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Beclin-1 suppresses gastric cancer progression by promoting apoptosis and reducing cell migration

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Abstract. To investigate Beclin-1 expression in gastric cancer and its clinical relevance, 60 samples were collected from patients with gastric carcinoma, which were subjected to immunohistochemical staining and analysis. Associations of Beclin-1 expression with the clinical parameters of the patients, including tumor size, histological differentiation and metastatic status, were examined by statistical analysis. The results demonstrated that Beclin-1 expression in gastric carcinoma tissue was significantly associated with the tumor, node, metastasis stage and tumor invasion status. Further experiments indicated that Beclin-1 overexpression promoted MKN-45 gastric cancer cell apoptosis and inhibited their migration. These data suggested that Beclin-1 was a suppressor of tumorigenesis in gastric cancer and a potential therapeutic target for patients with gastric cancer.

Introduction

Autophagy serves an important role in maintaining cell metabolism and homeostasis (1). Autophagy is a process of endogenous substrate digestion in cells; autophagosomes are formed, and mature proteins or damaged organelles in the cytoplasm are encased by lysosomes, in which lysosomal proteases degrade them.

During tumorigenesis and tumor progression, autophagy exerts its role as a tumor suppressor by removing abnormally folded proteins and dysfunctional organelles such as mitochondria, inhibiting cell stress responses (2). However, in instances of nutritional deficiencies and hypoxia, autophagy supports tumor cell survival, which promotes cell proliferation and suppresses cell death (3). Compromised autophagy promotes chromosomal instability, including increased DNA damage, gene amplification and aneuploidy (4).

As a key autophagy regulator associated with apoptosis and differentiation, the autophagy-associated protein Beclin-1 has been demonstrated to be involved in many types of cancer, including ovarian carcinoma (5), hepatocellular carcinoma (6), melanoma (7), rectal cancer (8) and tongue squamous cell carcinoma (9). It has been suggested that the endogenous Beclin-1 protein expression is frequently low in human breast epithelial carcinoma cell lines and tissues, whereas it is expressed ubiquitously at high levels in normal breast epithelia (10). Concomitantly, the autophagy-promoting activity in the MCF7 breast cancer cell line following transfection with Beclin-1 was observed to inhibit MCF7 proliferation, clonogenicity and tumorigenesis (10). A previous *in vivo* study demonstrated that mice with inactivated or deleted Beclin-1 were susceptible to tumors including lymphoma, lung cancer and liver cancer (11,12).

Beclin-1 modulates cancer initiation and progression by affecting a wide range of pathological events, including extracellular matrix degradation, epithelial-to-mesenchymal transition, tumor angiogenesis and alterations to the tumor microenvironment (13). However, the effect of Beclin-1 in cancer development is complex, as a number of reports have indicated the pro-neoplastic and anti-neoplastic functions for Beclin-1, as reviewed by Ozpolat and Benbrook (14). The present study aimed to address Beclin-1 expression and its clinical significance in gastric cancer, a type of cancer with one of the highest incidence rates worldwide, and to explore its primary potential mechanism.

Materials and methods

Clinical specimens and patient data. A total of 60 specimens of gastric carcinoma tumors and para-carcinoma tissues were sampled from patients during resection in Heilongjiang Province Land Reclamation Headquarter General Hospital (Harbin, China) between January 2014 and February 2016. The clinicopathological data of all cases were reviewed, including patient age, sex, tumor size, differentiation status, tumor node metastasis (TNM) stage, lymph node

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metastasis and invasion status (Table I) (15). No patients had received preoperative chemotherapy and radiotherapy, and those who succumbed to other diseases or accidents were excluded from the study. All specimens were fixed in 10% neutral formaldehyde at room temperature for 24-48 h, and embedded in paraffin at 65˚C and cut into 5-μm sections. All specimens were subjected to hematoxylin staining for 5 min and eosin staining for 1 min at room temperature and diagnosed as gastric carcinoma by two pathologists. Informed consent was obtained from all patients or their relatives. The experimental protocol was approved by the Ethics Committee of the King Medical Diagnostics Center (Shanghai, China).

Cell culture and transfection. MKN-45, MKN-28, and SGC-7901 gastric cancer cell lines (Cancer Cell Bank, Chinese Academy of Medical Sciences, Beijing, China) were cultured in Dulbecco's modified Eagle's medium (DMEM; PAN Biotech GmbH, Aidenbach, Germany) supplemented with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) at 37˚C with 5% CO_2. Western blot analysis determined that Beclin-1 expression was relatively low in the MKN-45 cell line (data not shown), thus the MKN-45 cell line was selected for all follow-up overexpression experiments.

The Beclin-1 sequence was cloned into a PCDNA3.0 plasmid (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). For transfection, the cells were seeded at a density of $1x10^5$/well in a 24-well plate and grown to >50-70% confluency. A total of 1 μl Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.) was added to 50 μl serum-free Opti-MEMI medium (Invitrogen; Thermo Fisher Scientific, Inc.) and mixed gently at room temperature for 5 min; 2 μg Beclin-1 plasmid was added to 50 μl serum-free Opti-MEMI medium and mixed gently. After 5 min, the diluted Lipofectamine 2000® was gently mixed with the diluted Beclin-1 plasmid at room temperature for 20 min. Subsequent to transfection with Beclin-1 or control plasmids (empty PCDNA3.0 vector), the 24-well plate was incubated at 37˚C with 5% CO_2 for 6 h, following which the culture medium was replaced with complete medium.

Immunohistochemical staining. The paraffin sections were incubated at 65˚C for 2 h and dewaxed in xylene for 20 min at room temperature. The specimens were washed with distilled water three times, incubated in 3% H_2O_2 at room temperature for 10 min and washed with distilled water again. Antigen retrieval was performed using Tris-EDTA buffer (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in a pressure cooker at medium to high pressure for 3 min. Subsequent to washing with PBS, the specimens were incubated with monoclonal primary antibody against Beclin-1 (1:500; cat. no. ab210498; Abcam, Cambridge, UK) at 4˚C overnight. The specimens were then rinsed with PBS buffer, and incubated in a 1:1,000 solution of biotin-labeled anti-Human Immunoglobulin G (IgG) secondary antibody (cat. no. SAB3701279; Sigma-Aldrich; Merck KGaA) at room temperature for 60 min. The specimens were washed with PBS buffer and treated with 3,3-Diaminobenzidine (Sigma-Aldrich; Merck, KGaA) for 2 min at room temperature. Slides were counterstained with hematoxylin for 1 min at room temperature, washed with distilled water, dehydrated sequentially with a graded ethanol series (80, 95 and 100% ethanol), transparentized with xylene and sealed with neutral gum at room temperature.

Immunohistochemical staining analysis. For each section, 10 high-power fields at magnification, x400 were randomly selected, and ~500 cells were counted in each field. Scores between 0-3 were assigned according to staining intensity and the number of positive cells. The cells were scored as follows: No staining, 0; light yellow, 1; pale brown, 2; dark brown, 3. The number of positive cells was scored as follows: <10%, 0; 10-45%, 1; 45-70%, 2; and >70%, 3. The two scores were totaled and the sum was divided into 4 levels: A total score of 1 was considered negative (-), a score of 2 as weakly positive (+), a score of 3-4 as positive (++), and a score of 5-6 as strongly positive (+++). Negative and weakly positive were regarded as low expression, while positive and strongly positive as high expression.

Western blotting analysis. Tissues were lysed in buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 1% protease cocktail inhibitor, followed by centrifugation at 12,000 x g for 10 min at 4˚C. The tissue lysis supernatants were collected and the protein concentrations were determined using BCA Protein Quantification kit (cat. no. 23227; Thermo Fisher Scientific, Inc.). A total of 20 μg protein samples were loaded in each well and separated by electrophoresis in a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). Subsequent to blocking with 5% milk in TBS containing 0.1% Tween-20 (TBST) at room temperature for 2 h, the membrane was incubated with primary antibodies against Beclin-1 (1:1,000; cat. no. ab210498; Abcam), light chain (LC) 3 II/I (1:500; cat. no. ab128025; Abcam), B-cell lymphoma-extra large (Bcl-xL; 1:800; cat. no. ab2568; Abcam) and β-actin (1:4,000; cat. no. A5441; Sigma-Aldrich; Thermo Fisher Scientific, Inc.). Following washing, membranes were incubated with a 1:3,000 solution of goat anti-rabbit IgG HRP-conjugated secondary antibody (cat. no. 7074; Cell Signaling Technology, Inc., Danvers, MA, USA) for 2 h at room temperature. The antigen-antibody complexes were detected using Western Lightening Plus enhanced chemiluminescence reagent (cat. no. NEL103E001EA; PerkinElmer, Inc., Waltham, MA, USA) with β-actin as the internal control protein. For immunoblotting quantification, the band intensity of the target protein was normalized to the internal control protein band from the same lane (ImageJ software, version 1.6; National Institutes of Health, Bethesda, MD, USA). Data were presented as the mean ± standard deviation of ≥3 replications for each group.

Apoptosis assay. At 48 h post-transfection, the cells were washed twice with cold PBS and dual staining was performed using the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Staining/Detection kit (cat. no. 55654; BD Biosciences, Franklin Lanes, NJ, USA). Briefly, cells were resuspended in 1X binding buffer at a concentration

Table I. Expression of Beclin-1 in gastric carcinoma and para-carcinoma tissue samples.

Group	Cases	Low expression	High expression
Para-carcinoma tissue	60	14	46
Gastric carcinoma tissue	60	38	22[a]

[a]P<0.01.

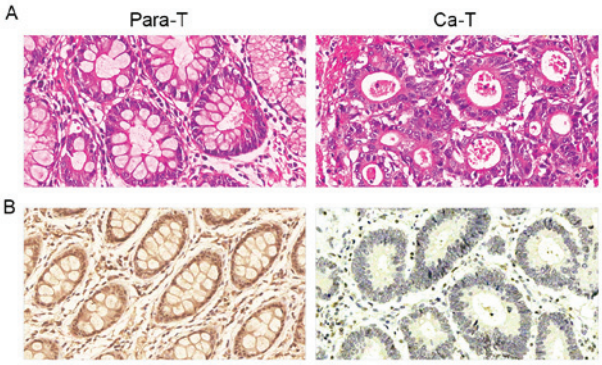

Figure 1. Expression of Beclin-1 in gastric Ca-T and Para-T samples. (A) Representative images of hematoxylin and eosin staining in Para-T and Ca-T samples (magnification, x400). (B) Representative images of Beclin-1 immunohistochemical staining to demonstrate that Beclin-1 protein expression was highly positive in Para-T samples, whereas gastric carcinoma tissue was predominantly negative for Beclin-1 (magnification, x400). Para-T, para-carcinoma tissue; Ca-T, gastric carcinoma tissue.

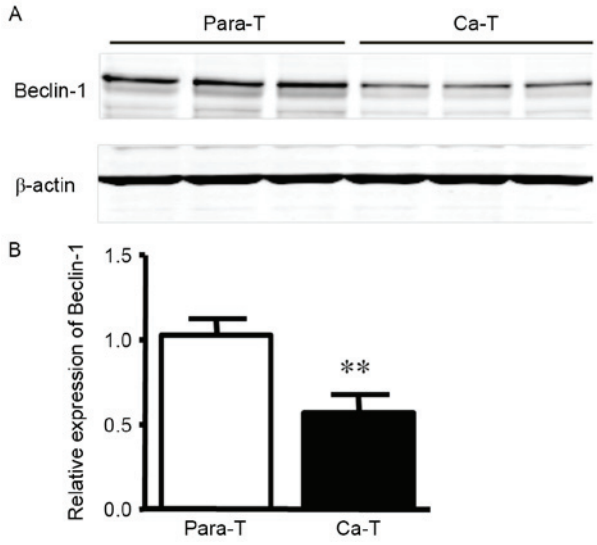

Figure 2. Expression of Beclin-1 protein in in Para-T and Ca-T samples as determined by western blotting. (A) Representative image of western blotting to demonstrate the expression level of Beclin-1 protein in in Para-T and Ca-T samples. (B) Quantification of the western blot analysis. **P<0.01. Para-T, para-carcinoma tissue; Ca-T, gastric carcinoma tissue.

of $1x10^6$ cells/ml. The 100 μl solution was transferred to a 5-ml culture tube. A total of 5 μl Annexin V-FITC and 5 μl propidium iodide were added to the tube and incubated for 15 min at 37˚C. A total of 400 μl 1X binding buffer was added to each tube. The cells were evaluated by flow cytometer (BD Biosciences) and data were analyzed by FCS Express software (version 3; De Novo Software, Los Angeles, CA, USA).

Cell migration assay. Following transfection, the cells were collected and resuspended in serum-free DMEM at a concentration of $1x10^5$ cells/ml. The lower chambers of Transwells (8 μm pore size; Corning Incorporated, Corning, NY, USA) were filled with 800 μl DMEM with 10% FBS, and 400 μl cell suspension was added to the upper chamber. Subsequent to incubation at 37˚C with 5% CO_2 in air for 48 h, cells on the lower surface were fixed with 75% ethanol for 30 min at room temperature and stained with 0.1% crystal violet for 2 min at room temperature. The migrated cells were counted at magnification, x60 using a light microscope (BX60; Olympus Corporation, Tokyo, Japan). Cells were counted in 3-5 fields of interest, which were randomly selected on each membrane, and the average number of cells was calculated.

Statistical analysis. Statistical analyses were performed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference. The results were analyzed with χ^2 tests.

Results

Detection of Beclin-1 protein expression in gastric tumor tissues. The immunohistochemical results demonstrated that positive Beclin-1 staining was primarily localized in the cytoplasm and occasionally in the nuclei (Fig. 1). Beclin-1 protein expression was positive in the majority of para-carcinoma tissue samples, with a positive expression rate of 76.67% (46/60). Gastric cancer tissues were primarily negative or weakly positive for Beclin-1, with a positive expression rate of 36.67% (22/60). The frequency of positive Beclin-1 expression in gastric carcinoma tissue samples was significantly lower compared with that in para-carcinoma samples (P<0.01; Table I).

Detection of Beclin-1 protein expression in gastric carcinoma tissues by western blotting. Western blot analysis indicated that Beclin-1 protein expression in para-carcinoma tissue samples was markedly higher than in tumor tissue (Fig. 2; 1.024±0.097 vs. 0.572±0.102; P<0.01).

Correlation between Beclin-1 protein expression and clinical characteristics. Based on the Beclin-1 protein expression levels detected by immunohistochemical staining, the gastric carcinoma specimens were divided into a low expression group (-, +) and a high expression group (++, +++). The clinical data of 60 cases were reviewed, and it was demonstrated that Beclin-1 protein expression was not associated with age, sex, tumor size, differentiation or lymph node metastasis, whereas it was associated with TNM stage (P=0.008) and invasion status (Table II; P=0.035).

Gastric cancer cell line apoptosis and migration in Beclin-1 overexpression. As Beclin-1 expression was low in the gastric

Table II. Association between Beclin-1 expression and clinical parameters in gastric carcinoma tissue samples.

Clinical parameters	Cases	Low expression (-, +)	High expression (++, +++)	χ^2	P-value
Age, years				1.279	0.258
<60	27	15	12		
≥60	33	23	10		
Sex				0.745	0.388
Male	37	25	12		
Female	23	13	10		
Tumor size, cm				0.191	0.662
<5	36	22	14		
≥5	24	16	8		
Differentiation				0.269	0.604
Low	38	25	13		
Moderate-high	22	13	9		
Tumor node metastasis stage				6.969	0.008[a]
I/II	39	20	19		
III/IV	21	18	3		
Lymph node metastasis					
Negative	36	26	10	3.060	0.080
Positive	24	12	12		
Invasion status					
Mucosa and muscle	42	23	19	4.429	0.035[b]
Serosa	18	15	3		

[a]P<0.01; [b]P<0.05.

carcinoma tissues and correlated with the TNM stage and invasion status of patients, the present study aimed to characterize the role of Beclin-1 in gastric cancer. A previous study indicated that Beclin-1 expression was associated with tumor cell apoptosis and migration (6); therefore, we hypothesized that Beclin-1 acted through a similar mechanism in gastric cancer. Beclin-1-overexpressing gastric cancer cells were produced by the plasmid transfection of MKN-45 gastric cancer cells. As indicated in Fig. 3A, the apoptosis rate was increased in the overexpression group (22.6±3.1%) compared with the control transfection group (8.4±1.4%) at 48 h post-transfection. A significant difference in the apoptosis rate between the groups was observed (Fig. 3B; P<0.01).

A Transwell assay was employed to evaluate the effect of Beclin-1 on MKN-45 cell invasion. The numbers of migrating cells in the Beclin-1 overexpression and control groups were 28.4±5.1 and 75.9±6.9, respectively, which was demonstrated to be a statistically significant difference (Fig. 3C; P<0.01).

To further explore the mechanisms for Beclin-1-regulated apoptosis resistance, alterations to the protein expression levels of the downstream autophagy effector LC 3 II/I and the pro-apoptotic factor Bcl-xL were examined by western blotting. Beclin-1 overexpression was associated with the increased expression of the autophagy effector LC 3 II/I and the reduced expression of the anti-apoptotic factor Bcl-xL (Fig. 3D). The data indicated that Beclin-1 promoted autophagy and apoptosis.

Discussion

The present study explored whether tumor Beclin-1 expression was associated with the clinical features of patients with gastric cancer. Reduced Beclin-1 expression levels were identified in gastric carcinoma tumor tissue, suggesting that Beclin-1 expression may be associated with the occurrence and development of gastric cancer. However, the association between Beclin-1 expression and the clinicopathological characteristics of different tumors is complex, and under debate. A number of studies have demonstrated that the high expression of Beclin-1, as confirmed by immunohistochemistry, is correlated with unfavorable clinicopathological parameters (3,16,17); however, other studies concluded that the pathogenesis and progression of cancer were associated with reduced Beclin-1 expression, which is consistent with the present study (6,9,18). These data indicated that Beclin-1 may induce different effects in different types of tumors, depending on the intrinsic characteristics of the tumors themselves.

In the present study, Beclin-1 expression in gastric carcinoma tissue samples was negatively associated with TNM stage (Table II; P=0.008), although the association between Beclin-1 expression and other clinical parameters of the patients, including tumor size, histological differentiation and metastatic status were not statistically significant. A previous study identified that Beclin-1 expression in stage 1 and 2 gastric cancer tissue samples was higher, whereas Beclin-1 expression levels in stage 3 gastric cancer tissue samples was

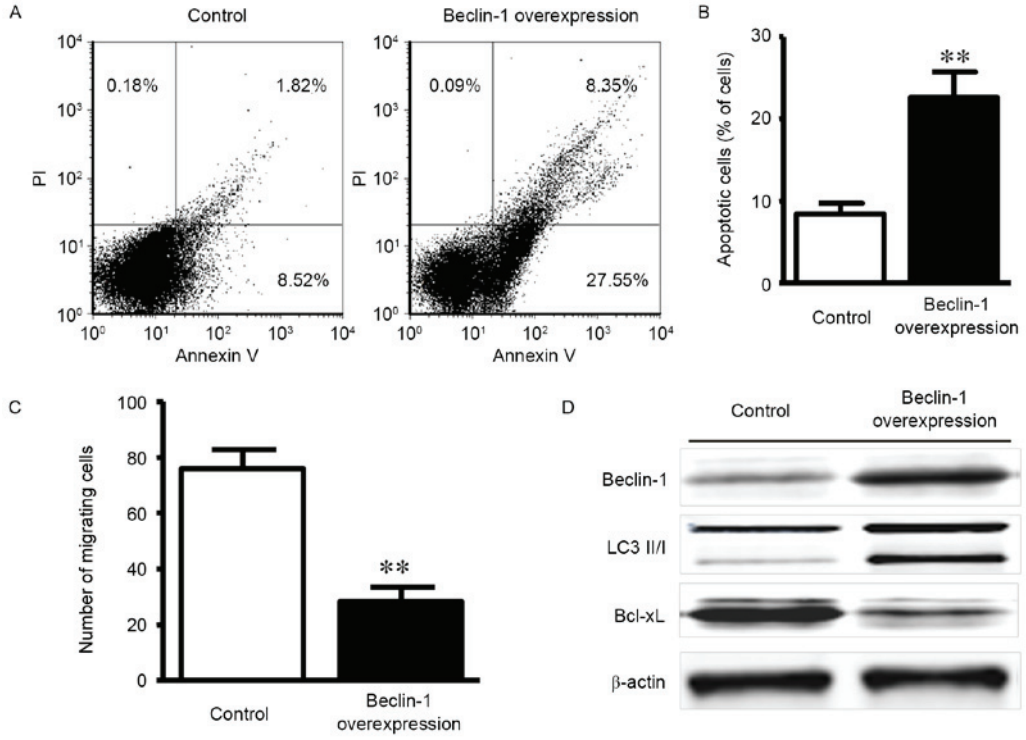

Figure 3. MKN-45 cell apoptosis and migration following Beclin-1 overexpression. (A) Apoptosis was promoted in the Beclin-1-overexpressing cells. A total of 3 independent experiments were performed and representative images are presented. (B) Statistical analysis of apoptosis in Beclin-1-overexpressing cells. **P<0.01. (C) Cell migration was inhibited in the Beclin-1-overexpressing cells. Each experiment was repeated three times. **P<0.01. (D) Beclin-1 overexpression increased the expression levels of the autophagy effector LC 3 II/I, and reduced that of the anti-apoptotic factor Bcl-xL. Representative western blotting images are presented. Bcl-xL, B-cell lymphoma 2-extra large; PI, propidium iodide.

significantly lower, than in normal adjacent tissues (4). These data were consistent with the data of the present study, which revealed that Beclin-1 expression was associated with gastric carcinoma TNM stage, implying that the detection of high Beclin-1 expression levels in patients with gastric cancer may be an effective strategy for predicting the tumor invasion and stage phenotype.

Autophagy and apoptosis serve a central role in maintaining homeostasis and disease progression (19), two processes that are regulated by complex biological processes including interactions between the autophagy-associated protein Beclin-1 and the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) (20,21). Previous studies identified that Beclin-1 possesses a BH3 domain near its N-terminus, which may combine with the BH3 domains of apoptosis-associated proteins including Bcl-2 and Bcl-xL (22,23). The binding of Bcl-2 family members to Beclin-1 may suppress the formation of the pro-autophagy Beclin-1/hVps34 complex formation and reduce Beclin-1-associated PI3 K activity, therefore inhibiting Beclin-1-induced autophagy (24). In addition, previous studies demonstrated that higher Bcl-2/Bcl-xL expression levels were significantly correlated with lower Beclin-1 expression levels in liver and lung cancer, and other types of tumor tissue (25,26).

However, other BH3-binding proteins, including Bcl-2-associated agonist of cell death, may also combine with the Beclin-1 BH3 domain, interrupting the interaction between Beclin-1 and Bcl-2/Bcl-xL, therefore increasing autophagy activity and inhibiting tumor growth (24,27). The present study identified that the upregulation of Beclin-1 promoted apoptosis in gastric cancer cells. A potential mechanism is that Beclin-1 overexpression inhibited the expression of the anti-apoptotic factor Bcl-xL, triggering the apoptosis pathway. These results suggest that the mutual regulation of autophagy and apoptosis serve a pivotal role in gastric tumorigenesis.

A previous study demonstrated that Beclin-1 lentivirus transfection may inhibit cell migration in tongue squamous cell carcinoma cell (28). Vascular endothelial growth factor, and matrix metalloproteinase-2 and -9 were identified as associated with the Beclin-1-mediated inhibition of migration and invasion (28). Another study suggested that increased Beclin-1 expression, as a tumor suppressor, contributed to the inhibition of tumor growth and metastasis in gastric adenocarcinoma through regulating the hedgehog signaling pathway (29). Therefore, it is reasonable to hypothesize that Beclin-1 interaction with other associated molecules regulated tumor cell migration. The data from the present study demonstrated that the overexpression of Beclin-1 may inhibit tumor cell migration, which also suggests that Beclin-1 expression is associated with tumor invasion in gastric cancer. Novel autophagy-based interventions, including Bcl-2 family regulation, caspase-dependent cleavage of autophagy-related gene protein and microRNA mimics to downregulate Beclin-1 have already been clinically or experimentally applied, suggesting promising approaches for novel clinical treatments (30-32).

In summary, the present study investigated the association between Beclin-1 expression, clinically relevant parameters and gastric carcinoma progression. Beclin-1 was associated with the invasion status and TNM stage of gastric carcinoma tissue samples, two major factors affecting the prognosis of

patients with gastric cancer. Increased apoptosis and reduced cell migration were observed in gastric cancer cells overexpressing Beclin-1, indicating a potential mechanism for the effect of Beclin-1 in suppressing gastric cancer progression.

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