

Expression and clinical significance of autophagic protein LC3B and EMT markers in gastric cancer

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Background and aim: Gastric cancer (GC) is a fatal malignancy with high rate of recurrence and metastasis. Here, we investigated the correlations between the expression of autophagic protein LC3B and 2 epithelial–mesenchymal transition-related proteins (E-cadherin and Vimentin) and the clinicopathological factors and prognosis of patients with GC.

Materials and methods: The expression of LC3B, E-cadherin, and Vimentin in GC samples (110 cases) and paracarcinoma tissues (40 cases) was analyzed using the Oncomine databases and further detected by immunohistochemistry. The correlations among these markers expression and clinicopathological features in GC were analyzed. The patients were followed for survival analysis.

Results: Compared to the nontumor tissues, the expression of LC3B and Vimentin proteins were significantly elevated in GC tissues, but the E-cadherin expression was decreased (all $p < 0.05$). Interestingly, LCB expression was positively correlated with Vimentin ($r = 0.320$, $p = 0.001$) and negatively associated with E-cadherin expression ($r = -0.484$, $p < 0.001$) in GC. The expression of these markers was closely related to tumor differentiation, T classification, TNM stage, and lymph node metastasis (all $p < 0.05$). Furthermore, survival analyses and screening Kaplan–Meier plotter database revealed that GC patients with high LC3B and Vimentin expression levels had a poorer clinical outcome than those with low expression. Conversely, high E-cadherin expression was linked with favorable overall survival (all $p < 0.05$, log-rank test). Multivariate survival analysis demonstrated that LC3B, E-cadherin, and Vimentin expression were independent prognostic factors of GC patients.

Conclusion: LC3B, E-cadherin, and Vimentin may play an important role in the tumorigenesis of GC, and these marker expressions may serve as additional prognostic indicators for overall survival of patients. The interactions of autophagy and epithelial–mesenchymal transition in GC merits further investigation.

Keywords: autophagy, epithelial–mesenchymal transition, gastric cancer, prognosis, Kaplan–Meier plotter database

Introduction

Gastric cancer (GC) represents one of the most aggressive malignant diseases with a high mortality rate globally. It still remains the leading causes of cancer-related death in Asia, in spite of its declining incidence worldwide.¹ Surgery is the cornerstone of therapy in early stage GC patients. However, most patients are in advanced or distant metastatic disease at diagnosis, and thus surgery is limited, resulting in a 5-year overall survival (OS) rate as low as 29%.² Clinicopathological parameters (TNM staging, grade of differentiation, and histological type) are used to predict patients with prognosis

and help guide therapeutic decisions, which, however, have limitation because of heterogeneity of patients.³ Therefore, it is of great clinical significance to identify new additional prognostic markers in order to improve individual patient care.

Autophagy is a self-degradative process for the degradation and recycling of cytoplasmic components such as proteins and damaged organelles. The process is tightly regulated and highly conserved to ensure homeostasis.⁴ Autophagy has complex and context-dependent roles in cancer, which depends on tumor stage, genetic context, and tumor microenvironment.⁵ The modulation of autophagy has been considered as a therapeutic approach for targeting cancer. In addition, studies reported that autophagy-related markers were associated with prognosis of cancer.^{6,7} LC3B is widely used as autophagy marker. Immunohistochemical LC3B staining is considered to be indicative of basal autophagy in patient tissue.^{8,9} LC3B protein has been proposed as potential prognostic biomarker for various tumors, including melanoma,¹⁰ esophageal adenocarcinomas,⁸ and breast cancer.⁶ However, there is sparse data on the prognostic role of LC3B in patients with GC.

The process whereby epithelial cells take on characteristics of mesenchymal cells is termed epithelial to mesenchymal transition (EMT).¹¹ The EMT process involves downregulation of the expression of E-cadherin, which is a well-established epithelial marker, and upregulation of Vimentin.¹² Studies have reported that EMT has a critical role for contributing to invasive and metastatic potential of cancer. Recent researches in ovarian cancer, nonsmall-cell lung cancer, colorectal cancer, and GC^{13–15} have shown that EMT-related markers were implicated as critical predictors for prognosis. Previously, EMT- and autophagy-related markers were separately reported regarding their prognostic effects on GC.^{15,16} Recent observations indicate that the interaction between autophagy and EMT exists in cancer.¹⁷ However, the complex relationship about the 2 processes are not definitive yet in GC. In addition, their mutual associations in a clinical setting remain elusive.

Therefore, we conducted the present study to determine the correlations between the expression of autophagic protein LC3B and 2 EMT-related proteins E-cadherin and Vimentin, and the clinicopathological factors and prognosis of GC.

Materials and methods

Patients

GC samples (110 cases) and paracarcinoma tissues (40 cases), obtained from 3201 Affiliated Hospital of Medical College of Xi'an Jiaotong University from 2010 to

2014, were used in the current study. All the patients with GC underwent a radical resection from the same surgical team and were histologically confirmed to have gastric adenocarcinoma. The patients did not receive preoperative chemotherapy and/or radiotherapy. Two independent gastroenterology pathologists assessed the tumor-related clinicopathologic factors by pathological examination. The clinicopathological parameters, including age, gender, tumor differentiation, stage, and lymph node metastasis, were retrospectively collected. These variables are listed in Table 1. Follow-up information was obtained by registered telephone, mail, or outpatient service. OS was calculated as the time from the date of surgery to death from any cause or the last follow-up date. Survival data were collected until October 2017 or until the date of patient death.

Immunohistochemistry and evaluation of immunostaining

Tissue samples were formalin fixed and paraffin embedded. streptavidin-peroxidase (SP) immunohistochemistry staining was performed in strict accordance with the instructions of the kit (SP Staining Kits; BLOSS Biotechnology Co., Ltd., Beijing, People's Republic of China). Antigen retrieval was done by a combination of heat and pressure in sodium citrate buffer (pH 6.0). Sections were incubated with rabbit polyclonal primary antibody anti-LC3B (dilution, 1:500; cat. no. bs-4843R; BLOSS), E-cadherin (dilution, 1:500; cat. no. bs-1519R; BLOSS), and Vimentin (dilution, 1:200; cat. no. bs-0756R; BLOSS) overnight at 4°C. After incubation with coordinate secondary antibody, staining was displayed with DBA solution. The nuclei were lightly counterstained with hematoxylin and sections were visualized through a light microscope (Olympus BH-12, Olympus Optical Co., Ltd., Tokyo, Japan) at magnification 400×. The immunoreactivity was evaluated according to the cell staining intensity and percentage of positive areas, as described previously.¹⁸ The percentage of staining was semiquantitatively scored as follows: 0% number of positive stained cell scored 0, <10% scored 1, 10%–50% scored 2, and >50% scored 3. The intensity of staining was as follows: no staining (colorless) scored 0, weak staining (pale yellow) scored 1, moderate staining (yellow) scored 2, and strong staining (brown) scored 3. The staining index was generated by multiplying the intensity score and the score of the percentage of positive cells. The final scores of <4 were noted as low expression, and the remaining were designated as high expression. Two pathologists who were blinded to patient clinicopathological data independently assessed the immunostaining results.

Table I Relationship between LC3B, E-cadherin, and Vimentin expression and clinicopathological characteristics in GC

Variables	N	LC3 expression		p-value	E-cadherin expression		p-value	Vimentin expression		p-value
		High (%)	Low (%)		High (%)	Low (%)		High (%)	Low (%)	
Gender										
Male	89	56 (62.9)	33 (37.1)	0.464	28 (31.5)	61 (68.5)	0.767	66 (74.2)	23 (25.8)	0.123
Female	21	15 (71.4)	6 (28.6)		6 (28.6)	15 (71.4)		12 (57.1)	9 (42.9)	
Age (years)										
≥60	48	33 (68.8)	15 (31.2)	0.417	17 (35.4)	31 (64.6)	0.368	32 (66.6)	16 (33.3)	0.628
<60	62	38 (61.3)	24 (38.7)		17 (27.4)	45 (72.6)		44 (72.6)	18 (27.4)	
T classification										
T1/T2	17	6 (35.3)	11 (64.7)	0.006	9 (52.9)	8 (47.1)	0.033	8 (47.1)	9 (52.9)	0.039
T3/T4	93	65 (69.9)	28 (30.1)		25 (26.9)	68 (73.1)		70 (75.3)	23 (24.7)	
Nodal involvement										
Negative	36	18 (50.0)	18 (50.0)	0.026	16 (44.4)	20 (55.6)	0.032	20 (55.6)	16 (44.4)	0.013
Positive	74	53 (71.6)	21 (28.4)		18 (24.3)	56 (73.7)		58 (78.4)	16 (21.6)	
TNM stage										
I+II	44	20 (45.5)	24 (54.5)	0.001	20 (45.5)	24 (54.5)	0.007	18 (40.9)	26 (59.1)	0.000
III+IV	66	51 (77.3)	15 (22.7)		14 (21.2)	52 (78.8)		60 (90.0)	6 (9.1)	
Tumor size										
≥5 cm	64	40 (62.5)	24 (37.5)	0.597	18 (28.1)	46 (71.9)	0.456	42 (65.6)	22 (34.4)	0.150
<5 cm	46	31 (67.4)	15 (32.6)		16 (34.8)	30 (65.2)		36 (78.3)	10 (21.7)	
Differentiation										
Low	50	39 (78.0)	11 (22.0)	0.007	10 (20.0)	40 (80.0)	0.024	31 (68.0)	19 (32.0)	0.060
High/moderate	60	32 (53.3)	28 (46.7)		24 (40.0)	36 (60.0)		47 (78.3)	13 (21.7)	

Note: Bold values have statistical significance.

Abbreviation: GC, gastric cancer.

Bioinformatics analysis

To determine the expression pattern of the LC3B, E-cadherin, and Vimentin genes in GC, a search of the publicly available Oncomine database (<http://www.oncomine.com>, Compendia Bioscience, Inc, Ann Arbor, MI, USA) was initially conducted. Briefly, the 3 genes were queried in the database, and the results were displayed by using Box chart. The data type was restricted to mRNA. GC versus normal tissues were compared in the differential analysis. We set up the *p*-value as 0.05 and gene rank to top 10%. The prognostic value of the LC3B, E-cadherin, and Vimentin mRNA expression in GC was also analyzed using the Kaplan–Meier Plotter (<http://kmplot.com/analysis/>). Currently, the database encompasses 54,675 genes, and their effect on survival is assessed using 10,461 cancer samples.¹⁹ The database was used as previously described.²⁰ Briefly, the genes were entered into the database of GC to obtain Kaplan–Meier survival plots. The Multivariate Cox regression model was used and HR, 95% CI, and log-rank *p*-value were calculated and displayed.

Statistical analysis

Categorical variables were compared using the chi-squared test or Fisher's exact test. Spearman's rank correlation analysis was applied to analyze the relationship between

LC3B- and EMT-related markers. OS curves were estimated using the Kaplan–Meier method and evaluated using the log-rank test. Multivariate analysis on prognostic variables was performed using the Cox proportional hazard regression model with stepwise forward selection. Statistical analyses were performed using SPSS 23.0 software (IBM Corporation, Armonk, NY, USA). Statistical significance was defined as *p*<0.05.

Ethics statements

The Institutional Review Board of 3201 Affiliated Hospital of Medical College of Xi'an Jiaotong University (Han-zhong, People's Republic of China) approved this research. Written informed consent was obtained from all participants.

Results

Expression of LC3B, E-cadherin, and Vimentin in GC and adjacent normal tissues

First, we evaluated transcription levels of LC3B, E-cadherin, and Vimentin in GC and normal gastric mucosa using the Oncomine database. In D'Errico et al's²¹ and Chen et al's²² data set, the results revealed that LC3B and Vimentin mRNA expression was higher in intestinal, diffuse, and mixed gastric adenocarcinoma compared with gastric mucosa

(Figure 1A–C and 1G–I, all $p < 0.05$). In Cho's data set,²³ E-cadherin mRNA expression in the gastric adenocarcinoma tissue was markedly lower than in normal tissue (Figure 1D–F, all $p < 0.05$). Then, as shown in Figure 2, we detected LC3B, E-cadherin, and Vimentin protein expression in GC and adjacent tissues using immunohistochemistry (IHC). Consistent with mRNA expression, as shown in Table 2, the high expression rates of LC3B and Vimentin in GC tissues were 64.5% (71/110) and 70.9% (78/110), respectively, which were higher than those in adjacent tissues (LC3B, 20% [8/40], $p < 0.001$; Vimentin, 30% [12/40], $p < 0.001$). The most adjacent normal tissues (77.5%, 31/40) showed higher levels of E-cadherin protein compared with the corresponding GC tissues (30.9%, 34/110, $p < 0.001$).

Correlations between LC3B, E-cadherin, and Vimentin expression and clinicopathological factors in GC

As listed in Table 1, the LC3B expression was associated with the T classification ($p = 0.006$), lymph node metastasis ($p = 0.026$), TNM stage ($p = 0.001$), and the degree of differentiation ($p = 0.007$). Significant associations between

E-cadherin expression and T classification ($p = 0.033$), nodal involvement ($p = 0.032$), TNM stage ($p = 0.007$), and histological differentiation ($p = 0.024$) were identified. Vimentin expression was closely related to T classification ($p = 0.039$), nodal involvement ($p = 0.013$), and TNM stage ($p = 0.000$). LC3B, E-cadherin, and Vimentin expression were unrelated to age, gender, and tumor size (all $p > 0.05$).

LC3B expression correlated with E-cadherin and Vimentin in GC

We next evaluated the correlations between LC3B and E-cadherin and Vimentin expression in GC tissues. As shown in Table 3, the Spearman rank correlation analysis indicated that the expression of LC3B was negatively correlated with E-cadherin expression ($r = -0.484$, $p < 0.001$) and was positively associated with Vimentin expression ($r = 0.320$, $p = 0.001$).

Survival analysis

We next examined the prognostic value of LC3B, E-cadherin, and Vimentin mRNA expression in GC using the Kaplan–Meier plotter database (<http://kmplot.com/analysis/>). The

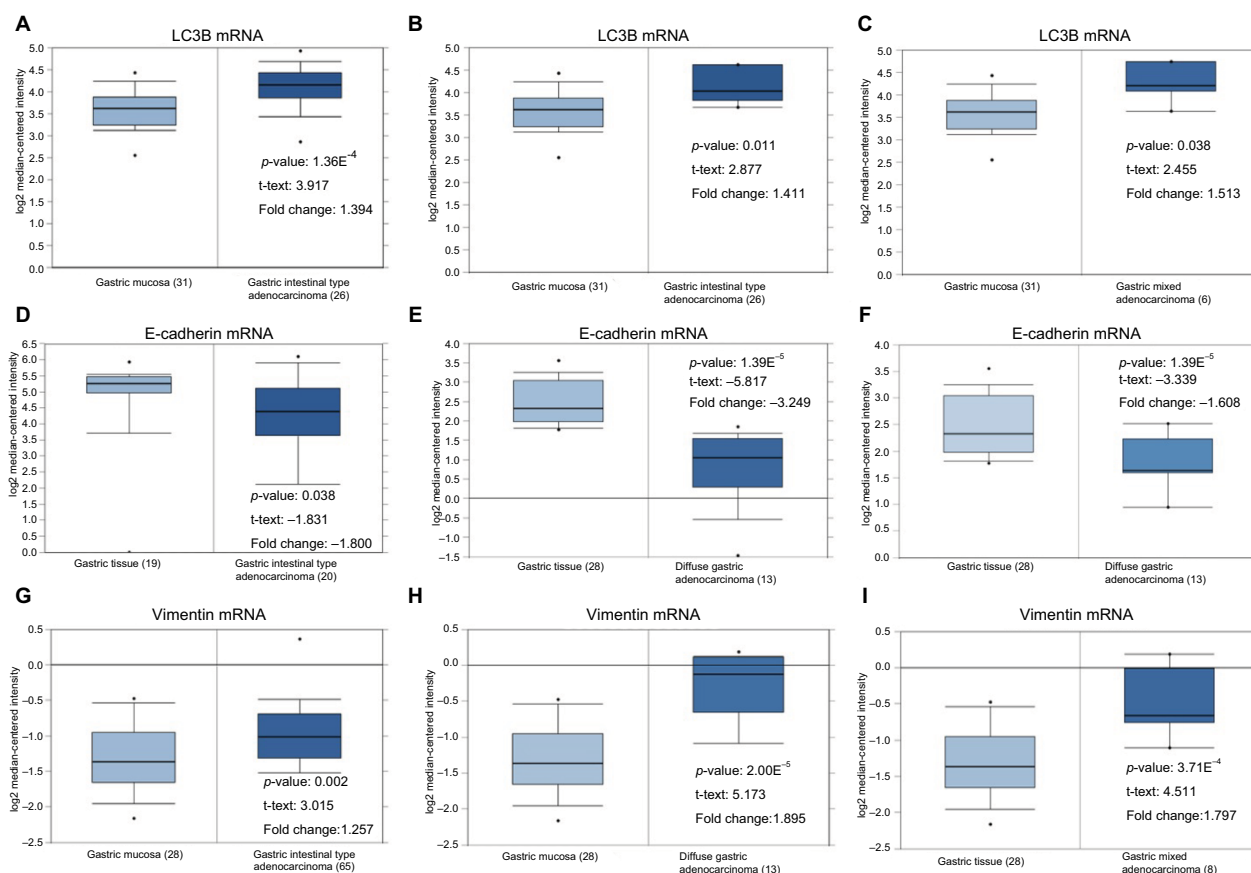


Figure 1 Box and whiskers plots of Oncomine data on LC3B (A–C), E-cadherin (D–F), and Vimentin (G–I) mRNA levels in GC and normal tissues. Abbreviation: GC, gastric cancer.

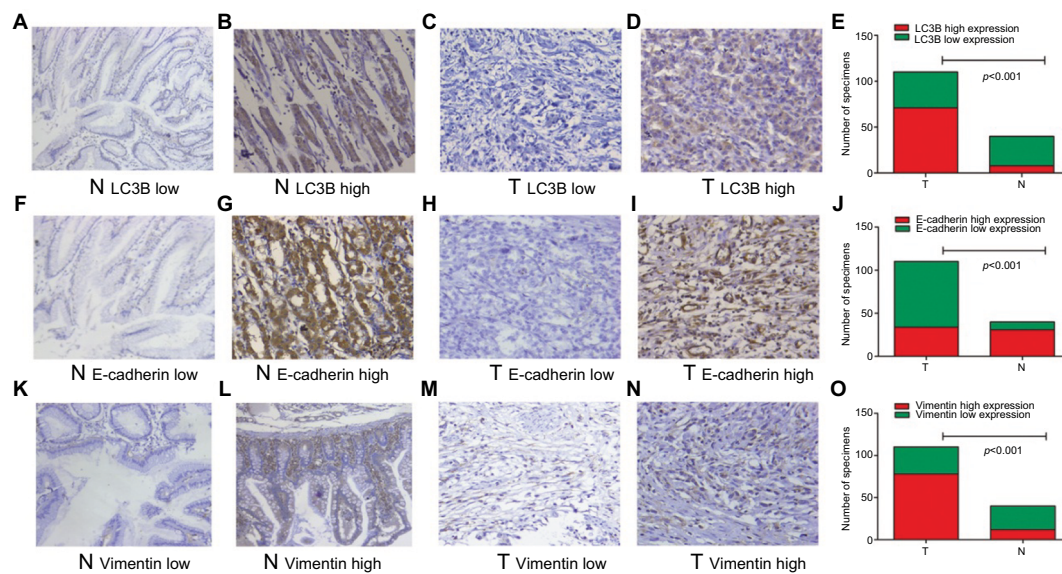


Figure 2 Representative examples of LC3B, E-cadherin, and Vimentin expression (200 \times).

Notes: Low LC3B expression in paired nontumor (A) and GC tissues (C), high LC3B expression in adjacent normal (B) and GC tissues (D), comparisons of LC3B expression in adjacent normal and GC tissues (E), low E-cadherin expression in adjacent normal (F) and GC tissues (H), high E-cadherin expression in adjacent normal (G) and GC tissues (I), comparisons of E-cadherin expression in adjacent normal and GC tissues (J), low Vimentin expression in adjacent normal (K) and GC tissues (M), high Vimentin expression in adjacent normal (L) and GC tissues (N), comparisons of Vimentin expression in adjacent normal and GC tissues (O).

Abbreviations: GC, gastric cancer; N, adjacent normal tissue; T, GC tissue.

Table 2 Expressions of LC3B, E-cadherin, and Vimentin in GC tissue and N tissue

Group	Samples	LC3B expression		p-value	E-cadherin expression		p-value	Vimentin expression		p-value
		High (%)	Low (%)		High (%)	Low (%)		High (%)	Low (%)	
GC	110	71 (64.5)	39 (35.5)	0.000	34 (30.9)	76 (69.1)	0.000	78 (70.9)	32 (29.1)	0.000
N	40	8 (20)	32 (80)		31 (77.5)	9 (22.5)		12 (30.0)	28 (70.0)	

Abbreviations: GC, gastric cancer; N, adjacent normal tissue.

Table 3 Correlations between LC3B with E-cadherin and Vimentin in GC

EMT markers	N	LC3B expression		r	p-value
		High (%)	Low (%)		
E-cadherin expression				-0.484	<0.001
High	34	10	24		
Low	76	61	15		
Vimentin expression				0.320	0.001
High	78	58	20		
Low	32	13	19		

Abbreviations: EMT, epithelial-mesenchymal transition; GC, gastric cancer.

following Affymetrix IDs are valid: 208786_s_at (LC3B), 201131_s_at (E-cadherin), and 201426_s_at (Vimentin). Survival analysis revealed that high expression of LC3B and Vimentin predicted worse OS in GC patients (N=876, HR=1.23 [95% CI: 1.04–1.46], $p=0.016$, Figure 3A; HR=1.29 [95% CI: 1.09–1.53], $p=0.003$; Figure 3C). However, high E-cadherin mRNA high expression was found to be correlated to better OS (N=876, HR=0.74 [95% CI:

0.62–0.87], $p<0.001$, Figure 3B). In our cohort, overall, 91 patients died and 19 survived after a median follow-up of 36 months (range, 8–71 months). Consistent with the aforementioned Kaplan–Meier analysis, patients with high LC3B and Vimentin expression exhibited shorter survival than those with low expression (all $p<0.05$, Figure 3D and F). High E-cadherin expression was associated with favorable prognosis in GC patients ($p<0.05$, Figure 3E). Multivariate Cox analysis identified tumor differentiation, TNM stage, T classification, and lymph node metastasis as significant prognostic factors for OS. In particular, LC3B, E-cadherin, and Vimentin expression were also independently prognostic factors for OS in patients with GC. These analyses are presented in Table 4.

Discussion

In current study, we evaluated the expression of LC3B- and EMT-related markers (E-cadherin and Vimentin) in GC and the corresponding adjacent normal tissues. In addition, we investigated their relationships with clinicopathological

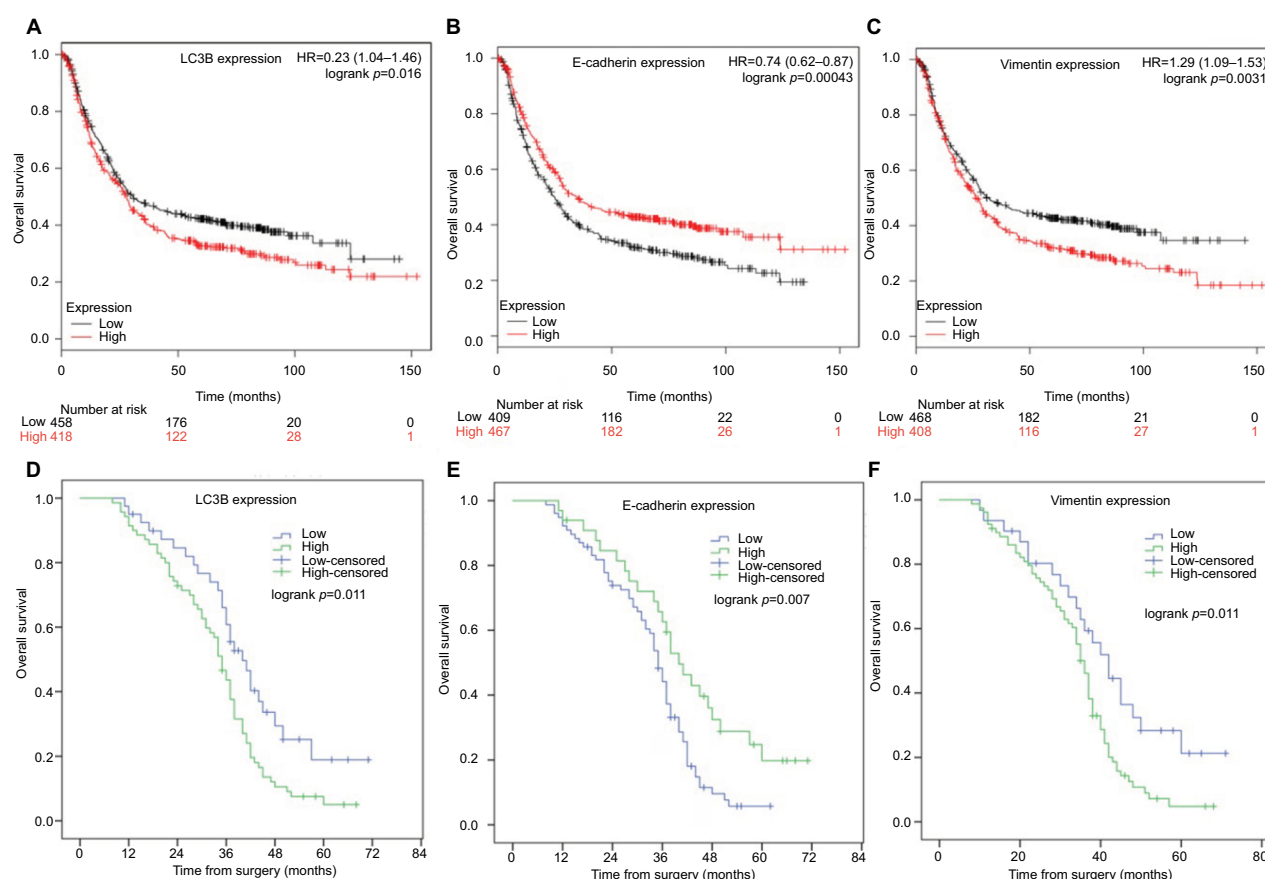


Figure 3 The prognostic value of LC3B, E-cadherin, and Vimentin expression in GC patients.

Notes: OS curves of are plotted for patients with tumors expressing low or high levels LC3B (A), E-cadherin (B), and Vimentin (C) mRNA in Kaplan–Meier plotter database (N=876). OS curves are plotted for patients with tumors expressing low or high levels LC3B (D), E-cadherin (E), and Vimentin (F) in our retrospective cohort (N=110). P-value was calculated by log-rank test and $p<0.05$ was regarded as statistically significant.

Abbreviations: GC, gastric cancer; OS, overall survival.

Table 4 Multivariate analysis of OS for GC patients

Variables	HR	95% CI	p-value
Differentiation			
Low vs high/moderate	2.078	(1.340–3.222)	0.001
Nodal involvement			
Negative vs positive	0.387	(0.189–0.792)	0.009
TNM stage			
I–II vs III–IV	0.216	(0.103–0.453)	0.000
T classification			
T1/T2 vs T2/T3	0.436	(0.203–0.936)	0.033
LC3 expression			
High vs low	1.696	(1.078–2.667)	0.022
Vimentin expression			
High vs low	1.707	(1.033–2.819)	0.037
E-cadherin expression			
High vs low	0.592	(0.363–0.967)	0.036

Abbreviations: GC, gastric cancer; OS, overall survival.

factors and clinical outcomes. Our data showed that LC3B, E-cadherin, and Vimentin were universally expressed in GC and corresponding normal tissues. Specifically, LC3B and

Vimentin expression were significantly higher in GC tissues than that in the counterpart normal tissues in both mRNA and protein levels. The expression of E-cadherin in GC tissues was significantly reduced in comparison with normal tissues. LC3B was positively associated with Vimentin and negatively correlated with E-cadherin in GC tissues by Spearman rank correlation analysis. The expression of these markers was closely related to tumor differentiation, T classification, TNM stage, and nodal involvement. Furthermore, Kaplan–Meier survival analyses revealed that GC patients with high LC3B and Vimentin expression levels had a worse clinical outcome than those with low expression levels. Conversely, high E-cadherin expression was linked with favorable OS. Multivariate survival analysis demonstrated that LC3B, E-cadherin, and Vimentin expression levels were independent prognostic factors of GC patients.

GC continues to pose a major challenge in clinical practice with a poor prognosis and limited treatment options. Therefore, it is urgent to identify new additional prognostic

markers to guide surveillance and improve individual treatment strategies. Mounting studies have indicated that autophagy has an important role in GC development. One study has investigated the prognostic value of autophagy-related proteins in GC and suggested LC3B was not associated with OS.²⁴ Yoshioka et al²⁵ has examined LC3 expression in gastrointestinal cancers and found that high protein expression of LC3 in 58% of GC (22/38 cases). A recent study by Masuda et al¹⁶ evaluated clinicopathological and prognostic significance of LC3 in GC. Consistent with the current study, Masuda et al¹⁶ report that LC3 was associated with T-stage (depth of tumor invasion) and lymph node metastasis, and suggested that LC3-positive indicated worse outcome in GC than that of patients negative by univariate analysis. Tumor metastasis renders most GC inoperable, resulting in a dismal prognosis. Studies found that autophagy facilitates tumor metastasis through affecting EMT,²⁶ tumor angiogenesis,²⁷ and inflammatory responses,²⁸ which may partially explain the LC3B high expression associated with more aggressive behavior of patients with GC. However, some studies^{29,30} revealed that high LC3B expression predicted the poor outcome of triple-negative breast cancer and oral squamous cell carcinoma patients. These findings suggest that autophagy serves multifaceted roles in different types of cancer.

EMT is a process by which epithelial cells dedifferentiate and acquire mesenchymal phenotype. EMT exhibits changes at the molecular level as observed by the decreased expression of E-cadherin and increased expression of Vimentin. Although the role of EMT in cancer is complicated and tissue-specific,³¹ numerous studies that have shown that EMT is a key GC progression driver and facilitates GC invasion and metastasis.³² In line with the previous report,³³ the present study showed that E-cadherin and Vimentin were prognostic factors of GC. Higher Vimentin and lower E-cadherin protein levels in GC patients predicted worse OS. Interestingly, the findings of current study suggested that LC3B was linked to EMT-related markers expression in GC. Remarkably, LC3B was positively associated with Vimentin and negatively correlated with E-cadherin in GC tissues. Recent evidence from basic research indicate that autophagy and EMT in cancer are linked in an intricate relationship.³⁴ The 2 processes share common molecular mediators and signaling pathways, such as TGF β , STAT3, and PI3K/AKT/mTOR signaling cascade. Autophagy is a survival-promoting pathway. On the one hand, aberrant EMT activation requires enhanced autophagy to survive during the cancer metastatic spreading under micro-environmental stress. On the other side, autophagy acting

as tumor suppression mechanism inhibits the early phases of cancer progression by destabilizing crucial mediators of EMT.³⁴ Qiang and He³⁵ reported that autophagy deficiency promotes EMT by SQSTM1-mediated TWIST1 stabilization. Zhao et al³⁶ found that the inhibition of autophagy promotes EMT through increasing ROS/HO-1 signaling pathway in ovarian cancer cells. At present, the precise interactions of autophagy and EMT in GC still remain obscure. Thus, the pivotal molecular mechanism behind the regulation of autophagy and EMT should be further explored.

Several potential limitations should be acknowledged for the current study. First, the numbers of samples are limited, which influences the power of statistical analysis. Second, this was a retrospective study using single-institutional medical information. Certain inherent biases exist. Future larger prospective studies with large samples size may be needed to validate our current data. Finally, the results of our study relied solely on histological examination, and many more in vivo interactions would need to be further researched.

Conclusion

In summary, LC3B, E-cadherin, and Vimentin may serve as potential prognostic biomarkers for GC; however, much more evidence is needed to prove this. There are correlations between autophagy and EMT in GC based on our study, but their complex interactions require further studies to explore the molecular mechanisms involved. The modulation of autophagy and EMT could be promising targets for the treatment of GC.

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Disclosure

The authors report no conflicts of interest in this work.

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