usually the 10th slice) with a size of 1.5 cm × 1.0 cm × 3 μm from each tissue block for immunohistochemistry as described elsewhere (Bravou et al., 2006; Sun et al., 2010; Pan et al., 2011; Zuo et al., 2012).

Immunostaining for LC3A

A purified rabbit polyclonal antibody MAP1LC3A (AP1805a) (Abgent, San Diego, CA) was used to detect both the cytosolic form (LC3A-I) and the membrane-bound form (LC3A-II) of LC3A.

Tissues were stained as described previously (Sivridis et al., 2010b). Briefly, after being deparaffinized in xylene and rehydrated through a graded alcohol series, the sections were heated at 120°C for 3 min in citrate buffer (pH = 7.2) for antigen retrieval. Endogenous peroxidase was quenched by incubation with 3% hydrogen peroxidase for 10 min and blocking was done by preincubation with 10% normal goat serum at room temperature for 30 min. Subsequently, the sections were incubated with the primary antibody MAP1LC3A (AP1805a, 1:20 dilution) overnight at 4°C in a moist chamber. Then, the sections were washed with PBS (2 × 5 min) and incubated with the secondary antibody (Envision, Dako, Glostrup, Denmark) for 1 hr at room temperature. Next, the sections were washed in PBS again and were stained with 3,3-diaminobenzidine. Finally, the sections were counterstained with hematoxylin, dehydrated, and mounted.

The primary antibody was replaced with normal rabbit immunoglobulin-G for the sections processed as negative controls.

Western Blot

Western blotting was performed according to the guidelines in a previous report (Jensen, 2012) with some modifications. Briefly, cellular protein was extracted from mouse liver using lysis buffer, and protein concentration was determined with a BCA protein assay kit

(KeyGEN, China). Denatured protein was separated on a 12% SDS-PAGE along with 170 kDa to 10 kDa as molecular weight markers (Prestained Dual Color Protein Molecular Weight Marker, Beyotime, China). To save antibodies and improve the efficiency of immunoblotting, the part of the gel containing protein of the molecular weights of interest (10–17 kDa) was selected and cut, and then transferred to a polyvinylidene difluoride membrane (PVDF) (Beyotime, China). The PVDF membrane was blocked overnight in 5% skim milk in Tris-buffered saline and Tween 20 (TBST) (10 mmol/L Tris–HCl, 150 mmol/L NaCl, 0.1% Tween 20). For immunoblotting, the PVDF membrane was incubated for 2 hr with the antibody against LC3A (AP1805a, 1:500 dilution). Then, it was washed by TBST and incubated with anti-rabbit-IgG conjugated to horseradish peroxidase (1:5,000 Dako) for 1 hr. Finally, bands were visualized with X film (Fuji, Japan) by ECL-Plus detection reagents (Santa Cruz, USA).

Evaluation of Staining Patterns

After immunohistochemical staining for LC3A protein, two distinct patterns (diffuse cytoplasmic and SLS) were recognized in GC. Diffuse cytoplasmic staining for LC3A was expressed as the percentage of the positive tumor cells in the total number of tumor cells in the entire section at a magnification of 100× (Giatromanolaki et al., 2010), and scores were assigned in 5% increments (0%–100%). The median value for all of the tumors was used as the cut-off value to classify tumors into low (< median) and high (≥ median) cytoplasmic LC3A staining groups. The number of LC3A-positive SLS was counted in the entire section at a magnification of 400×, and then, the number in each section was divided by the total number of fields included in the entire section to correct for different section sizes (Sivridis et al., 2013). The 67th percentile of the number of LC3A-positive SLS per field was used as the cut-off value to classify tumors into low (<67th percentile) and high (≥67th percentile) LC3A-positive SLS groups.

Statistical Analysis

Statistical analysis was performed with the SPSS 16.0 statistical software package. The chi-square test was used to assess the relationship between the pattern of LC3A expression and clinicopathological features of the patients. Kaplan-Meier survival curves were drawn and the log-rank test was performed to analyze differences of the recurrence and survival rates. Hazard ratios (HR) were determined using the Cox proportional hazards model. Statistical significance was accepted at $P < 0.05$.

RESULTS
MAP1LC3A Antibody Validation

The specificity of the MAP1LC3A antibody was validated by western blot. The two bands corresponded to the LC3A-I (cytosolic 16 kDa) and LC3A-II (membrane-bound 14 kDa) forms (Fig. 1).

Patterns of LC3A Staining

The pattern of LC3A staining was examined by immunohistochemistry in the 188 GC specimens, which
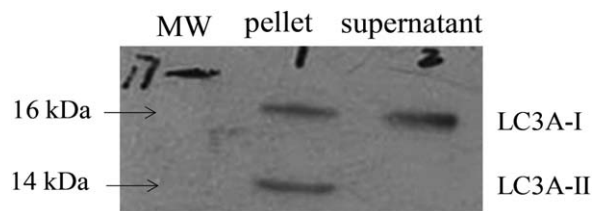

Fig. 1. LC3A antibody qualification by western blot analysis using mouse liver extracts. MW, molecular weight marker showing 17 kDa band.

included 62 paired specimens of GC tissue and adjacent noncancerous tissue from the same patient. Of the three previously described staining patterns for LC3A in several cancers, the GC tissues showed either diffuse cytoplasmic staining or had LC3A-positive SLS, whereas no juxtanuclear staining was found in either GC tissues or the adjacent noncancerous tissues.

The diffuse cytoplasmic pattern of LC3A positivity was seen as a diffuse or finely granular cytoplasmic staining in both cancerous and noncancerous tissues (Fig. 2A,B). When we examined the 62 GC specimens and the corresponding adjacent noncancerous tissues, cells showing the diffuse cytoplasmic LC3A staining were significantly more frequent in GC tissues ($P = 0.040$) compared with the adjacent noncancerous tissues (Fig. 2C). The median percentage of tumor cells showing diffuse cytoplasmic LC3A positivity was 40% (range, 5%–90%) per section. A total of 90 out of the 188 tumors were above the median and were defined as having high levels of diffuse cytoplasmic LC3A staining.

LC3A-positive SLS were observed only in cancer tissues. Figure 3 shows that SLS in different types of GC. These structures were large, spheroidal, densely stained dark brown or black, and were typically enclosed within cytoplasmic vacuoles. The number of LC3A-positive SLS per section ranged from 0 to 32 (the 67th percentile was 3), and 62 of the 188 tumors were in the high LC3A-positive SLS group.

Intra-Observer and Inter-Observer Variation

Evaluation of the LC3A staining pattern was assessed by two pathologists for intra-observer and inter-observer variation. Both pathologists were blinded to the clinicopathological data. They were asked to evaluate the slides separately and to repeat their evaluation 1 week later. The second evaluation was closely correlated with the first for both pathologists (r = 0.90, $P < 0.001$). Similarly, the scores assigned by the two pathologists were highly correlated for the two staining patterns, with a correlation coefficient (r) of 0.92 ($P < 0.001$) for the diffuse cytoplasmic pattern and 0.95 ($P < 0.001$) for the number of positive SLS.

Relationship between LC3A Expression and Clinical Parameters

The association of the two patterns of LC3A staining with the clinical variables of GC patients was investigated. In patients from stages I–III, a high number of LC3A-positive SLS was positively correlated with gastric wall invasion ($P = 0.006$), the number of metastatic lymph
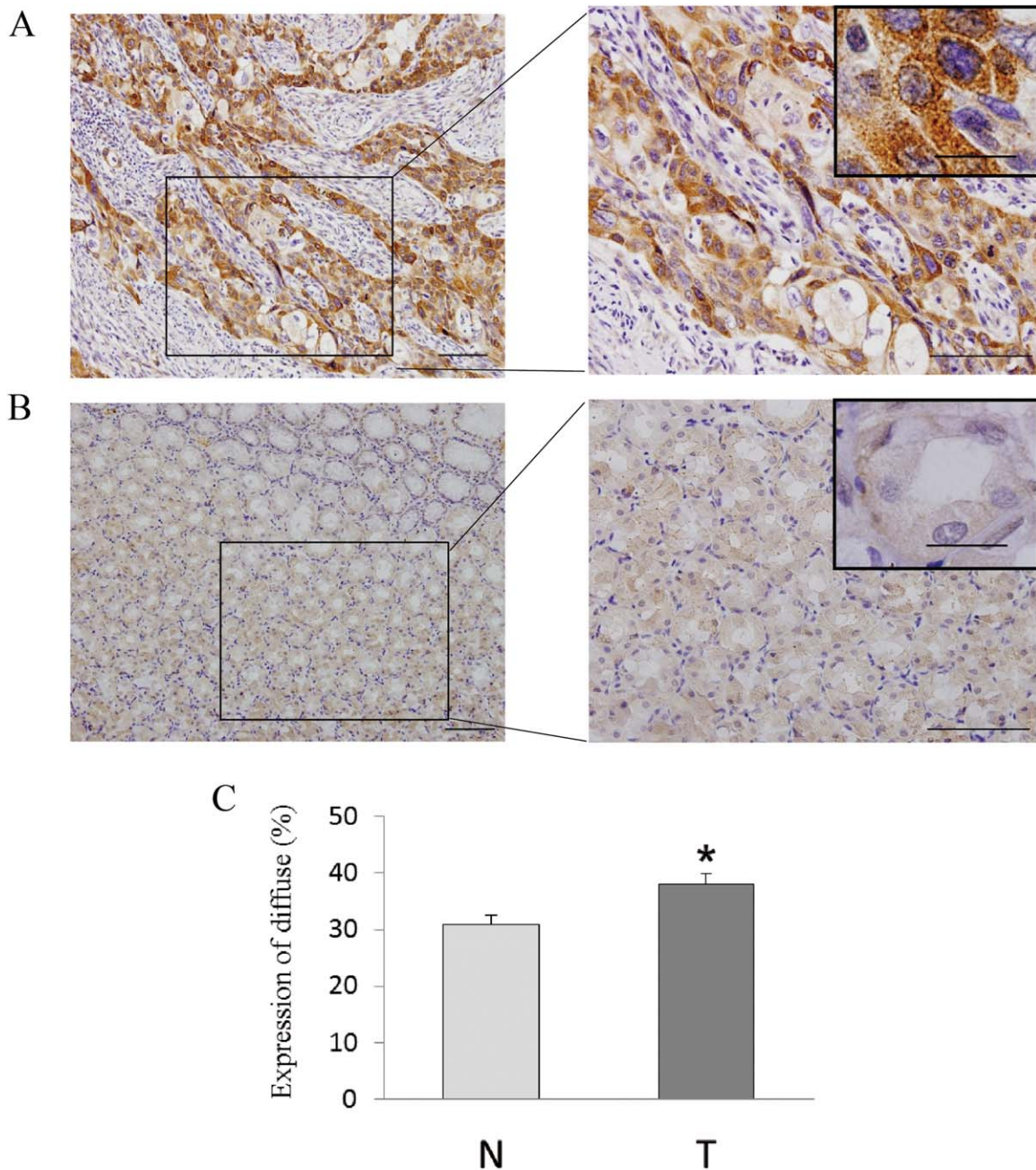
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Fig. 2. The diffuse cytoplasmic pattern of LC3A staining in GC tissues and adjacent noncancerous tissues. Representative images of the diffuse cytoplasmic pattern of LC3A staining in GC tissue (**A**) and in adjacent noncancerous tissue (**B**). There is diffuse or finely granular cytoplasmic staining for LC3A (left: ×200, bar = 100 μm; right: ×400, bar = 50 μm; the right upper corner: ×1,000, bar = 20 μm). **C**: Comparison of diffuse cytoplasmic LC3A staining between GC tissues and matched adjacent noncancerous tissues, as evaluated by LC3A expression. Expression of diffuse staining (%) for LC3A was weaker in the noncancerous tissues (N) and was significantly stronger in GC tissues (T) (n = 62). The results are mean ± SE, *$P < 0.05$.

nodes ($P = 0.002$), and postoperative recurrence (including local recurrence and distant metastasis) ($P < 0.001$). In stage IV, a high number of LC3A-positive SLS was more frequent among patients with metastasis ($P = 0.025$) and patients with ascites ($P = 0.009$) (Table 1).

We also examined the association between the two patterns of LC3A expression and tumor type according to the classification of Lauren, finding that LC3A-positive SLS numbers varied between tumor types. Diffuse tumors had more LC3A-positive SLS than the intestinal type ($P = 0.038$) (Fig. 4A), but there was no significant difference for these two types as compared with mixed tumors. In contrast, there was no significant difference among diffuse, intestinal, and mixed tumors
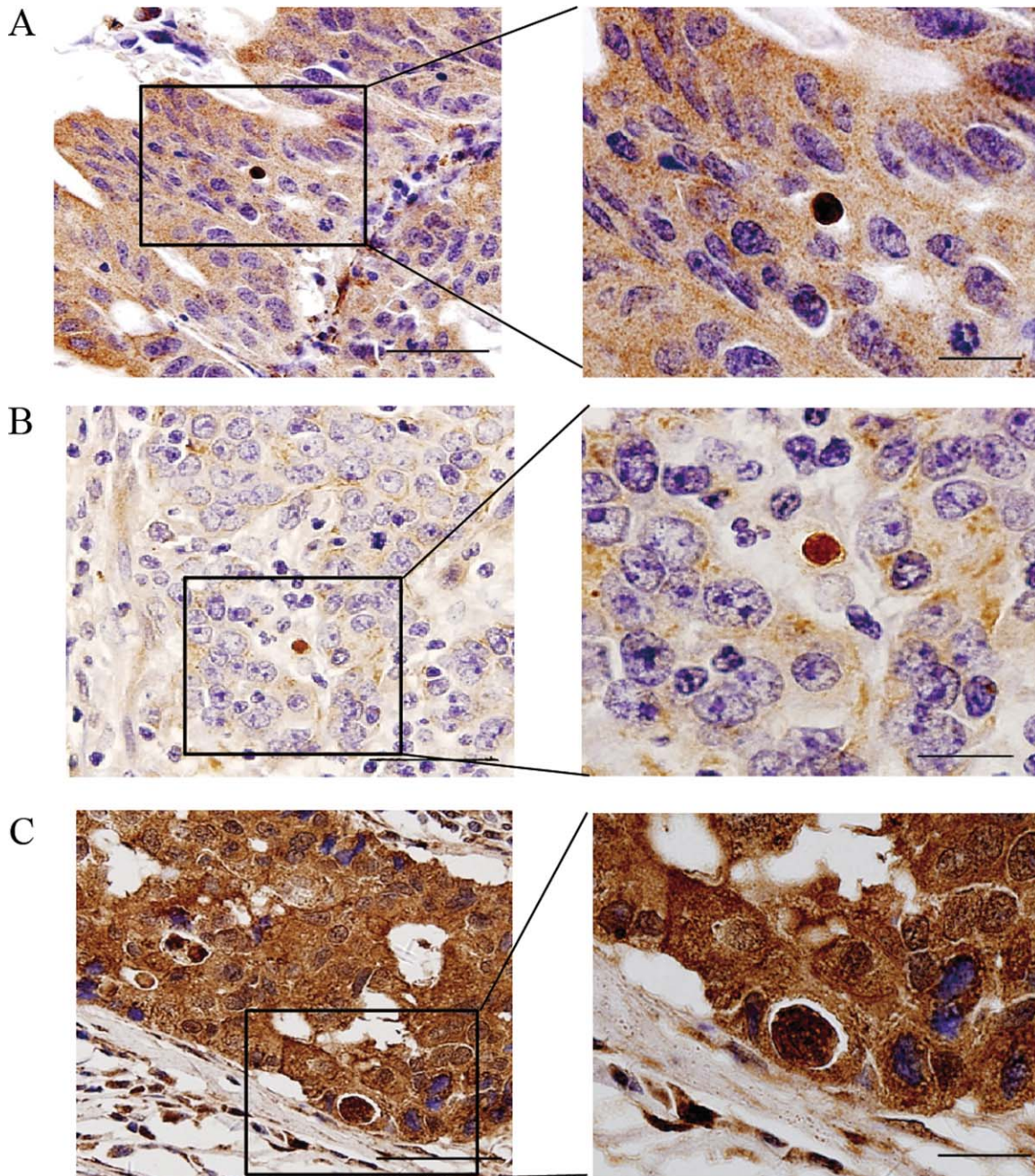
Fig. 3. The "stone-like" structures (SLS) of LC3A in the three different types of GC. **A**: The intestinal type; (**B**) the diffuse type; (**C**) the mixed type. The SLS are densely stained masses, which are typically enclosed within cytosolic vacuoles (left: ×400, bar = 50 μm; right: ×1,000, bar = 20 μm).

with respect to diffuse cytoplasmic staining for LC3A (Fig. 4B). Because tumor stage was also associated with postoperative recurrence ($P < 0.001$) (Fig. 4C), we investigated the relationship between LC3A-positive SLS and the disease stage. The number of LC3A-positive SLS was compared among patients in stages I–IV, revealing a higher number of these structures in more advanced tumors ($P < 0.001$) (Fig. 4D). In contrast, tumor stage showed no significant relationship with the diffuse cytoplasmic pattern of LC3A staining (Fig. 4E).

Multivariate Analysis

On the basis of multivariate analysis using the Cox regression model, the LC3A-positive SLS status ($P < 0.001$; HR = 3.273, 95% CI 2.111–5.075) and tumor stage ($P < 0.001$; HR = 5.208, 95% CI 2.074–13.087) were found to be independent factors predicting the risk of recurrence after radical surgery in stage I-III patients, whereas the LC3A-positive SLS status ($P = 0.018$; HR = 2.514, 95% CI 1.168–5.411) was also an independent predictor of death for stage IV patients (Table 2).

TABLE 1. Occurrence of light chain 3 expression patterns categorized by clinical parameters in patients with gastric cancer

	Stone-like expression			Diffuse cytoplasmic expression		
	Low	High	P value	Low	High	P value
Stages I–III GC (n = 148)						
Age (years)						
≥55	56 (66.7%)	28 (33.3%)	0.144	34 (40.5%)	50 (59.5%)	0.317
<55	50 (78.1%)	14 (21.9%)		32 (50.0%)	32 (50.0%)	
Sex						
Male	67 (73.6%)	24 (26.4%)	0.575	40 (44.0%)	51 (56.0%)	0.866
Female	39 (68.4%)	18 (31.6%)		26 (45.6%)	31 (54.4%)	
Gastric wall invasion						
T1–3	33 (89.2%)	4 (10.8%)	**0.006**	13 (35.1%)	24 (64.9%)	0.252
T4	73 (65.8%)	38 (34.2%)		53 (47.7%)	58 (52.3%)	
Nodal metastasis						
N1–2	94 (77.0%)	28 (23.0%)	**0.002**	52 (42.6%)	70 (57.4%)	0.385
N3	12 (46.2%)	14 (53.8%)		14 (53.8%)	12 (46.2%)	
Recurrence						
No	52 (86.7%)	8 (13.3%)	**<0.001**	23 (38.3%)	37(61.7%)	0.240
Yes	50 (56.8%)	38 (43.2%)		43 (48.9%)	45 (51.1%)	
Stage IV GC (n = 40)						
Age (years)						
≥55	8 (40.0%)	12 (60.0%)	1.000	7 (35.0%)	13 (65.0%)	0.748
<55	8 (40.0%)	12 (60.0%)		9 (45.0%)	11(55.0%)	
Sex						
Male	9 (39.1%)	14 (60.9%)	1.000	7 (30.4%)	16 (69.6%)	0.199
Female	7 (41.2%)	10 (58.8%)		9 (52.9%)	8 (47.1%)	
Metastasis lesions						
≥3	6 (25.0%)	18 (75.0%)	**0.025**	9 (37.5%)	15 (62.5%)	0.750
<3	10 (62.5%)	6 (37.5%)		7 (43.8%)	9 (56.3%)	
Ascites						
No	11 (64.7%)	6 (35.3%)	**0.009**	7 (41.2%)	10 (58.8%)	1.000
Yes	5 (21.7%)	18 (78.3%)		9 (39.1%)	14 (60.9%)	

GC, gastric cancer; *P* value, chi-square test between high and low expression groups.
Bold font indicates *P* values (when *P* < 0.05).

Univariate Analysis of the Prognostic Significance of LC3A

To evaluate the prognosis of the GC patients in relation to LC3A-positive SLS and the diffuse cytoplasmic LC3A staining, Kaplan-Meier survival analysis was performed. This showed that LC3A-positive SLS were associated with an increased risk of postoperative recurrence in stages I–III ($P < 0.001$; HR = 0.205). The median time to recurrence was 12 months in stages I–III patients with a high number of LC3A-positive SLS versus 40 months in those with a low number (Fig. 5A). Stratified analysis of postoperative recurrence was also performed to assess the influence of LC3A-positive SLS in stage II patients (Fig. 5B) and stage III patients (Fig. 5C). The results indicated that LC3A-positive SLS predicted postoperative recurrence in both stage II ($P < 0.001$; HR = 0.025) and stage III ($P < 0.001$; HR = 0.067). Due to the limited number of stage I patients, stratified analysis of postoperative recurrence was not performed. In stage IV patients, a high number of LC3A-positive SLS was associated with lower overall survival rate ($P < 0.001$; HR = 0.364) (Fig. 5D). The median survival time of stage IV patients with a high number of LC3A-positive SLS was 3.55 months versus 13.6 months for those with a low number. In contrast, there was no significant association between the diffuse cytoplasmic pattern of LC3A staining and the survival of GC patients (Fig. 5E,F).

DISCUSSION

Tumor growth requires an abundant supply of energy, which is usually supported by anaerobic glycolysis (DeBerardinis et al., 2008). However, continuous and rapid tumor growth sometimes exceeds the capacity of this mechanism to provide energy for tumor cells. Autophagy is a conserved metabolic process that recycles long-life cellular proteins and disposes of excess or defective organelles to maintain the cell's energy supply. Increased autophagic activity is often found in aggressively growing tumors, especially in the less-perfused regions (Liu and Ryan, 2012), which suggests that autophagy may be an indicator of tumor malignancy and an important mechanism that provides energy for tumor survival.

Therefore, assessing autophagic activity in tumors could be important; however, because it is a dynamic process, autophagy is currently difficult to measure accurately (Mizushima et al., 2010). Transmission electron microscopy has been an accepted method of identifying autophagy in tissue sections, but it is not easy to distinguish autophagic vacuoles from other cellular vacuoles on the basis of morphology alone (Mizushima, 2004). However, the recent introduction of LC3A immunohistochemistry has provided a new method of assessing autophagic activity (Sivridis et al., 2010a). Previous immunohistochemical studies using LC3A have analyzed
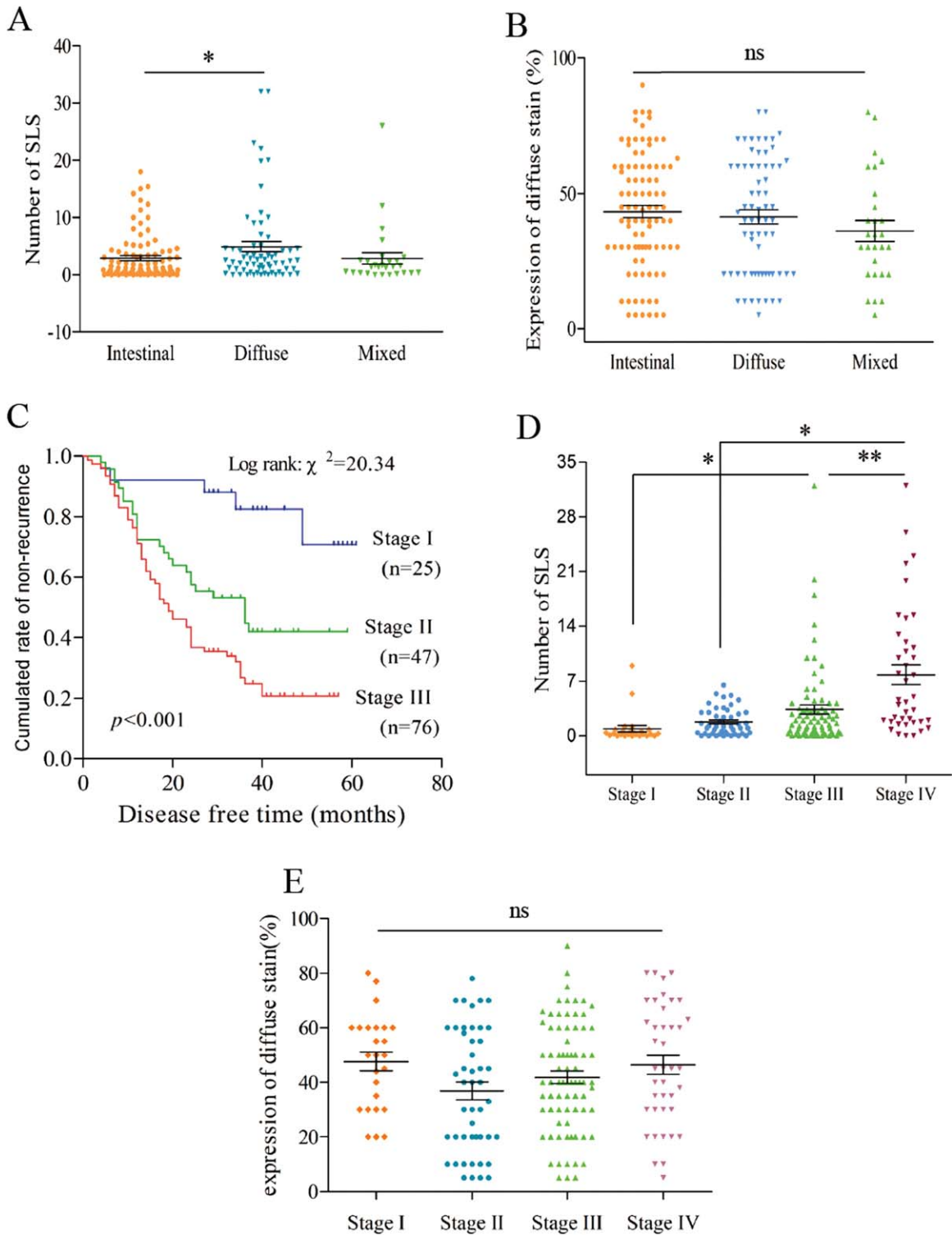
Fig. 4. Association between the pattern of LC3A expression and tumor type or stage. **A**: Comparison of LC3A-positive SLS with tumor type reveals a higher number of these structures in diffuse tumors than in the intestinal type of tumor (intestinal: n = 93, diffuse: n = 67, mixed: n = 28) (*diffuse vs. intestinal). **B**: Comparison of the diffuse cytoplasmic pattern of LC3A staining between tumor types. **C**: Rela-tionship between tumor stage and disease-free survival. **D**: Compari-son of the number of LC3A-positive SLS between tumor stages: a higher number of SLS is associated with a more advanced stage. **E**: Comparison of the diffuse cytoplasmic pattern of LC3A staining between tumor stages. The results are mean ± SE, ns: no significance, *$P < 0.05$, **$P < 0.001$.

TABLE 2. Hazard analysis for recurrence incidence and overall survival rate

			95% CI for HR	
Covariates	Hazard ratio (HR)	*P* value	Lower	Upper
Recurrence in stages I–III GC				
Gender (vs. female)	1.076	0.750	0.591	1.460
Age (vs. <55 years)	1.193	0.441	0.762	1.867
Stage(II–III vs. I)	5.208	**<0.001**	2.074	13.087
Diffuse (vs. low)	1.472	0.079	0.957	2.266
SLS (vs. low)	3.273	**<0.001**	2.111	5.075
Survival in stage IV GC				
Gender (vs. female)	1.075	0.854	0.432	2.004
Age (vs. <55 years)	1.443	0.302	0.719	2.899
Metastasis lesions (vs. <3)	1.620	0.208	0.764	3.436
Diffuse (vs. low)	1.004	0.992	0.490	2.053
SLS (vs. low)	2.514	**0.018**	1.168	5.411

SLS, stone-like structure.
Bold font indicates *P* values (when $P < 0.05$).

autophagic activity in ovarian, colorectal, and lung cancer as well as have shown that immunostaining for LC3A is a novel biomarker for predicting the prognosis of these tumors.

In this study, immunohistochemistry was used, for the first time, to investigate the role of LC3A in GC. Two patterns of LC3A staining (diffuse cytoplasmic and SLS) were found in GC tissues, while juxtanuclear LC3A staining was not detected. This was similar to the findings obtained for liver cancer (Xi et al., 2013), urothelial cancer (Sivridis et al., 2013), and squamous cell cancer of the skin (Sivridis et al., 2011). Currently, the reason for the lack of juxtanuclear staining is unclear, but it may be related to differences of the cell type and organ (Miracco et al., 2007). In this study, the diffuse cytoplasmic pattern of LC3A staining was more frequently observed in GC tissues than in the adjacent noncancerous tissues, suggesting that autophagy was upregulated in the tumors. This result was in agreement with the reports that LC3 expression is upregulated in gastrointestinal cancer (Yoshioka et al., 2008) and pancreatic cancer (Fujii et al., 2008). However, there was no correlation between cytoplasmic LC3A staining and the prognosis of GC, probably because diffuse cytoplasmic staining indicates basal autophagic activity (Sivridis et al., 2010a).

In this study, LC3A-positive SLS were detected only in cancer tissues, suggesting that these structures may be unique to tumor cells. The number of LC3A-positive SLS was higher in diffuse cancers compared with the intestinal type. Previous studies have demonstrated that diffuse tumors are associated with a more advanced stage and a worse prognosis (Qiu et al., 2013). Accordingly, it seems that LC3A-positive SLS numbers are higher in more aggressive tumors. In our study, a high number of LC3A-positive SLS was associated with a more advanced stage, whereas a higher stage predicted an increased risk of recurrence. Moreover, a high number of LC3A-positive SLS was correlated with more metastatic lesions and ascites in stage IV patients, in agreement with previous reports that LC3A-positive SLS numbers are associated with distant metastasis of lung cancer and colorectal cancer. Taken together, these results suggest that a high number of LC3A-positive SLS reflects a population of tumor cells with high invasiveness, so that these SLS are associated with recurrence and mortality.

Although the actual structure of LC3A-positive SLS is unknown, it has been reported that the number of such SLS is positively associated with markers of hypoxia in ovarian cancer (Spowart et al., in press) and colorectal cancer (Giatromanolaki et al., 2010). There is increasing evidence that hypoxia stimulates autophagic activity. Therefore, it is reasonable to speculate that LC3A-positive SLS are associated with upregulation of autophagic activity that confers a survival advantage on cancer by improving resistance to stress and consequently promotes tumor progression (Sivridis et al., 2010b).

Our study demonstrated a close association between the upregulation of autophagic activity and progression of GC. It has previously been reported that inhibition of autophagy sensitizes GC cells to the antiproliferative effect of a proteasome inhibitor (Wu et al., 2010). Similarly, knockdown of autophagy related genes or pharmacologic blockade of autophagy inhibits the proliferation of pancreatic cancer (Guo et al., 2011), colorectal cancer (Sato et al., 2007), and liver cancer (Guo et al., 2013). On the other hand, the activation of autophagy increases resistance to cisplatin and 5-fluorouracil in esophageal cancer (O'Donovan et al., 2011). Clinical trials to evaluate the therapeutic effect of modulating autophagy in combination with chemotherapy are underway in patients with prostate, breast, and lung cancer (White and DiPaola, 2009). Taken together, these results suggest that targeting aberrant autophagy might be a novel strategy for the treatment of cancer. Therefore, we need to establish reliable biomarkers for identification of patients who are likely to benefit from the modulation of autophagy. In view of the strong association between upregulation of autophagy and tumor progression, and considering that LC3A-positive SLS reflect excessive autophagy activity, these structures might be a candidate marker and deserve further study.

Overall, our results suggest that LC3A-positive SLS could be a useful prognostic indicator for GC. The present studies have adopted methods similar to those used in other recent studies of the pattern of LC3A expression (Sivridis et al., 2010b; Karpathiou et al., 2011). However, considering the limitation of SLS detection (currently
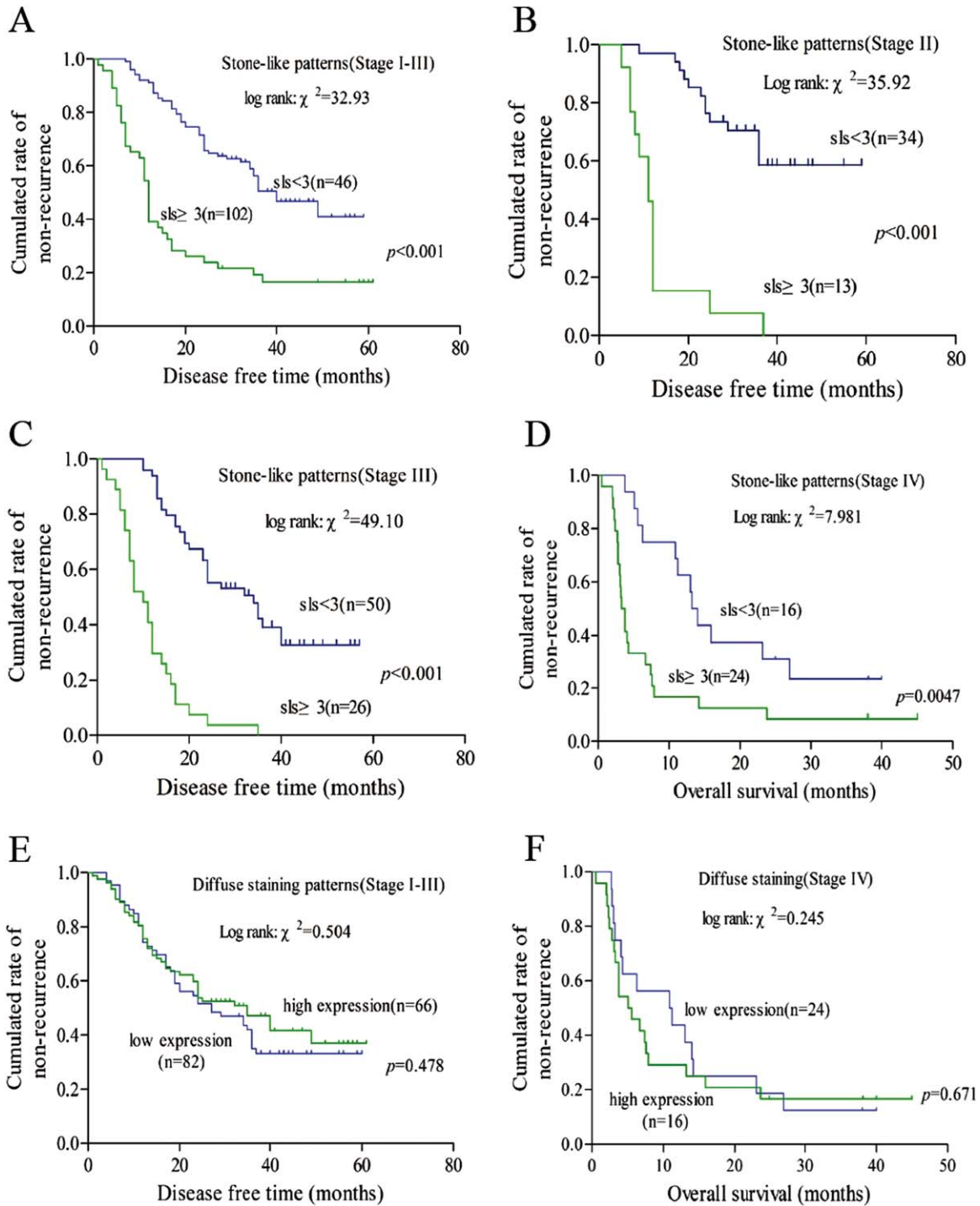
Fig. 5. Kaplan-Meier analysis of the influence of the two patterns of LC3A staining in patients with GC at different stages. **A**: Postoperative recurrence of stages I–III disease was analyzed in relation to the number of LC3A-positive SLS. Separate analyses were also carried out for patients from stage II (**B**) or stage III (**C**). **D**: Overall survival of stage IV patients according to the number of LC3A-positive SLS. **E**: Postoperative recurrence of stages I–III disease in relation to diffuse cytoplasmic LC3A staining. **F**: Overall survival of stage IV GC patients in relation to diffuse cytoplasmic staining for LC3A.

only by immunohistochemistry), a combination of other autophagy biomarkers, such as Atg5 and Atg7 (Yang et al., 2011), might be used to further evaluate autophagy in future studies.

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