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Original Article

Prognostic value of autophagy related proteins ULK1, Beclin 1, ATG3, ATG5, ATG7, ATG9, ATG10, ATG12, LC3B and p62/SQSTM1 in gastric cancer

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Abstract: Autophagy-related (ATG) genes contributed to tumorigenesis and cancer progression. This study aims to investigate the expression of ATG proteins and their clinicopathological significance in gastric cancer. Nine well-known ATG proteins, (ULK1, Beclin 1, ATG3, ATG5, ATG7, ATG9, ATG10, ATG12 and LC3B) and p62/SQSTM1, which represented key regulators that participated in whole autophagosomes stepwise processes, were detected in a large cohort of 352 primary gastric cancer patients. Among these 352 patients, 117 cases were randomly assigned to the training set to detect the clinicopathological value of ATG proteins, and another 235 patients were used as the testing set for further validation. Except for Beclin 1, ATG9 and ATG10, another six ATG proteins and p62/SQSTM1 were closely correlated with histological types for gastric cancer. Moreover, low expression of ULK1, Beclin 1 and ATG10 were associated with lymph node metastasis. In addition, down-regulation of ULK1, Beclin 1, ATG7 and ATG10, up-regulation of ATG12 correlated with advanced TNM stage. Importantly, multivariate cox analysis identified ULK1, Beclin 1, ATG3 and ATG10 as favorable independent prognostic factors for overall survival. Combination analysis of ULK1, Beclin 1, ATG3, ATG10 revealed the improved prognostic accuracy for gastric cancer. Our study showed that ATG proteins might serve as novel prognostic biomarkers in gastric cancer, and supply a new valuable insight into cancer treatment targeting autophagy for patients.

Keywords: ATG proteins, autophagosomes formation steps, prognostic marker, gastric cancer

Introduction

Gastric cancer, a chronic *Helicobacter pylori* infection related malignancy, remains the second leading cause of cancer mortality worldwide [1]. Although the fluoropyrimidine and platinum-based combination therapies have partially benefited the early-stage gastric cancer patients, local recurrence and distant metastasis are still the major reasons for the poor survival of advanced subgroups [2]. Supported by accumulated clinical and prognostic biomarkers, such as peritoneal metastases, plasma alkaline phosphatase, ERCC1, HER2 and HER3 [3, 4], the risk classification of patient outcome was defined more accurately. HER2 and HER3 amplification, for example, were detected in 12-64% of patients with gastric cancer and predicted a poor overall survival (OS) [3, 4]. Importantly, Trastuzumab, a recombinant monoclonal antibody against HER2, had greatly improved progression free survival and overall survival for advanced gastric cancer in the phase III ToGA trial [5]. Therefore, it will be of great clinical value to identify more novel HER2-alike molecular markers, that not only predicting the prognosis individually but also providing promising therapeutic molecular targets for gastric cancer.

The modulation of autophagy is considered as one of the hallmarks of cancer cells. There are three primary types of autophagy: macroautophagy (commonly referred to as autophagy),

microautophagy and chaperone-mediated autophagy. As described in detail previously [6-9], the most distinguishing feature of macroautophagy is the formation of the double-membrane bound phagophore and autophagosome. Autophagosomes undergo a stepwise maturation process, including initiation, nucleation, elongation, maturation and degradation. Simultaneously, a number of key signaling events that orchestrate the sequestration process have been identified, which has led to the discovery of more than 30 autophagy-related (ATG) genes. The key autophagy regulatory ATG genes are listed in Figure S1. At the autophagosomes initiation step, the dephosphorylation of mTOR-dependent sites on the ULK1/Atg13/FIP200 complex releases ULK1's activity, and auto-phosphorylation of the ULK1/ATG13/FIP200 complex by localization of ATG9 to phagophore [10-12]. This leads to the formation of autophagy specific Beclin 1/ATG14L/VPS34/VPS15 complex and coats a cup-shaped isolation membrane, which serves as a recruitment signal for the isolation membrane elongation [13]. Two protein conjugation systems, which are respectively triggered by ubiquitin-like molecules ATG12 and LC3, are required to autophagosome elongation and maturation. Firstly, ATG12 is activated by ATG7 (E1) and transferred to ATG10 (E2) following by covalent linking to ATG5 [14, 15]. Secondly, LC3 is cleaved by ATG4, and formed a cleaved LC3-I with a C-terminal glycine residue. ATG7 (E1) further activates LC3-I and transfers it to ATG3 (E2), subsequent conversion of LC3-I into LC3-II [16-18]. With the assistance of ATG5/ATG12 conjugates, p62/SQSTM1 bound LC3-II is then conjugated to phosphatidylethanolamine and coated the outer surfaces of the autophagosome [19-21]. The autophagosome ultimately fuses with a lysosome that facilitates the turnover of engulfed material by lysosomal/vacuolar acid hydrolases.

Importantly, the aberrant ATG genes were crucial for tumorigenesis and cancer progression. As the central player indispensable for the first phases of autophagy, Beclin 1 is monoallelically deleted in human ovarian, breast and prostate cancers, and might be a prognostic marker in a variety of solid tumors [22]. Ectopic ATG10 and ULK1 were significantly correlated with lymph node metastasis and a poor OS for colorectal and breast cancers [23, 24]. Nevertheless, the clinicopathological values of ATG proteins in cancer remain debatable. Beclin 1 high expression, for example, a favorable biomarker to predict outcome for gastric cancer, hepatocellular carcinoma and high-grade gliomas [25-29] and also as an inferior prognostic biomarker for nasopharyngeal carcinoma [30]. For gastric cancer, the clinicopathological significance of ATG proteins, except Beclin 1 [27-29] had not yet been characterized.

Collected data suggesting that key autophagy regulatory ATG proteins contribute to tumorigenesis and tumor progression, and may predict prognosis in human cancers. In order to find out the relationship between ATG proteins and clinicopathological parameters, especially, the prognostic value in gastric cancer, we detected the clinicopathological value of 9 known ATG proteins (ULK1, Beclin 1, ATG3, ATG5, ATG7, ATG9, ATG10, ATG12 and LC3B) and p62/SQSTM1, which represented key regulators that participated in whole autophagosomes stepwise processes in a large cohort of 352 gastric cancers.

Materials and methods

This study was approved by the Human Ethics Committee of the Sixth Affiliated Hospital, Sun Yat-sen University. A written informed consent was obtained from all the patients at the time of admission, with which the tissue, blood and other samples might be used for scientific research but did not relate to patient's privacy.

Patients

A total of 352 primary gastric cancer patients who underwent initial surgical resection were recruited in the present study. The archived formalin-fixed, paraffin-embedded tissues were collected in both the First Affiliated Hospital and the Sixth Affiliated Hospital of Sun Yat-sen University from January 2002 to October 2006. Of these patients, 117 cases were randomly assigned to the training set to assess the clinicopathological value of these 10 proteins, and another independent cohort of 235 patients were used as the testing set for further validation. Patients were selected by the following inclusion criteria: pathologically confirmed as gastric adenocarcinoma; without oncological surgery, chemotherapy, and radiotherapy history; completed follow-up information and par-

affin-embedded specimens; and received post-surgical chemotherapy depending on the severity of the disease and according to the National Comprehensive Cancer Network (NCCN) guidelines. Moreover, patient would be excluded for any of the following reasons: previously received any anticancer therapy; prior malignancy; and pregnancy. The patient stage was redefined according to 2010 AJCC staging system for gastric cancer [31]. The pathological differentiation of intestinal subtype denoted to papillary and/or tubular adenocarcinomas, and diffuse subtype included of poorly differentiated, signet-ring cell, and/or mucinous adenocarcinomas [32].

Tissue microarrays (TMAs) construction

TMAs were constructed in accordance with a previously described method [30]. For each case, two cores taken from the selected tumor area and additional one core from normal adjacent mucosa (within 2 cm distance from the tumor margin) were used to construct the TMAs. Briefly, a hollow needle was utilized to punch and remove bipartite cylinders tissue cores (1.0 mm in diameter) from selected donor tissues regions. Further, the punched tissue cores were inserted into a recipient paraffin block with a precisely spaced, array pattern, using an automatic tissue arraying instrument (Beecher Instruments, Silver Spring, Maryland, USA).

Immunohistochemical (IHC) staining

TMAs containing 352 gastric cancers were detected by IHC. The sections (4 μm thick) were dewaxed in xylene and rehydrated in gradient ethanol solutions. Endogenous peroxidase activity and nonspecific binding of antibodies were blocked by hydrogen peroxide and goat serum albumin, respectively. Antigenic retrieval was performed in EDTA antigenic retrieval buffer (pH 8.0) or citrate buffer (pH 6.0) with a microwave. Sections were then incubated with primary antibodies at 4°C overnight. A total of 10 proteins representing the key players in autophagosomes formation processes, consisting of initiation and nucleation: Beclin 1 (Cell Signaling, #3738, 1:100 dilution), ULK1 (Sigma-Aldrich, #A7481, 1:200 dilution) and ATG9 (Abcam, #ab108338, 1:100 dilution); elongation: ATG12 (Sigma-Aldrich, #A8731, 1:200 dilution), ATG7 (Sigma-Aldrich, #A2856, 1:200 dilution), ATG10 (MBL International, #M151-3, 1:300 dilution), ATG5 (Sigma-Aldrich, #A0731, 1:400 dilution); formation: ATG3 (Sigma-Aldrich, #A3606, 1:300 dilution), p62/SQSTM1 (MBL International, #PM045, 1:200 dilution) and LC3B (Sigma-Aldrich, #L7543, 1:300 dilution), were detected in this study. Then, the sections were washed in PBS buffer for 5 min 3 times, treated with 100 μl secondary antibody for 30 min at room temperature and stained with DAB (Dako, Envision System/DAB-chromogen, K5007, DK-2600 Glostrup, Denmark) according to the manufacturer's instructions. The sections were washed in PBS buffer for 10 min 3 times and counterstained with hematoxylin for 2 min at room temperature. Finally, the sections were washed in dH_2O for 5 min 3 times and mounted the coverslips. A negative control was utilized by changing the specific primary antibody with non-immune serum immunoglobulins at the 1:200 dilutions.

Semiquantitative assessment of IHC staining

The expression level of each protein was evaluated by combined assessment of staining intensity and extent as we previously described [33]. We scored the staining intensity as following: negative (score 0), bordering (score 1), weak (score 2), moderate (score 3) and strong (score 4). Staining extent was graded into five parts according to the percentage of elevated staining cells in the field: negative (score 0), 0-25% (score 1), 26-50% (score 2), 51-75% (score 3) and 76-100% (score 4). The multiplied overall score was subjected to further clinicopathological analysis. Immunohistochemical staining was evaluated and scored by two independent pathologists (Fan XJ and Cao QH) blinded to clinical follow-up data. In case completely different results occurred, they would work together to confirm the score.

Selection of cutoff score for each biomarker

The receiver operating characteristic (ROC) curve analysis was used to selection of cutoff score in the training set as we previously reported [33]. Briefly, the sensitivity and specificity for patient outcome at each score were plotted to generate a ROC curve. The score localized closest to the point at both maximum sensitivity and specificity, ie., the point (0.0, 1.0) on the curve, was identified as the cutoff score

Table 1. Expression status of autophagy related proteins in relation to patient characteristics in 352 gastric cancers

	ULK1		Beclin 1		ATG3		ATG5		ATG7		ATG9		ATG10		ATG12		P62/SQSTM1		LC3B	
	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High
Gender																				
Male	105	130	136	99	86	149	47	188	63	172	89	146	152	83	44	191	112	123	85	150
Female	65	52	72	45	38	79	29	88	34	83	56	61	68	49	18	99	57	60	38	79
P value[a]	0.070		0.565		0.479		0.336		0.704		0.085		0.244		0.462		0.910		0.553	
Age																				
≥ 58[b]	79	95	90	84	59	115	42	132	43	131	54	120	106	68	27	147	75	99	49	125
< 58	91	87	118	60	65	123	34	144	54	124	91	87	114	64	35	143	94	84	74	104
P value	0.288		0.007		0.912		0.300		0.283		< 0.001		0.583		0.330		0.071		0.010	
Location																				
Upper third	54	46	59	41	27	73	21	79	20	80	32	68	68	32	17	83	44	56	41	59
Middle third	34	62	59	37	34	62	14	82	20	76	38	58	53	43	15	81	48	48	36	60
Lower third	72	69	78	63	57	84	35	106	51	90	67	74	86	55	28	113	65	76	40	101
Whole	10	5	12	3	6	9	6	9	6	9	8	7	13	2	2	13	12	3	6	9
P value	0.017		0.288		0.188		0.083		0.010		0.077		0.062		0.809		0.067		0.192	
Size																				
≥ 5 cm	111	101	131	81	72	140	50	162	66	146	94	118	138	74	43	169	105	107	82	130
< 5 cm	59	81	77	63	53	87	26	114	31	109	51	89	82	58	19	121	64	76	41	99
P value	0.065		0.224		0.495		0.291		0.069		0.151		0.219		0.117		0.514		0.087	
Histological type																				
Intestinal	126	152	162	116	81	197	50	228	63	215	108	170	170	108	38	240	119	159	85	193
Diffuse	44	30	46	28	43	31	26	48	34	40	37	37	50	24	24	50	50	24	38	36
P value	0.036		0.596		< 0.001		0.002		< 0.001		0.086		0.346		< 0.001		< 0.001		0.001	
Lymph node metastasis																				
With	143	102	161	84	94	151	58	187	73	172	106	139	176	69	49	196	125	120	91	154
Without	27	80	47	60	30	77	18	89	24	83	39	68	44	63	13	94	44	63	32	75
P value	< 0.001		< 0.001		0.069		0.162		0.194		0.242		< 0.001		0.094		0.104		0.224	
Tumor-Node-Metastasis stage																				
I	9	32	11	30	23	28	6	39	8	37	17	28	23	22	7	38	18	27	12	33
II	21	63	40	44	26	58	14	66	17	63	27	53	31	49	7	73	31	49	23	57
III	112	77	124	65	67	122	44	145	56	133	84	105	140	29	39	150	98	91	73	116
IV	28	10	33	5	18	20	12	26	16	22	17	21	26	12	9	29	22	16	15	23
P value	< 0.001		< 0.001		0.195		0.160		0.042		0.381		< 0.001		0.040		0.089		0.243	

[a]Chi-square test or Fisher's exact test. [b]median age.

that could be correctly classified patient outcome as death or alive.

Western blot analysis

Gastric cancer tissue samples preserved in liquid nitrogen were subsequently ground and lysed with the RIPA buffer (Sigma-Aldrich, R0278) for Western blot analysis. The protein concentration was tested by the Bradford method with BSA (Sigma-Aldrich, A4503) as the standard protein. Equal amounts of tissue extract were electrophilic run in SDS-polyacrylamide gel and transferred to nitrocellulose membrane (BioRad Laboratories, 162-0094) for antibody blotting. The membrane was further blocked and incubated with according primary antibodies (ULK1, Sigma-Aldrich, #A7481, 1:1000 dilution; Beclin 1, Cell Signaling, #3738, 1:1000 dilution; ATG3, Sigma-Aldrich, #A3606, 1:3000 dilution; ATG5, Sigma-Aldrich, #A0731, 1:1000 dilution; ATG7, Sigma-Aldrich, #A2856, 1:2000 dilution; ATG9, Abcam, #ab108338, 1:2000 dilution; ATG10, MBL International, #M151-3, 1:500 dilution; ATG12, Sigma-Aldrich, #A8731, 1:500 dilution; p62/SQSTM1, MBL International, #PM045, 1:1000 dilution and LC3B, Sigma-Aldrich, #L7543, 1:1000 dilution). The HRP labeled secondary antibody was diluted in 1% BBB buffer (1:15000). The membrane was then incubated in diluted secondary antibody for 1 hour at room temperature on an

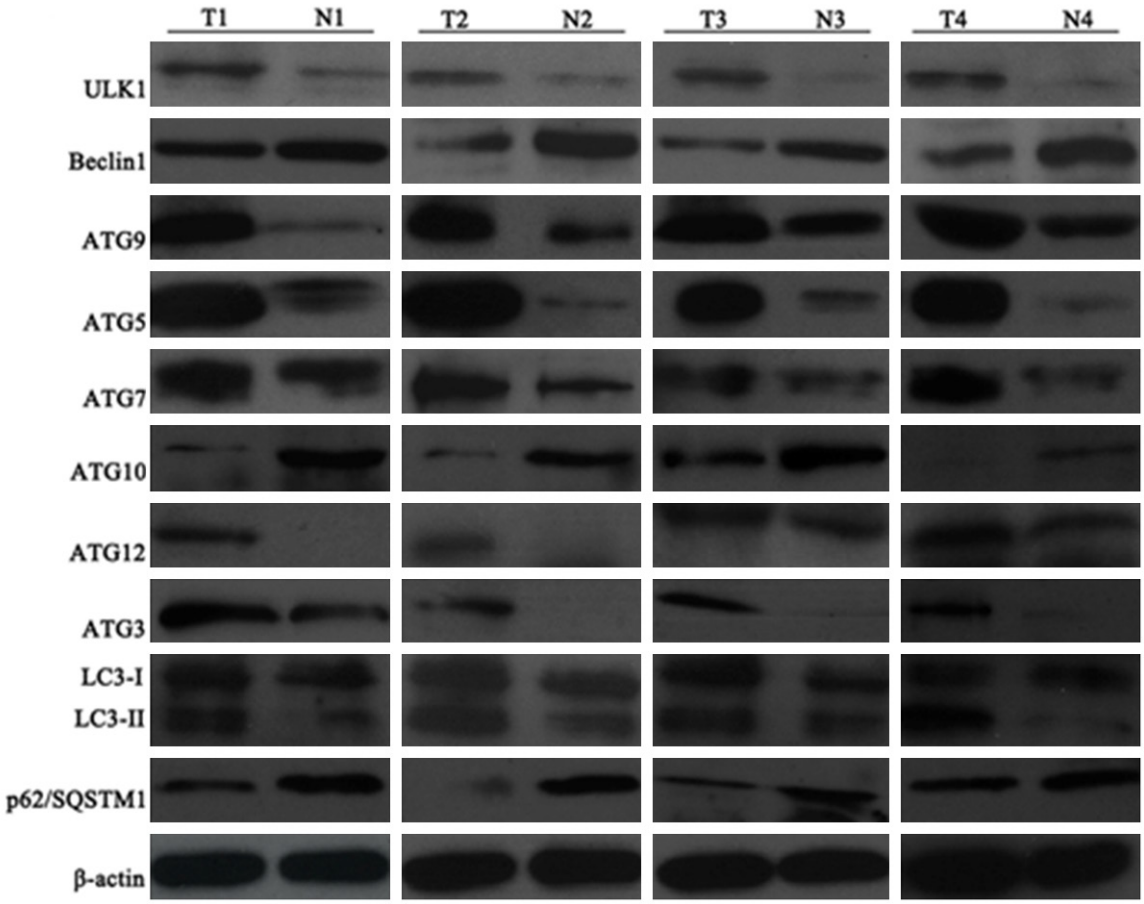

Figure 1. ATG proteins expression in human gastric cancer and non-tumor tissues. Western blot analysis of ATG expression in four pairs of matched gastric tumor (T) and normal adjacent epithelia (N). Equal loading of ATG proteins was determined by β-actin.

orbital shaker. The membrane was washed in the 1X PBST for 5 min 3 times. Finally, the membrane was incubated in 10 ml SignalFire™ ECL Reagent (CST, #6883) with gentle agitation for 1 min at room temperature, drained off excess developing solution, wrapped in plastic wrap and exposed to x-ray film.

Statistical analysis

In the training set, ROC analysis was employed to generate the IHC cutoff score for each ATG protein (SPSS-Analyze-ROC Curve). For validation, the clinicopathological value of each ATG proteins were evaluated in the testing set and overall patients. The chi-square test or Fisher's exact test was used to evaluate the relationship between each ATG proteins and clinicopathological variables (SPSS-Analyze-Descriptive Statistics--Crosstabs). The multivariate Cox proportional hazards model was utilized to estimate the hazard ratio (HR) and 95% confidence interval (CI) (SPSS-Survival-Cox Regression). The OS difference between patients subsets was analyzed by Kaplan-Meier analysis and log-rank tests (SPSS-Analyze-Kaplan-Meier). Statistically significant difference was considered if the *P* value from a two-tailed test was less than 0.05. Statistical analysis was performed using SPSS v. 17.0 (SPSS, Inc., Chicago, IL).

Results

Patient characteristics

Of these 352 patients, the median duration of overall survival (OS) for training set and testing set was 33.34 ± 2.75 months and 31.82 ± 1.74 months, respectively (P = 0.613). The clinico-

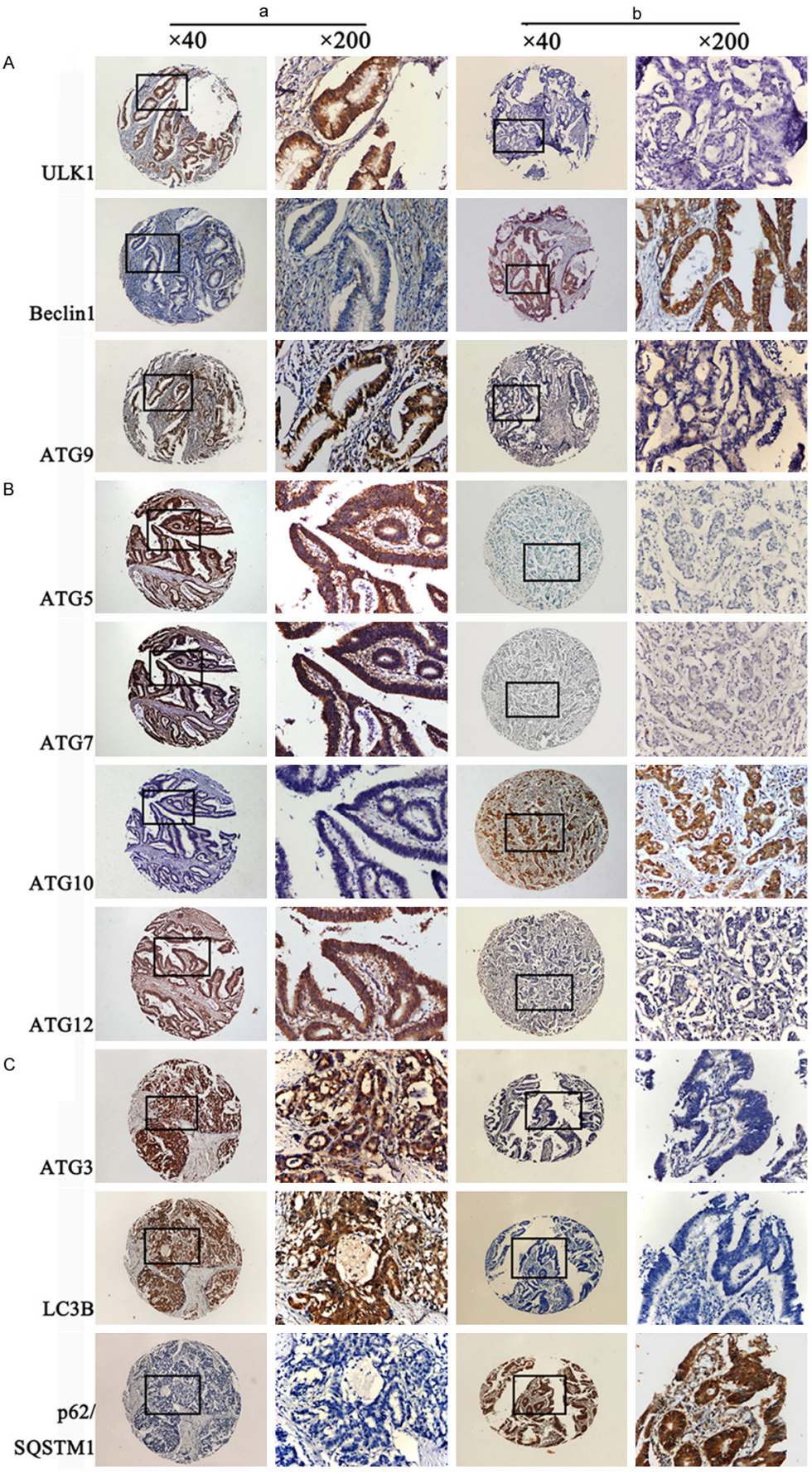
a
b
×40
×200
×40
×200
A
ULK1
Beclin1
ATG9
B
ATG5
ATG7
ATG10
ATG12
C
ATG3
LC3B
p62/
SQSTM1

Figure 2. The expression of ATG proteins in gastric cancer. A. The expression of representative ATG proteins at initiation step, containing ULK1, Beclin 1 and ATG9, In left panel a, immunohistochemistry (IHC) revealed high expression of ULK1, ATG9 and low expression of Beclin 1 in the case 36. In right panel b, IHC demonstrated low expression of ULK1, ATG9 and high expression of Beclin 1 in the case 157. B. The expression of representative ATG proteins at elongation step, including ATG5, ATG7, ATG10, ATG12. In left panel a, IHC showed high expression of ATG5, ATG7 ATG12 and low expression of ATG10 in the case 239. In right panel b, high expression of ATG10 and low expression of ATG5, ATG7, ATG12 were displayed in the same case 12. C. The expression of representative ATG proteins at maturation step containing ATG3, LC3B, p62/SQSTM1. In left panel a, IHC showed that ATG3 and LC3B were highly expressed, while p62/SQSTM1 was lowly expressed in the same case 65. In right panel b, the low expression of ATG3 and LC3B and high expression of p62/SQSTM1 in case 112. In panel a and b, the right figures displayed representative ATGs proteins expression in selected tumor zone with enlarged view.

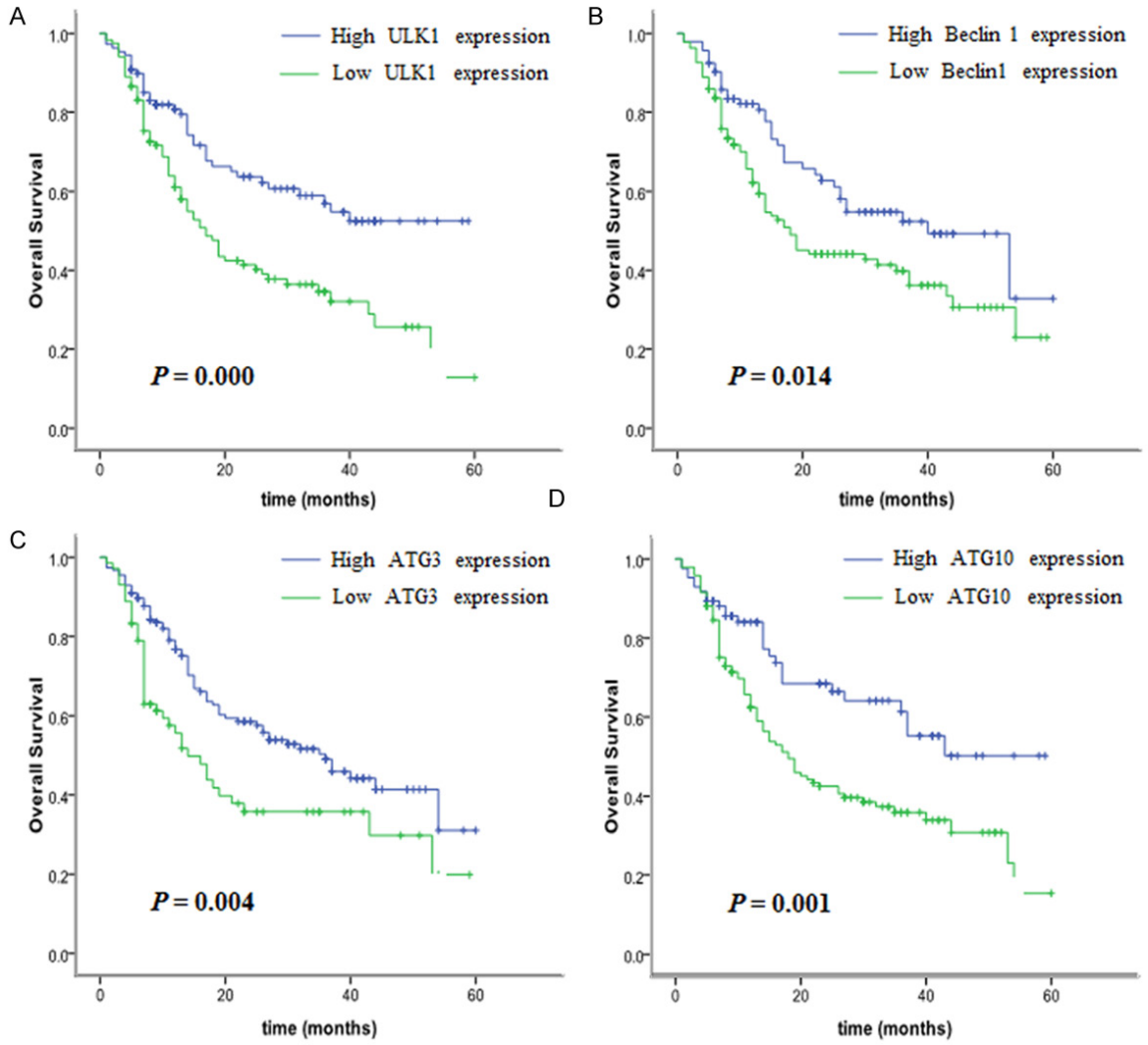

Figure 3. Kaplan-Meier estimated of overall survival according to ATG proteins expression level in testing set. In testing set, high expression of ULK1 (A), Beclin 1 (B), ATG3 (C) and ATG10 (D) were closely correlated with favorable overall survival.

pathological features of these two subgroups, including gender, age, tumor location, tumor size, histological type, TNM stage, and lymph node metastases status, were all comparable (**Table 1** and Table S1). Moreover, the Kaplan-Meier survival analysis showed that the training set and testing set had the similar overall survival probability (data not shown), indicating a balanced clinicopathological features of both cohorts for further analysis.

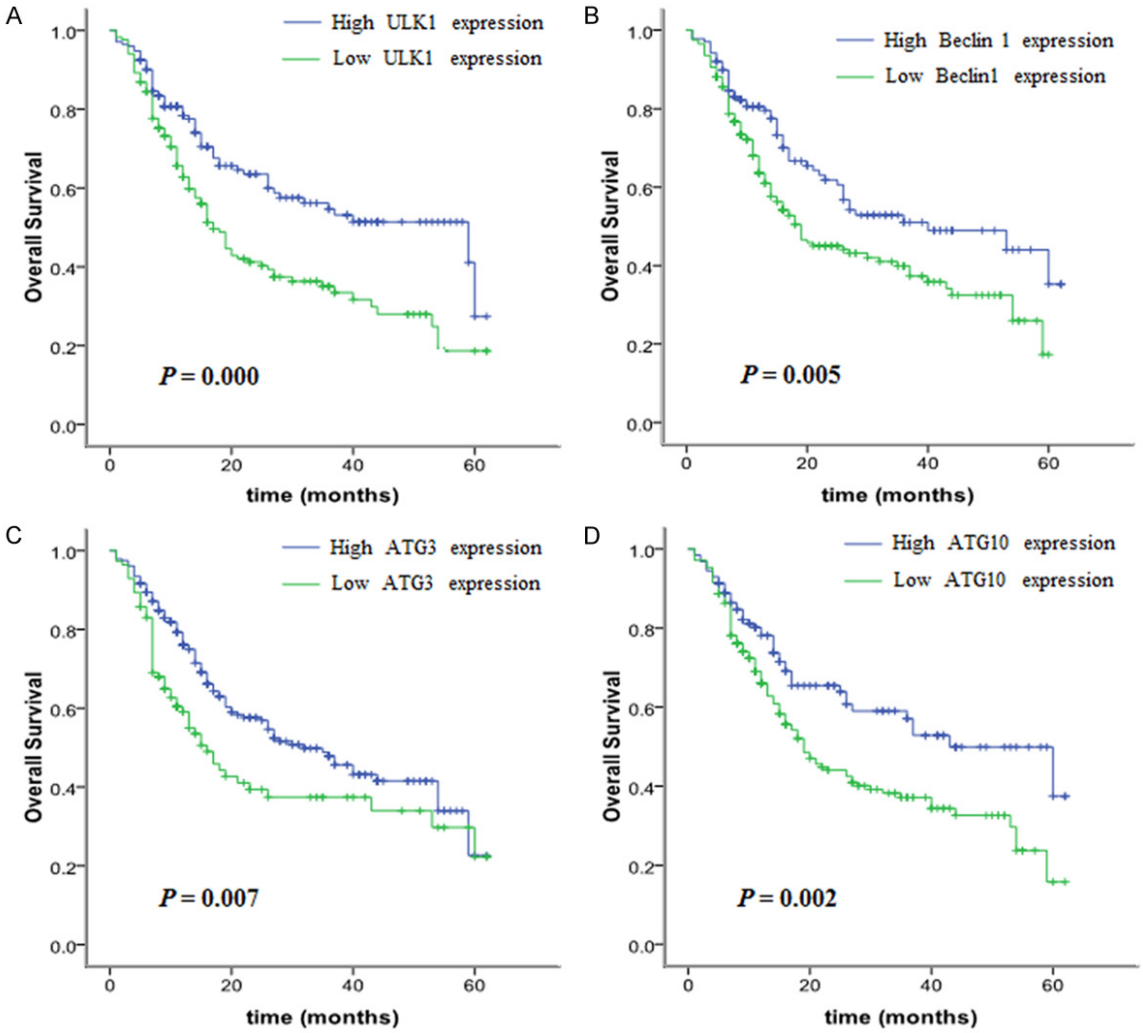

Figure 4. Kaplan-Meier estimated of overall survival according to ATG proteins expression level in overall patients. In overall patients, high expression of ULK1 (A), Beclin 1 (B), ATG3 (C) and ATG10 (D) were closely correlated with favorable overall survival.

ATG proteins expression status in gastric cancer tumor and normal adjacent tissue

As shown in **Figure 1** and Figure S2, Beclin 1, ATG10 and p62/SQSTM1 were respectively weakly expressed in gastric cancer tissues, whereas strongly expressed in normal adjacent tissues. Conversely, the other 7 markers, including ULK1, ATG3, ATG5, ATG7, ATG9, ATG12 and LC3B, were highly expressed in the gastric cancer tissues, and lowly or moderately expressed in adjacent non-tumor tissues. In addition, the western blot analysis showed that the LC3-II expression level was up-regulated in the tumor tissues than adjacent normal tissues. Interestingly, we also observed an increased LC3-II to LC3-I ratio in the tumor tissues, indicating an activation of autophagy (**Figure 1**).

Correlations between ATG proteins expression and clinicopathological features

In the training set, ROC analysis showed that the IHC cutoff scores of ULK1, Beclin 1, ATG3, ATG5, ATG7, ATG9, ATG10, ATG12, LC3B and p62/SQSTM1 were 8, 6, 6, 8, 8, 6, 6, 6, 8 and 8, respectively. Dichotomized these proteins according to their own cutoff scores, we found that ULK1, Beclin 1, ATG3, ATG5, ATG7, ATG9, ATG10, ATG12, LC3B and p62/SQSTM1 were respectively highly expressed in 51.7%, 40.9%, 66.8%, 78.4%, 72.4%, 58.8%, 37.5%, 82.4%, 65.1% and 52.0% of overall patients (**Figure 2**;

Table 2. Cox multivariate regression analyses of ATG proteins, p62/SQSTM1 expression and clinicophathological variables on overall survival in testing set and overall patients

Variable	Testing set			Overall patients		
	HR	95% CI	*P* value	HR	95% CI	*P* value
ULK1 (low VS high)	0.626	0.405-0.966	0.034	0.620	0.435-0.884	0.008
Beclin 1 (low VS high)	0.625	0.401-0.974	0.038	0.617	0.437-0.872	0.006
ATG3 (low VS high)	0.591	0.384-0.910	0.017	0.703	0.498-0.992	0.045
ATG5 (low VS high)	0.945	0.601-1.486	0.807	1.218	0.835-1.778	0.306
ATG7 (low VS high)	0.926	0.560-1.532	0.764	0.977	0.645-1.480	0.913
ATG9 (low VS high)	1.226	0.789-1.905	0.366	1.210	0.845-1.735	0.298
ATG10 (low VS high)	0.577	0.360-0.923	0.022	0.642	0.440-0.938	0.022
ATG12 (low VS high)	1.437	0.804-2.659	0.222	1.164	0.740-1.831	0.512
P62/SQSTM1 (low VS high)	1.054	0.591-1.879	0.859	1.056	0.658-1.695	0.822
LC3B (low VS high)	1.065	0.625-1.814	0.817	1.214	0.750-1.963	0.430
Gender (male VS female)	1.012	0.659-1.554	0.957	1.022	0.721-1.450	0.902
Age (≥ 58 VS < 58)	1.366	0.894-2.088	0.149	1.288	0.915-1.811	0.146
Location (upper third & middle third VS lower third & whole)	0.909	0.603-1.372	0.650	0.867	0.622-1.210	0.403
Size (≥ 5 cm VS < 5 cm)	0.992	0.654-1.504	0.96	1.012	0.727-1.408	0.944
Histological type (intestinal VS diffuse)	0.535	0.329-0.862	0.010	0.501	0.331-0.759	0.001

Abbreviation: TNM, Tumor-Node-Metastasis stage.

Table 1). Moreover, we also detected the similar expression status of these 10 markers in the training set and testing set (Table S1).

As shown in **Table 1**, Beclin 1, ATG9 and LC3B were closely correlated with age in overall patients ($P = 0.007$, $P < 0.001$ and $P = 0.010$, respectively). In addition, ULK1 and ATG7 expression levels were significantly varied among different tumor locations ($P = 0.017$ and $P = 0.010$, respectively). In addition, comparing intestinal with diffuse gastric cancer subtypes, significantly different ULK1, ATG3, ATG5, ATG7, ATG12, p62/SQSTM1 and LC3B expression levels were detected ($P = 0.036$, $P < 0.001$, $P = 0.002$, $P < 0.001$, $P < 0.001$, $P < 0.001$ and $P = 0.001$, respectively). Importantly, low expression of ULK1, Beclin 1 and ATG10 were associated with lymph node metastasis ($P < 0.001$, $P < 0.001$ and $P < 0.001$, respectively) and advanced TNM stage ($P < 0.001$, $P < 0.001$ and $P < 0.001$, respectively). Moreover, low expression of ATG7 and ATG10 correlated with high TNM stage ($P = 0.042$ and $P = 0.040$, respectively).

ATG proteins expression and survival analysis

As shown in **Figure 3A**, the median OS duration for the subgroup with high ULK1 expression was 38.6 months, whereas was 26.2 months for the subgroup with low ULK1 expression in testing set ($P < 0.001$). Moreover, the similar OS difference between high and low ULK expression subgroups was also obtained in overall patients (median OS: 39.1 VS 27.7 months, $P < 0.001$, **Figure 4A**). Similarly, the statistically significant OS differences between the highly and lowly expressed subgroups were also observed for Beclin 1 (**Figures 3B** and **4B**), ATG3 (**Figures 3C** and **4C**) and ATG10 (**Figures 3D** and **4D**) in the testing set and overall patients. However, we failed to detect the prognostic values for ATG5 (Figures S3A and S4A), ATG7 (Figures S3B and S4B), ATG9 (Figures S3C and S4C), ATG12 (Figures S3D and S4D), LC3B (Figures S3E and S4E) and p62/SQSTM1 (Figures S3F and S4F) in both testing set and overall patients (all $P > 0.05$).

Importantly, Cox multivariate regression analyses demonstrated that ULK1 (HR: 0.620, $P = 0.008$), Beclin 1 (HR: 0.617, $P = 0.006$), ATG3 (HR: 0.703, $P = 0.045$) and ATG10 (HR: 0.642, $P = 0.022$) were indeed the independent indicators to predict the prognosis for overall patients with gastric cancer (**Table 2**). Interestingly, we found that histological type (HR: 0.501, $P = 0.001$), other than gender, age, tumor location and size, was also displayed a significantly predictive value for OS (**Table 2**).

Combination analysis of ULK1, Beclin 1, ATG3, ATG10 expression and survival

To analyze the prognostic value of combining ULK1, Beclin 1, ATG3, ATG10 for gastric cancer, we divided the patients into five groups: without

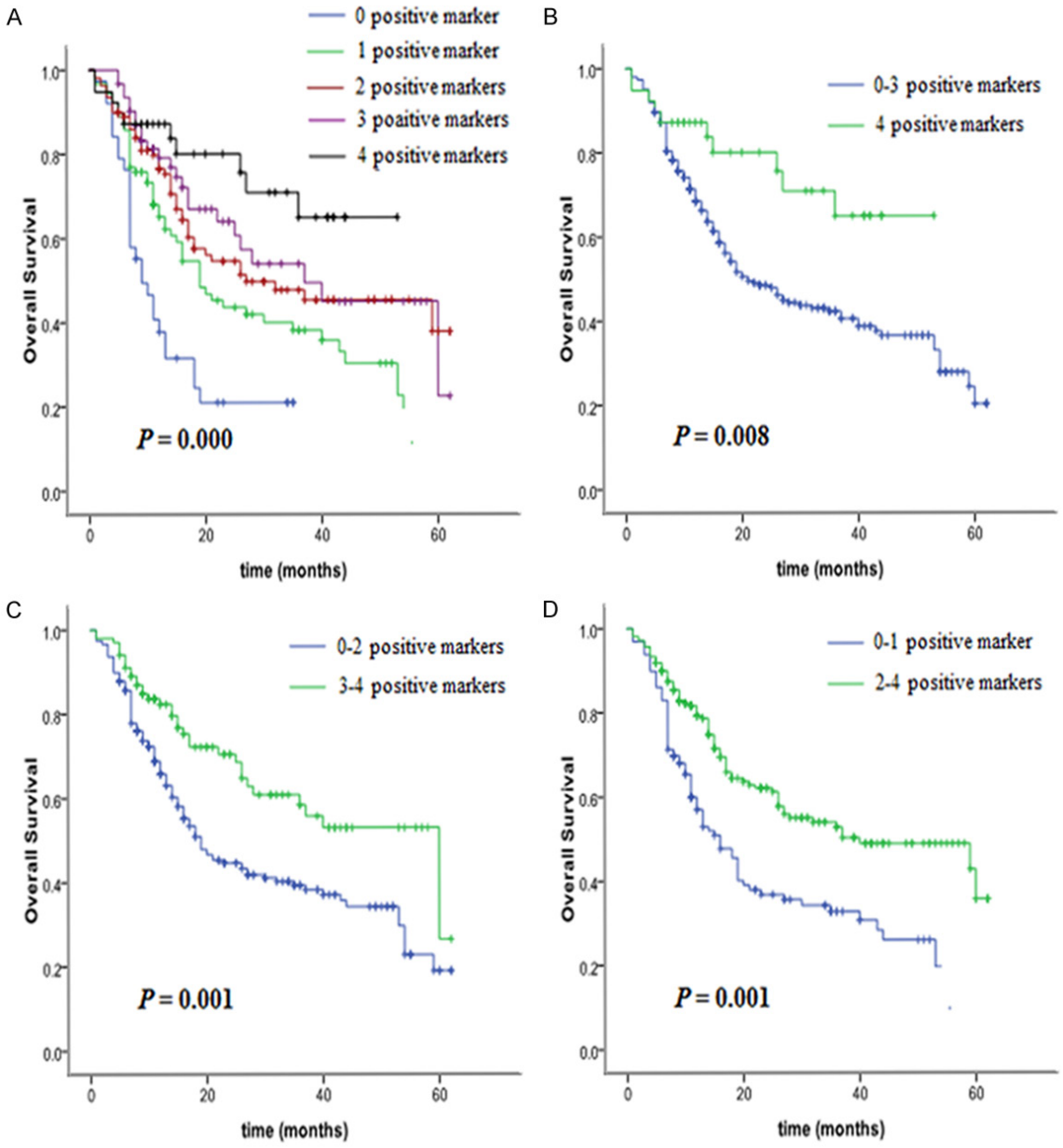

Figure 5. Combination of ULK1, Beclin 1, ATG3, ATG10 expression and overall survival. A. Survival curve of patients without positive marker (0), with only one positive marker (1), with two (2), three (3) and four (4) positive markers. B. Patients with four positive markers (4) displayed a superior prognosis compared with those with zero-to-three positive makers (0-3). C. Patients with three-to-four positive markers (3-4) displayed a favorable prognosis compared with those with zero-to-two positive makers (0-2). D. Patients with two-to-four positive markers (2-4) displayed a better survival compared with those with zero-to-one positive maker (0-1).

positive maker (0), with only one positive marker (1), with two (2), three (3) and four (4) positive markers. The OS curve of these five subgroups was showed respectively in **Figure 5A**. More importantly, patients with four positive markers displayed a superior survival compared with those with 0-3 positive makers (P = 0.008, **Figure 5B**). The same difference was found between patients with 0-2 positive makers and those with 3-4 positive markers (P = 0.001, **Figure 5C**), patients with 0-1 positive makers and those with 2-4 positive markers (P < 0.001, **Figure 5D**).

Discussion

In this study, we assessed the expression pattern of 9 ATG proteins and p62/SQSTM1

involved the autophagosomes formation processes (ULK1, Beclin 1 and ATG9), elongation steps (ATG5, ATG7, ATG10 and ATG12), and maturation (ATG3, p62/SQSTM1 and LC3B). Our results revealed that low expression of ULK1, Beclin 1, ATG3 and ATG10 predicted a favorable prognosis in a 5-year follow-up analysis. More importantly, the four-biomarker-based combination had a significantly better prognosis compared with one to three-protein-based combination. Furthermore, multivariate analysis revealed that ULK1, Beclin 1, ATG3 and ATG10 were indeed the significant independent prognostic factors for alive, not the death.

ULK1, Beclin 1 and ATG9 play important roles at the autophagosomes formation processes [10, 12, 34]. In non-small cell lung cancer, activation of ULK1 preserved cancer cell cytoskeletal dynamics and released the cell motility effector FAK, leading to enhanced metastatic dissemination to bone/liver [35]. Beclin 1 is classified as a haplo-insufficient tumor suppressor gene [22, 36]. Knockdown of Beclin 1 enhanced chemotherapy-induced apoptosis and reduced clone formation and impaired cell growth of hepatocellular carcinoma cells [37]. Indeed, accumulated studies demonstrated that ULK1 and Beclin 1 deficiency were closely correlated with poor clinicopathological features, including lymph node metastasis, advanced TNM stage and unfavorable outcome, in a variety of cancers [24-26, 30, 38, 39]. Consistent with previous studies, low expressions of ULK1 and Beclin 1 were correlated with lymph node metastasis, advanced TNM stage and poor prognosis. Interestingly, ULK1 expression was up-regulated in gastric tumor compared to adjacent normal tissues, and predicted a favorable OS, suggesting ULK1 playing a tumor suppressor gene role in the gastric cancer. However, the underlying mechanism is not clear and need to be further investigated. Unfortunately, we did not find any association between ATG9 with clinicopathological variables except age. Importantly, multivariate analysis confirmed that ULK1 and Beclin 1 were the independent prognostic biomarker for gastric cancer, suggesting that autophagosomes initiation step played an essential role in regulating cancer cell metastasis and predicting patient prognosis for gastric cancer.

At autophagosome elongation steps, ATG12 is activated by ATG7 (E1) and ATG10 (E2), and followed by covalent linkage to an internal lysine on ATG5 [14, 15]. Functionally, inhibition of Atg5/7 by siRNA or chloroquine suppressed cancer cell epithelial-mesenchymal transition (EMT) and diminished invasiveness for hepatocellular carcinoma [40]. Serum deprivation of mesenchymal stem cells (MSCs) upregulated ATG10 and ATG12, and further activated autophagy to facilitate MCF-7 breast cancer cell survival and progression [41], indicating that autophagosome elongation step contributed to tumorigenesis and tumor progression. In the present study, the associations between the expression of ATG5, ATG7, ATG10, ATG12 and the clinicopathological parameters were investigated. We further confirmed that deficiency of ATG10 and ATG12 is associated with lymph node metastasis in gastric cancer (**Table 1** and Table S1). Significantly, our multivariate analysis demonstrated that ATG10 was an independent favorable prognostic biomarker for gastric cancer. Similar to the controversial role of autophagy and Beclin 1 in tumorigenesis and predicting prognosis, our study showed an opposite outcome of the prognostic impact of ATG10 when compared with the previous study. Jo YK et al [23] reported that increased expression of ATG10 was associated with lymphovascular invasion and lymph node metastasis, and predicted an unfavorable OS for colorectal cancer. The possibility was that ATG10 may have different roles in different types of cancer, the expression of ATG10 was also depending upon the cancer cell types. However, we did not find any association of ATG5, ATG7, ATG12 expression with patients' OS.

At the last step of autophagosome maturation, ATG7 (E1) activates LC3 and transfers it to ATG3 (E2), and subsequent converses LC3-I into LC3-II [16, 17, 42]. Together with ATG5/ATG12, p62/SQSTM1 bound LC3-II coats the outer surfaces of the autophagosome. In the present study, we found that high expression of ATG3 predicted a favorable OS for gastric cancer. To our best of knowledge, this is the first report about the positive prognostic value of ATG3 in human cancers. Consistent with reported results, an in vitro study found that siRNA targeting ATG3 and ATG7 evidently decreased AKT inhibitor AZD5363 treated prostate cancer cell proliferation and survival [43]. Inversely, high-expressed ATG3 gene and protein, the myelodysplastic syndrome SKM-1 cell line was inhibited cell proliferation and vitality. The

malignancy of SKM-1 cell line was decreased after transfected with ATG3 [44]. Similar to ULK1, the expression of ATG3 was upregulated in tumor tissues, but played a role as putative suppressor gene. The potential reason may be complicated. Our ongoing study will focus on the mechanisms research. These data demonstrated that ATG3 may play discriminatory role in different types of cancer and/or phases of tumorigenesis. The underlying mechanism linking ATG3 and tumor progression will be further investigated. However, the statistically OS difference was not shown between the subgroups with high or low expression of LC3 and p62/SQSTM1. Nonetheless, previous studies showed that increased LC3 expression typically has poor prognosis in lung and pancreatic cancers and melanoma [45, 46] while decreased LC3 expression has been correlated with tumorigenesis and an inferior prognosis in breast cancer and early stage cervical squamous cell carcinoma [47, 48]. When it comes to p62/SQSTM1, the results of prognostic value were also divers depending on different cancer types. The role of p62/SQSTM1 as an inferior prognostic marker has been reported in lung adenocarcinoma and prostate cancer [49]. In contrast to this, Ellis RA et al showed that high p62 expression status predict longer disease-free survival in cutaneous malignant melanoma [50]. Combined, these results indicated that ATG proteins play different roles in different cancer types and stage, and autophagosome maturation ATG may mediate tumor progression in gastric cancer.

Moreover, we interestingly found that, histological type, other than gender, age, tumor location and size, was also an independent factor for OS. The patients with diffuse type had an inferior OS compared to those with intestinal type [51]. We founded that different tumor locations did not show any predictive value for gastric survival. Some reports revealed that the patients with upper third gastric cancer showed a poorer prognosis than those with other locations due to the stomach anatomy, complex lymphatic systems, or technical difficulties during surgery [52, 53].

In addition, there are limitations in the present study. The absence of data for metastasis and progression analyses was one limitation. Furthermore, another independent validation cohort with larger sample size from multicenter was needed to evaluate the prognostic value of ATG proteins in patients with gastric cancer.

Taken together, our study demonstrated that autophagosomes formation processes related ATG proteins are associated with histological differentiation, refined the risk to lymph node metastasis, and might to be the prognostic indicators for gastric cancer. Also, these findings supply a comprehensive understanding of correlation between autophagy and gastric cancer, and new valuable insight into cancer treatment targeting autophagy for gastric cancer patients.

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Disclosure of conflict of interest

None.

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References

[1] Cunningham D and Chua YJ. East meets west in the treatment of gastric cancer. N Engl J Med 2007; 357: 1863-1865.

[2] Macdonald JS, Smalley SR, Benedetti J, Hundahl SA, Estes NC, Stemmermann GN, Haller DG, Ajani JA, Gunderson LL, Jessup JM and Martenson JA. Chemoradiotherapy after surgery compared with surgery alone for adenocarcinoma of the stomach or gastroesophageal junction. N Engl J Med 2001; 345: 725-730.

[3] Terashima M, Kitada K, Ochiai A, Ichikawa W, Kurahashi I, Sakuramoto S, Katai H, Sano T, Imamura H, Sasako M; ACTS-GC Group. Impact of expression of human epidermal growth factor receptors EGFR and ERBB2 on survival in stage II/III gastric cancer. Clin Cancer Res 2012; 18: 5992-6000.

[4] Begnami MD, Fukuda E, Fregnani JH, Nonogaki S, Montagnini AL, da Costa WL Jr and Soares FA. Prognostic implications of altered human epidermal growth factor receptors (HERs) in gastric carcinomas: HER2 and HER3 are predictors of poor outcome. J Clin Oncol 2011; 29: 3030-3036.

[5] Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Ruschoff J, Kang YK; ToGA Trial Investigators. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. Lancet 2010; 376: 687-697.

[6] Xie Z and Klionsky DJ. Autophagosome formation: core machinery and adaptations. Nat Cell Biol 2007; 9: 1102-1109.

[7] Bodemann BO, Orvedahl A, Cheng T, Ram RR, Ou YH, Formstecher E, Maiti M, Hazelett CC, Wauson EM, Balakireva M, Camonis JH, Yeaman C, Levine B and White MA. RalB and the exocyst mediate the cellular starvation response by direct activation of autophagosome assembly. Cell 2011; 144: 253-267.

[8] Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, Agholme L, Agnello M, Agostinis P, Aguirre-Ghiso JA, Ahn HJ, Ait-Mohamed O, Ait-Si-Ali S, Akematsu T, Akira S, Al-Younes HM, Al-Zeer MA, Albert ML, Albin RL, Alegre-Abarrategui J, Aleo MF, Alirezaei M, Almasan A, Almonte-Becerril M, Amano A, Amaravadi R, Amarnath S, Amer AO, Andrieu-Abadie N, Anantharam V, Ann DK, Anoopkumar-Dukie S, Aoki H, Apostolova N, Arancia G, Aris JP, Asanuma K, Asare NY, Ashida H, Askanas V, Askew DS, Auberger P, Baba M, Backues SK, Baehrecke EH, Bahr BA, Bai XY, Bailly Y, Baiocchi R, Baldini G, Balduini W, Ballabio A, Bamber BA, Bampton ET, Banhegyi G, Bartholomew CR, Bassham DC, Bast RC Jr, Batoko H, Bay BH, Beau I, Bechet DM, Begley TJ, Behl C, Behrends C, Bekri S, Bellaire B, Bendall LJ, Benetti L, Berliocchi L, Bernardi H, Bernassola F, Besteiro S, Bhatia-Kissova I, Bi X, Biard-Piechaczyk M, Blum JS, Boise LH, Bonaldo P, Boone DL, Bornhauser BC, Bortoluci KR, Bossis I, Bost F, Bourquin JP, Boya P, Boyer-Guittaut M, Bozhkov PV, Brady NR, Brancolini C, Brech A, Brenman JE, Brennand A, Bresnick EH, Brest P, Bridges D, Bristol ML, Brookes PS, Brown EJ, Brumell JH, Brunetti-Pierri N, Brunk UT, Bulman DE, Bultman SJ, Bultynck G, Burbulla LF, Bursch W, Butchar JP, Buzgariu W, Bydlowski SP, Cadwell K, Cahova M, Cai D, Cai J, Cai Q, Calabretta B, Calvo-Garrido J, Camougrand N, Campanella M, Campos-Salinas J, Candi E, Cao L, Caplan AB, Carding SR, Cardoso SM, Carew JS, Carlin CR, Carmignac V, Carneiro LA, Carra S, Caruso RA, Casari G, Casas C, Castino R, Cebollero E, Cecconi F, Celli J, Chaachouay H, Chae HJ, Chai CY, Chan DC, Chan EY, Chang RC, Che CM, Chen CC, Chen GC, Chen GQ, Chen M, Chen Q, Chen SS, Chen W, Chen X, Chen X, Chen X, Chen YG, Chen Y, Chen Y, Chen YJ, Chen Z, Cheng A, Cheng CH, Cheng Y, Cheong H, Cheong JH, Cherry S, Chess-Williams R, Cheung ZH, Chevet E, Chiang HL, Chiarelli R, Chiba T, Chin LS, Chiou SH, Chisari FV, Cho CH, Cho DH, Choi AM, Choi D, Choi KS, Choi ME, Chouaib S, Choubey D, Choubey V, Chu CT, Chuang TH, Chueh SH, Chun T, Chwae YJ, Chye ML, Ciarcia R, Ciriolo MR, Clague MJ, Clark RS, Clarke PG, Clarke R, Codogno P, Coller HA, Colombo MI, Comincini S, Condello M, Condorelli F, Cookson MR, Coombs GH, Coppens I, Corbalan R, Cossart P, Costelli P, Costes S, Coto-Montes A, Couve E, Coxon FP, Cregg JM, Crespo JL, Cronje MJ, Cuervo AM, Cullen JJ, Czaja MJ, D'Amelio M, Darfeuille-Michaud A, Davids LM, Davies FE, De Felici M, de Groot JF, de Haan CA, De Martino L, De Milito A, De Tata V, Debnath J, Degterev A, Dehay B, Delbridge LM, Demarchi F, Deng YZ, Dengjel J, Dent P, Denton D, Deretic V, Desai SD, Devenish RJ, Di Gioacchino M, Di Paolo G, Di Pietro C, Diaz-Araya G, Diaz-Laviada I, Diaz-Meco MT, Diaz-Nido J, Dikic I, Dinesh-Kumar SP, Ding WX, Distelhorst CW, Diwan A, Djavaheri-Mergny M, Dokudovskaya S, Dong Z, Dorsey FC, Dosenko V, Dowling JJ, Doxsey S, Dreux M, Drew ME, Duan Q, Duchosal MA, Duff K, Dugail I, Durbeej M, Duszenko M, Edelstein CL, Edinger AL, Egea G, Eichinger L, Eissa NT, Ekmekcioglu S, El-Deiry WS, Elazar Z, Elgendy M, Ellerby LM, Eng KE, Engelbrecht AM, Engelender S, Erenpreisa J, Escalante R, Esclatine A, Eskelinen EL, Espert L, Espina V, Fan H, Fan J, Fan QW, Fan Z, Fang S, Fang Y, Fanto M, Fanzani A, Farkas T, Farre JC, Faure

M, Fechheimer M, Feng CG, Feng J, Feng Q, Feng Y, Fesus L, Feuer R, Figueiredo-Pereira ME, Fimia GM, Fingar DC, Finkbeiner S, Finkel T, Finley KD, Fiorito F, Fisher EA, Fisher PB, Flajolet M, Florez-McClure ML, Florio S, Fon EA, Fornai F, Fortunato F, Fotedar R, Fowler DH, Fox HS, Franco R, Frankel LB, Fransen M, Fuentes JM, Fueyo J, Fujii J, Fujisaki K, Fujita E, Fukuda M, Furukawa RH, Gaestel M, Gailly P, Gajewska M, Galliot B, Galy V, Ganesh S, Ganetzky B, Ganley IG, Gao FB, Gao GF, Gao J, Garcia L, Garcia-Manero G, Garcia-Marcos M, Garmyn M, Gartel AL, Gatti E, Gautel M, Gawriluk TR, Gegg ME, Geng J, Germain M, Gestwicki JE, Gewirtz DA, Ghavami S, Ghosh P, Giammarioli AM, Giatromanolaki AN, Gibson SB, Gilkerson RW, Ginger ML, Ginsberg HN, Golab J, Goligorsky MS, Golstein P, Gomez-Manzano C, Goncu E, Gongora C, Gonzalez CD, Gonzalez R, Gonzalez-Estevez C, Gonzalez-Polo RA, Gonzalez-Rey E, Gorbunov NV, Gorski S, Goruppi S, Gottlieb RA, Gozuacik D, Granato GE, Grant GD, Green KN, Gregorc A, Gros F, Grose C, Grunt TW, Gual P, Guan JL, Guan KL, Guichard SM, Gukovskaya AS, Gukovsky I, Gunst J, Gustafsson AB, Halayko AJ, Hale AN, Halonen SK, Hamasaki M, Han F, Han T, Hancock MK, Hansen M, Harada H, Harada M, Hardt SE, Harper JW, Harris AL, Harris J, Harris SD, Hashimoto M, Haspel JA, Hayashi S, Hazelhurst LA, He C, He YW, Hebert MJ, Heidenreich KA, Helfrich MH, Helgason GV, Henske EP, Herman B, Herman PK, Hetz C, Hilfiker S, Hill JA, Hocking LJ, Hofman P, Hofmann TG, Hohfeld J, Holyoake TL, Hong MH, Hood DA, Hotamisligil GS, Houwerzijl EJ, Hoyer-Hansen M, Hu B, Hu CA, Hu HM, Hua Y, Huang C, Huang J, Huang S, Huang WP, Huber TB, Huh WK, Hung TH, Hupp TR, Hur GM, Hurley JB, Hussain SN, Hussey PJ, Hwang JJ, Hwang S, Ichihara A, Ilkhanizadeh S, Inoki K, Into T, Iovane V, Iovanna JL, Ip NY, Isaka Y, Ishida H, Isidoro C, Isobe K, Iwasaki A, Izquierdo M, Izumi Y, Jaakkola PM, Jaattela M, Jackson GR, Jackson WT, Janji B, Jendrach M, Jeon JH, Jeung EB, Jiang H, Jiang H, Jiang JX, Jiang M, Jiang Q, Jiang X, Jiang X, Jimenez A, Jin M, Jin S, Joe CO, Johansen T, Johnson DE, Johnson GV, Jones NL, Joseph B, Joseph SK, Joubert AM, Juhasz G, Juillerat-Jeanneret L, Jung CH, Jung YK, Kaarniranta K, Kaasik A, Kabuta T, Kadowaki M, Kagedal K, Kamada Y, Kaminskyy VO, Kampinga HH, Kanamori H, Kang C, Kang KB, Kang KI, Kang R, Kang YA, Kanki T, Kanneganti TD, Kanno H, Kanthasamy AG, Kanthasamy A, Karantza V, Kaushal GP, Kaushik S, Kawazoe Y, Ke PY, Kehrl JH, Kelekar A, Kerkhoff C, Kessel DH, Khalil H, Kiel JA, Kiger AA, Kihara A, Kim DR, Kim DH, Kim DH, Kim EK, Kim HR, Kim JS, Kim JH, Kim JC, Kim JK, Kim PK, Kim SW, Kim YS, Kim Y, Kimchi A, Kimmelman AC, King JS, Kinsella TJ, Kirkin V, Kirshenbaum LA, Kitamoto K, Kitazato K, Klein L, Klimecki WT, Klucken J, Knecht E, Ko BC, Koch JC, Koga H, Koh JY, Koh YH, Koike M, Komatsu M, Kominami E, Kong HJ, Kong WJ, Korolchuk VI, Kotake Y, Koukourakis MI, Kouri Flores JB, Kovacs AL, Kraft C, Krainc D, Kramer H, Kretz-Remy C, Krichevsky AM, Kroemer G, Kruger R, Krut O, Ktistakis NT, Kuan CY, Kucharczyk R, Kumar A, Kumar R, Kumar S, Kundu M, Kung HJ, Kurz T, Kwon HJ, La Spada AR, Lafont F, Lamark T, Landry J, Lane JD, Lapaquette P, Laporte JF, Laszlo L, Lavandero S, Lavoie JN, Layfield R, Lazo PA, Le W, Le Cam L, Ledbetter DJ, Lee AJ, Lee BW, Lee GM, Lee J, Lee JH, Lee M, Lee MS, Lee SH, Leeuwenburgh C, Legembre P, Legouis R, Lehmann M, Lei HY, Lei QY, Leib DA, Leiro J, Lemasters JJ, Lemoine A, Lesniak MS, Lev D, Levenson VV, Levine B, Levy E, Li F, Li JL, Li L, Li S, Li W, Li XJ, Li YB, Li YP, Liang C, Liang Q, Liao YF, Liberski PP, Lieberman A, Lim HJ, Lim KL, Lim K, Lin CF, Lin FC, Lin J, Lin JD, Lin K, Lin WW, Lin WC, Lin YL, Linden R, Lingor P, Lippincott-Schwartz J, Lisanti MP, Liton PB, Liu B, Liu CF, Liu K, Liu L, Liu QA, Liu W, Liu YC, Liu Y, Lockshin RA, Lok CN, Lonial S, Loos B, Lopez-Berestein G, Lopez-Otin C, Lossi L, Lotze MT, Low P, Lu B, Lu B, Lu B, Lu Z, Luciano F, Lukacs NW, Lund AH, Lynch-Day MA, Ma Y, Macian F, MacKeigan JP, Macleod KF, Madeo F, Maiuri L, Maiuri MC, Malagoli D, Malicdan MC, Malorni W, Man N, Mandelkow EM, Manon S, Manov I, Mao K, Mao X, Mao Z, Marambaud P, Marazziti D, Marcel YL, Marchbank K, Marchetti P, Marciniak SJ, Marcondes M, Mardi M, Marfe G, Marino G, Markaki M, Marten MR, Martin SJ, Martinand-Mari C, Martinet W, Martinez-Vicente M, Masini M, Matarrese P, Matsuo S, Matteoni R, Mayer A, Mazure NM, McConkey DJ, McConnell MJ, McDermott C, McDonald C, McInerney GM, McKenna SL, McLaughlin B, McLean PJ, McMaster CR, McQuibban GA, Meijer AJ, Meisler MH, Melendez A, Melia TJ, Melino G, Mena MA, Menendez JA, Menna-Barreto RF, Menon MB, Menzies FM, Mercer CA, Merighi A, Merry DE, Meschini S, Meyer CG, Meyer TF, Miao CY, Miao JY, Michels PA, Michiels C, Mijaljica D, Milojkovic A, Minucci S, Miracco C, Miranti CK, Mitroulis I, Miyazawa K, Mizushima N, Mograbi B, Mohseni S, Molero X, Mollereau B, Mollinedo F, Momoi T, Monastyrska I, Monick MM, Monteiro MJ, Moore MN, Mora R, Moreau K, Moreira PI, Moriyasu Y, Moscat J, Mostowy S, Mottram JC, Motyl T, Moussa CE, Muller S, Muller S, Munger K, Munz C, Murphy LO, Murphy ME, Musaro A,

Mysorekar I, Nagata E, Nagata K, Nahimana A, Nair U, Nakagawa T, Nakahira K, Nakano H, Nakatogawa H, Nanjundan M, Naqvi NI, Narendra DP, Narita M, Navarro M, Nawrocki ST, Nazarko TY, Nemchenko A, Netea MG, Neufeld TP, Ney PA, Nezis IP, Nguyen HP, Nie D, Nishino I, Nislow C, Nixon RA, Noda T, Noegel AA, Nogalska A, Noguchi S, Notterpek L, Novak I, Nozaki T, Nukina N, Nurnberger T, Nyfeler B, Obara K, Oberley TD, Oddo S, Ogawa M, Ohashi T, Okamoto K, Oleinick NL, Oliver FJ, Olsen LJ, Olsson S, Opota O, Osborne TF, Ostrander GK, Otsu K, Ou JH, Ouimet M, Overholtzer M, Ozpolat B, Paganetti P, Pagnini U, Pallet N, Palmer GE, Palumbo C, Pan T, Panaretakis T, Pandey UB, Papackova Z, Papassideri I, Paris I, Park J, Park OK, Parys JB, Parzych KR, Patschan S, Patterson C, Pattingre S, Pawelek JM, Peng J, Perlmutter DH, Perrotta I, Perry G, Pervaiz S, Peter M, Peters GJ, Petersen M, Petrovski G, Phang JM, Piacentini M, Pierre P, Pierrefite-Carle V, Pierron G, Pinkas-Kramarski R, Piras A, Piri N, Platanias LC, Poggeler S, Poirot M, Poletti A, Pous C, Pozuelo-Rubio M, Praetorius-Ibba M, Prasad A, Prescott M, Priault M, Produit-Zengaffinen N, Progulske-Fox A, Proikas-Cezanne T, Przedborski S, Przyklenk K, Puertollano R, Puyal J, Qian SB, Qin L, Qin ZH, Quaggin SE, Raben N, Rabinowich H, Rabkin SW, Rahman I, Rami A, Ramm G, Randall G, Randow F, Rao VA, Rathmell JC, Ravikumar B, Ray SK, Reed BH, Reed JC, Reggiori F, Regnier-Vigouroux A, Reichert AS, Reiners JJ Jr, Reiter RJ, Ren J, Revuelta JL, Rhodes CJ, Ritis K, Rizzo E, Robbins J, Roberge M, Roca H, Roccheri MC, Rocchi S, Rodemann HP, Rodriguez de Cordoba S, Rohrer B, Roninson IB, Rosen K, Rost-Roszkowska MM, Rouis M, Rouschop KM, Rovetta F, Rubin BP, Rubinsztein DC, Ruckdeschel K, Rucker EB 3rd, Rudich A, Rudolf E, Ruiz-Opazo N, Russo R, Rusten TE, Ryan KM, Ryter SW, Sabatini DM, Sadoshima J, Saha T, Saitoh T, Sakagami H, Sakai Y, Salekdeh GH, Salomoni P, Salvaterra PM, Salvesen G, Salvioli R, Sanchez AM, Sanchez-Alcazar JA, Sanchez-Prieto R, Sandri M, Sankar U, Sansanwal P, Santambrogio L, Saran S, Sarkar S, Sarwal M, Sasakawa C, Sasnauskiene A, Sass M, Sato K, Sato M, Schapira AH, Scharl M, Schatzl HM, Scheper W, Schiaffino S, Schneider C, Schneider ME, Schneider-Stock R, Schoenlein PV, Schorderet DF, Schuller C, Schwartz GK, Scorrano L, Sealy L, Seglen PO, Segura-Aguilar J, Seiliez I, Seleverstov O, Sell C, Seo JB, Separovic D, Setaluri V, Setoguchi T, Settembre C, Shacka JJ, Shanmugam M, Shapiro IM, Shaulian E, Shaw RJ, Shelhamer JH, Shen HM, Shen WC, Sheng ZH, Shi Y, Shibuya K, Shidoji Y, Shieh JJ, Shih CM, Shimada Y, Shimizu S, Shintani T, Shirihai OS, Shore GC, Sibirny AA, Sidhu SB, Sikorska B, Silva-Zacarin EC, Simmons A, Simon AK, Simon HU, Simone C, Simonsen A, Sinclair DA, Singh R, Sinha D, Sinicrope FA, Sirko A, Siu PM, Sivridis E, Skop V, Skulachev VP, Slack RS, Smaili SS, Smith DR, Soengas MS, Soldati T, Song X, Sood AK, Soong TW, Sotgia F, Spector SA, Spies CD, Springer W, Srinivasula SM, Stefanis L, Steffan JS, Stendel R, Stenmark H, Stephanou A, Stern ST, Sternberg C, Stork B, Stralfors P, Subauste CS, Sui X, Sulzer D, Sun J, Sun SY, Sun ZJ, Sung JJ, Suzuki K, Suzuki T, Swanson MS, Swanton C, Sweeney ST, Sy LK, Szabadkai G, Tabas I, Taegtmeyer H, Tafani M, Takacs-Vellai K, Takano Y, Takegawa K, Takemura G, Takeshita F, Talbot NJ, Tan KS, Tanaka K, Tanaka K, Tang D, Tang D, Tanida I, Tannous BA, Tavernarakis N, Taylor GS, Taylor GA, Taylor JP, Terada LS, Terman A, Tettamanti G, Thevissen K, Thompson CB, Thorburn A, Thumm M, Tian F, Tian Y, Tocchini-Valentini G, Tolkovsky AM, Tomino Y, Tonges L, Tooze SA, Tournier C, Tower J, Towns R, Trajkovic V, Travassos LH, Tsai TF, Tschan MP, Tsubata T, Tsung A, Turk B, Turner LS, Tyagi SC, Uchiyama Y, Ueno T, Umekawa M, Umemiya-Shirafuji R, Unni VK, Vaccaro MI, Valente EM, Van den Berghe G, van der Klei IJ, van Doorn W, van Dyk LF, van Egmond M, van Grunsven LA, Vandenabeele P, Vandenberghe WP, Vanhorebeek I, Vaquero EC, Velasco G, Vellai T, Vicencio JM, Vierstra RD, Vila M, Vindis C, Viola G, Viscomi MT, Voitsekhovskaja OV, von Haefen C, Votruba M, Wada K, Wade-Martins R, Walker CL, Walsh CM, Walter J, Wan XB, Wang A, Wang C, Wang D, Wang F, Wang F, Wang G, Wang H, Wang HG, Wang HD, Wang J, Wang K, Wang M, Wang RC, Wang X, Wang X, Wang YJ, Wang Y, Wang Z, Wang ZC, Wang Z, Wansink DG, Ward DM, Watada H, Waters SL, Webster P, Wei L, Weihl CC, Weiss WA, Welford SM, Wen LP, Whitehouse CA, Whitton JL, Whitworth AJ, Wileman T, Wiley JW, Wilkinson S, Willbold D, Williams RL, Williamson PR, Wouters BG, Wu C, Wu DC, Wu WK, Wyttenbach A, Xavier RJ, Xi Z, Xia P, Xiao G, Xie Z, Xie Z, Xu DZ, Xu J, Xu L, Xu X, Yamamoto A, Yamamoto A, Yamashina S, Yamashita M, Yan X, Yanagida M, Yang DS, Yang E, Yang JM, Yang SY, Yang W, Yang WY, Yang Z, Yao MC, Yao TP, Yeganeh B, Yen WL, Yin JJ, Yin XM, Yoo OJ, Yoon G, Yoon SY, Yorimitsu T, Yoshikawa Y, Yoshimori T, Yoshimoto K, You HJ, Youle RJ, Younes A, Yu L, Yu L, Yu SW, Yu WH, Yuan ZM, Yue Z, Yun CH, Yuzaki M, Zabirnyk O, Silva-Zacarin E, Zacks D, Zacksenhaus E, Zaffaroni N, Zakeri Z, Zeh HJ 3rd, Zeitlin SO, Zhang H, Zhang HL, Zhang J, Zhang JP, Zhang L, Zhang L, Zhang MY, Zhang XD, Zhao M, Zhao YF, Zhao

Y, Zhao ZJ, Zheng X, Zhivotovsky B, Zhong Q, Zhou CZ, Zhu C, Zhu WG, Zhu XF, Zhu X, Zhu Y, Zoladek T, Zong WX, Zorzano A, Zschocke J and Zuckerbraun B. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 2012; 8: 445-544.

[9] Feng Y, He D, Yao Z and Klionsky DJ. The machinery of macroautophagy. Cell Res 2014; 24: 24-41.

[10] Hosokawa N, Hara T, Kaizuka T, Kishi C, Takamura A, Miura Y, Iemura S, Natsume T, Takehana K, Yamada N, Guan JL, Oshiro N and Mizushima N. Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. Mol Biol Cell 2009; 20: 1981-1991.

[11] Jung CH, Jun CB, Ro SH, Kim YM, Otto NM, Cao J, Kundu M and Kim DH. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. Mol Biol Cell 2009; 20: 1992-2003.

[12] Mack HI, Zheng B, Asara JM and Thomas SM. AMPK-dependent phosphorylation of ULK1 regulates ATG9 localization. Autophagy 2012; 8: 1197-1214.

[13] Suzuki K, Kirisako T, Kamada Y, Mizushima N, Noda T and Ohsumi Y. The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. EMBO J 2001; 20: 5971-5981.

[14] Mizushima N, Noda T, Yoshimori T, Tanaka Y, Ishii T, George MD, Klionsky DJ, Ohsumi M and Ohsumi Y. A protein conjugation system essential for autophagy. Nature 1998; 395: 395-398.

[15] Mizushima N, Sugita H, Yoshimori T and Ohsumi Y. A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy. J Biol Chem 1998; 273: 33889-33892.

[16] Tanida I, Tanida-Miyake E, Komatsu M, Ueno T and Kominami E. Human Apg3p/Aut1p homologue is an authentic E2 enzyme for multiple substrates, GATE-16, GABARAP, and MAP-LC3, and facilitates the conjugation of hApg12p to hApg5p. J Biol Chem 2002; 277: 13739-13744.

[17] Tanida I, Ueno T and Kominami E. Human light chain 3/MAP1LC3B is cleaved at its carboxyl-terminal Met121 to expose Gly120 for lipidation and targeting to autophagosomal membranes. J Biol Chem 2004; 279: 47704-47710.

[18] Nakatogawa H, Ichimura Y and Ohsumi Y. Atg8, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion. Cell 2007; 130: 165-178.

[19] Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y and Yoshimori T. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 2000; 19: 5720-5728.

[20] Hanada T, Noda NN, Satomi Y, Ichimura Y, Fujioka Y, Takao T, Inagaki F and Ohsumi Y. The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy. J Biol Chem 2007; 282: 37298-37302.

[21] Fujita N, Itoh T, Omori H, Fukuda M, Noda T and Yoshimori T. The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. Mol Biol Cell 2008; 19: 2092-2100.

[22] Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y, Cattoretti G and Levine B. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Invest 2003; 112: 1809-1820.

[23] Jo YK, Kim SC, Park IJ, Park SJ, Jin DH, Hong SW, Cho DH and Kim JC. Increased expression of ATG10 in colorectal cancer is associated with lymphovascular invasion and lymph node metastasis. PLoS One 2012; 7: e52705.

[24] Tang J, Deng R, Luo RZ, Shen GP, Cai MY, Du ZM, Jiang S, Yang MT, Fu JH and Zhu XF. Low expression of ULK1 is associated with operable breast cancer progression and is an adverse prognostic marker of survival for patients. Breast Cancer Res Treat 2012; 134: 549-560.

[25] Shi YH, Ding ZB, Zhou J, Qiu SJ and Fan J. Prognostic significance of Beclin 1-dependent apoptotic activity in hepatocellular carcinoma. Autophagy 2009; 5: 380-382.

[26] Pirtoli L, Cevenini G, Tini P, Vannini M, Oliveri G, Marsili S, Mourmouras V, Rubino G and Miracco C. The prognostic role of Beclin 1 protein expression in high-grade gliomas. Autophagy 2009; 5: 930-936.

[27] Chen YB, Hou JH, Feng XY, Chen S, Zhou ZW, Zhang XS and Cai MY. Decreased expression of Beclin 1 correlates with a metastatic phenotypic feature and adverse prognosis of gastric carcinomas. J Surg Oncol 2012; 105: 542-547.

[28] Zhou WH, Tang F, Xu J, Wu X, Yang SB, Feng ZY, Ding YG, Wan XB, Guan Z, Li HG, Lin DJ, Shao CK and Liu Q. Low expression of Beclin 1, associated with high Bcl-xL, predicts a malignant phenotype and poor prognosis of gastric cancer. Autophagy 2012; 8: 389-400.

[29] Yu M, Gou WF, Zhao S, Xiao LJ, Mao XY, Xing YN, Takahashi H, Takano Y and Zheng HC. Beclin 1 expression is an independent prognostic factor for gastric carcinomas. Tumour Biol 2013; 34: 1071-1083.

[30] Wan XB, Fan XJ, Chen MY, Xiang J, Huang PY, Guo L, Wu XY, Xu J, Long ZJ, Zhao Y, Zhou WH, Mai HQ, Liu Q and Hong MH. Elevated Beclin 1 expression is correlated with HIF-1alpha in predicting poor prognosis of nasopharyngeal carcinoma. Autophagy 2010; 6: 395-404.

[31] Edge SB and Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Ann Surg Oncol 2010; 17: 1471-1474.
[32] Ochiai T, Hayashi H, Suzuki T, Nakajima K, Shimada H, Hishikawa E, Yasumoto A, Takeda A and Isono K. Evaluation of a new staging system by the Japanese Research Society for Gastric Cancer. Surg Today 1998; 28: 1015-1021.
[33] Fan XJ, Wan XB, Huang Y, Cai HM, Fu XH, Yang ZL, Chen DK, Song SX, Wu PH, Liu Q, Wang L and Wang JP. Epithelial-mesenchymal transition biomarkers and support vector machine guided model in preoperatively predicting regional lymph node metastasis for rectal cancer. Br J Cancer 2012; 106: 1735-1741.
[34] Russell RC, Tian Y, Yuan H, Park HW, Chang YY, Kim J, Kim H, Neufeld TP, Dillin A and Guan KL. ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. Nat Cell Biol 2013; 15: 741-750.
[35] Caino MC, Chae YC, Vaira V, Ferrero S, Nosotti M, Martin NM, Weeraratna A, O'Connell M, Jernigan D, Fatatis A, Languino LR, Bosari S and Altieri DC. Metabolic stress regulates cytoskeletal dynamics and metastasis of cancer cells. J Clin Invest 2013; 123: 2907-2920.
[36] Yue Z, Jin S, Yang C, Levine AJ and Heintz N. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc Natl Acad Sci U S A 2003; 100: 15077-15082.
[37] Guo XL, Li D, Hu F, Song JR, Zhang SS, Deng WJ, Sun K, Zhao QD, Xie XQ, Song YJ, Wu MC and Wei LX. Targeting autophagy potentiates chemotherapy-induced apoptosis and proliferation inhibition in hepatocarcinoma cells. Cancer Lett 2012; 320: 171-179.
[38] Huang JJ, Li HR, Huang Y, Jiang WQ, Xu RH, Huang HQ, Lv Y, Xia ZJ, Zhu XF, Lin TY and Li ZM. Beclin 1 expression: a predictor of prognosis in patients with extranodal natural killer T-cell lymphoma, nasal type. Autophagy 2010; 6: 777-783.
[39] Dong LW, Hou YJ, Tan YX, Tang L, Pan YF, Wang M and Wang HY. Prognostic significance of Beclin 1 in intrahepatic cholangiocellular carcinoma. Autophagy 2011; 7: 1222-1229.
[40] Takamura A, Komatsu M, Hara T, Sakamoto A, Kishi C, Waguri S, Eishi Y, Hino O, Tanaka K and Mizushima N. Autophagy-deficient mice develop multiple liver tumors. Genes Dev 2011; 25: 795-800.
[41] Sanchez CG, Penfornis P, Oskowitz AZ, Boonjindasup AG, Cai DZ, Dhule SS, Rowan BG, Kelekar A, Krause DS and Pochampally RR. Activation of autophagy in mesenchymal stem cells provides tumor stromal support. Carcinogenesis 2011; 32: 964-972.
[42] Amar N, Lustig G, Ichimura Y, Ohsumi Y and Elazar Z. Two newly identified sites in the ubiquitin-like protein Atg8 are essential for autophagy. EMBO Rep 2006; 7: 635-642.
[43] Lamoureux F and Zoubeidi A. Dual inhibition of autophagy and the AKT pathway in prostate cancer. Autophagy 2013; 9: 1119-1120.
[44] Wang L, Song J, Zhang J, Zhu C, Ma Y and Xu X. Lentiviral vector-mediate ATG3 overexpression inhibits growth and promotes apoptosis of human SKM-1 cells. Mol Biol Rep 2014; 41: 2093-2099.
[45] Han C, Sun B, Wang W, Cai W, Lou D, Sun Y and Zhao X. Overexpression of microtubule-associated protein-1 light chain 3 is associated with melanoma metastasis and vasculogenic mimicry. Tohoku J Exp Med 2011; 223: 243-251.
[46] Karpathiou G, Sivridis E, Koukourakis MI, Mikroulis D, Bouros D, Froudarakis ME and Giatromanolaki A. Light-chain 3A autophagic activity and prognostic significance in non-small cell lung carcinomas. Chest 2011; 140: 127-134.
[47] He JH, Luo RZ, Cai MY, Li M, Lu JB and Yuan ZY. Decreased expression of light chain 3 (LC3) increased the risk of distant metastasis in triple-negative breast cancer. Med Oncol 2013; 30: 468.
[48] Zhu W, Pan X, Li F, Zhang Y and Lu X. Expression of Beclin 1 and LC3 in FIGO stage I-II cervical squamous cell carcinoma and relationship to survival. Tumour Biol 2012; 33: 1653-1659.
[49] Kitamura H, Torigoe T, Asanuma H, Hisasue SI, Suzuki K, Tsukamoto T, Satoh M and Sato N. Cytosolic overexpression of p62 sequestosome 1 in neoplastic prostate tissue. Histopathology 2006; 48: 157-161.
[50] Ellis RA, Horswell S, Ness T, Lumsdon J, Tooze SA, Kirkham N, Armstrong JL and Lovat PE. Prognostic impact of p62 expression in cutaneous malignant melanoma. J Invest Dermatol 2014; 134: 1476-1478.
[51] Wang W, Li YF, Sun XW, Chen YB, Li W, Xu DZ, Guan XX, Huang CY, Zhan YQ and Zhou ZW. Prognosis of 980 patients with gastric cancer after surgical resection. Chin J Cancer 2010; 29: 923-930.
[52] Kajiyama Y, Tsurumaru M, Udagawa H, Tsutsumi K, Kinoshita Y, Ueno M and Akiyama H. Prognostic factors in adenocarcinoma of the gastric cardia: pathologic stage analysis and multivariate regression analysis. J Clin Oncol 1997; 15: 2015-2021.
[53] Jang JH, Beron RI, Ahn HS, Kong SH, Lee HJ, Kim WH, Lee KU and Yang HK. Clinicopathological Features of Upper Third Gastric Cancer during a 21-Year Period (Single Center Analysis). J Gastric Cancer 2010; 10: 212-218.

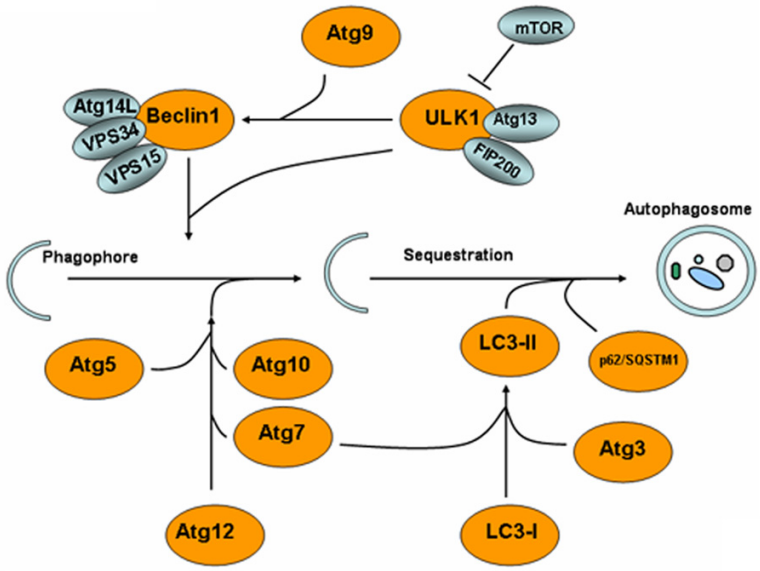

Figure S1. Principal ATG proteins involved in the process of autophagy.

Table S1. Expression status of autophagy related proteins in relation to patient characteristics in training set and testing set

Variables	ULK1				Beclin 1				ATG3				ATG5				ATG7			
	Training set (n = 117)		Testing set (n = 235)		Training set (n = 117)		Testing set (n = 235)		Training set (n = 117)		Testing set (n = 235)		Training set (n = 117)		Testing set (n = 235)		Training set (n = 117)		Testing set (n = 235)	
	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High
Gender																				
Male	34	43	76	82	44	33	92	66	31	46	53	105	18	59	31	127	18	59	46	112
Female	22	18	45	32	26	14	47	30	12	28	22	55	11	29	19	58	7	33	27	50
P value[a]	0.330		0.165		0.434		0.778		0.316		0.461		0.656		0.398		0.635		0.371	
Age																				
≥ 58[b]	31	34	52	60	31	34	62	50	25	40	35	77	16	49	27	85	14	51	32	80
< 58	25	27	69	54	39	13	77	46	18	34	40	83	13	39	23	100	11	41	41	82
P value	0.967		0.152		0.004		0.289		0.703		0.889		0.962		0.341		0.960		0.481	
Location																				
Upper 1/3	16	12	37	35	17	11	42	30	7	21	17	55	7	22	15	57	8	20	12	60
Middle 1/3	11	24	23	39	21	13	38	24	13	21	19	43	5	29	9	53	2	32	18	44
Lower 1/3	24	25	55	37	27	22	51	41	18	31	37	55	14	35	21	71	14	36	38	54
Whole	4	2	6	3	4	2	8	1	2	4	4	5	0	6	6	3	1	5	5	4
P value	0.134		0.037		0.898		0.263		0.696		0.125		0.256		0.460		0.067		0.003	
Size																				
≥ 5 cm	40	32	75	66	46	26	87	54	26	46	43	98	15	57	36	105	15	57	53	88
< 5 cm	16	29	46	48	24	21	52	42	17	28	32	62	14	31	14	80	10	35	20	74
P value	0.039		0.594		0.333		0.346		0.856		0.571		0.272		0.053		0.858		0.010	
Histological type																				
Intestinal	47	55	83	94	66	36	99	78	33	69	43	134	20	82	32	145	17	84	47	130
Diffuse	9	6	38	20	4	11	40	18	10	5	32	26	9	6	18	40	7	9	26	32
P value	0.409		0.016		0.009		0.091		0.019		< 0.001		0.002		0.043		0.021		0.014	
TNM stage																				
I	4	13	6	18	7	10	5	19	5	13	5	19	5	13	3	21	3	14	4	20
II	5	12	16	48	9	8	30	34	5	12	17	47	5	12	9	55	2	15	15	49
III	38	30	88	44	44	24	81	43	27	41	42	82	16	52	30	94	18	50	40	84
IV	9	6	19	4	10	5	23	0	7	8	11	12	4	11	8	15	2	13	14	9
P value	0.029		< 0.001		0.288		< 0.001		0.597		0.165		0.953		0.101		0.440		0.003	
Lymph node metastasis																				
With	47	42	102	58	55	34	107	53	38	51	58	102	22	67	40	120	21	68	54	106
Without	9	19	19	56	15	13	32	43	5	23	17	58	7	21	10	65	4	24	19	56
P value	0.082		< 0.001		0.509		0.001		0.008		0.053		0.976		0.059		0.429		0.227	

Variables	ATG9				ATG10				ATG12				P62/SQSTM1				LC3B			
	Training set (n = 117)		Testing set (n = 235)		Training set (n = 117)		Testing set (n = 235)		Training set (n = 117)		Testing set (n = 235)		Training set (n = 117)		Testing set (n = 235)		Training set (n = 117)		Testing set (n = 235)	
	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High
Gender																				
Male	30	47	62	96	53	24	101	57	8	69	37	121	29	48	85	73	18	59	68	90
Female	16	24	42	35	23	17	47	30	5	35	14	63	19	21	38	39	13	27	26	51
P value[a]	0.913		0.036		0.229		0.669		0.762		0.403		0.328		0.579		0.377		0.202	
Age																				
≥ 58[b]	22	43	35	77	40	25	69	43	7	58	21	91	23	42	55	57	14	51	37	75
< 58	24	28	69	54	36	16	79	44	6	46	30	93	25	27	68	55	17	35	57	66
P value	0.188		< 0.001		0.439		0.687		0.895		0.343		0.189		0.362		0.208		0.052	
Location																				
Upper 1/3	11	17	21	51	21	7	47	25	5	23	12	60	11	17	33	39	10	18	31	41
Middle 1/3	12	22	26	36	16	18	37	25	2	32	13	49	10	24	38	24	6	28	30	32
Lower 1/3	19	30	53	38	33	16	57	35	6	43	24	68	23	26	44	48	13	36	29	63
Whole	4	2	4	5	6	0	7	2	0	6	2	7	4	2	8	1	2	4	4	5
P value	0.548		0.003		0.025		0.720		0.385		0.545		0.234		0.032		0.438		0.177	
Size																				
≥ 5 cm	30	42	67	74	51	21	89	52	7	65	36	105	29	43	77	64	21	51	62	79
< 5 cm	16	29	37	57	25	20	59	35	6	39	15	79	19	26	46	48	10	35	32	62
P value	0.563		0.230		0.112		0.956		0.559		0.106		0.849		0.425		0.519		0.137	
Histological type																				
Intestinal	38	64	73	104	64	38	108	69	8	94	32	145	38	64	83	94	24	78	63	114
Diffuse	8	7	31	27	12	3	40	18	5	10	19	39	10	5	40	18	7	8	31	27
P value	0.266		0.128		0.253		0.347		0.012		0.027		0.047		0.004		0.068		0.020	
TNM stage																				
I	6	11	10	14	10	7	12	12	1	16	5	19	2	15	12	12	3	14	7	17
II	5	12	24	40	7	10	25	39	2	15	7	57	6	11	28	38	4	13	20	44
III	30	38	58	66	52	16	92	32	9	59	31	93	33	35	68	56	21	47	55	69
IV	5	10	12	11	7	8	19	4	1	14	8	15	7	8	15	8	3	12	12	11
P value	0.638		0.541		0.014		< 0.001		0.780		0.058		0.065		0.215		0.624		0.132	
Lymph node metastasis																				
With	36	53	75	85	64	25	117	43	11	78	39	121	40	49	88	72	24	65	70	90
Without	10	18	29	46	12	16	31	44	2	26	12	63	8	20	35	40	7	21	24	51
P value	0.825		0.262		0.007		< 0.001		0.731		0.176		0.186		0.263		0.837		0.116	

NM stage, Tumor-Node-Metastasis stage. [a]Chi-suare test or Fisher's exact test; [b]Median age.

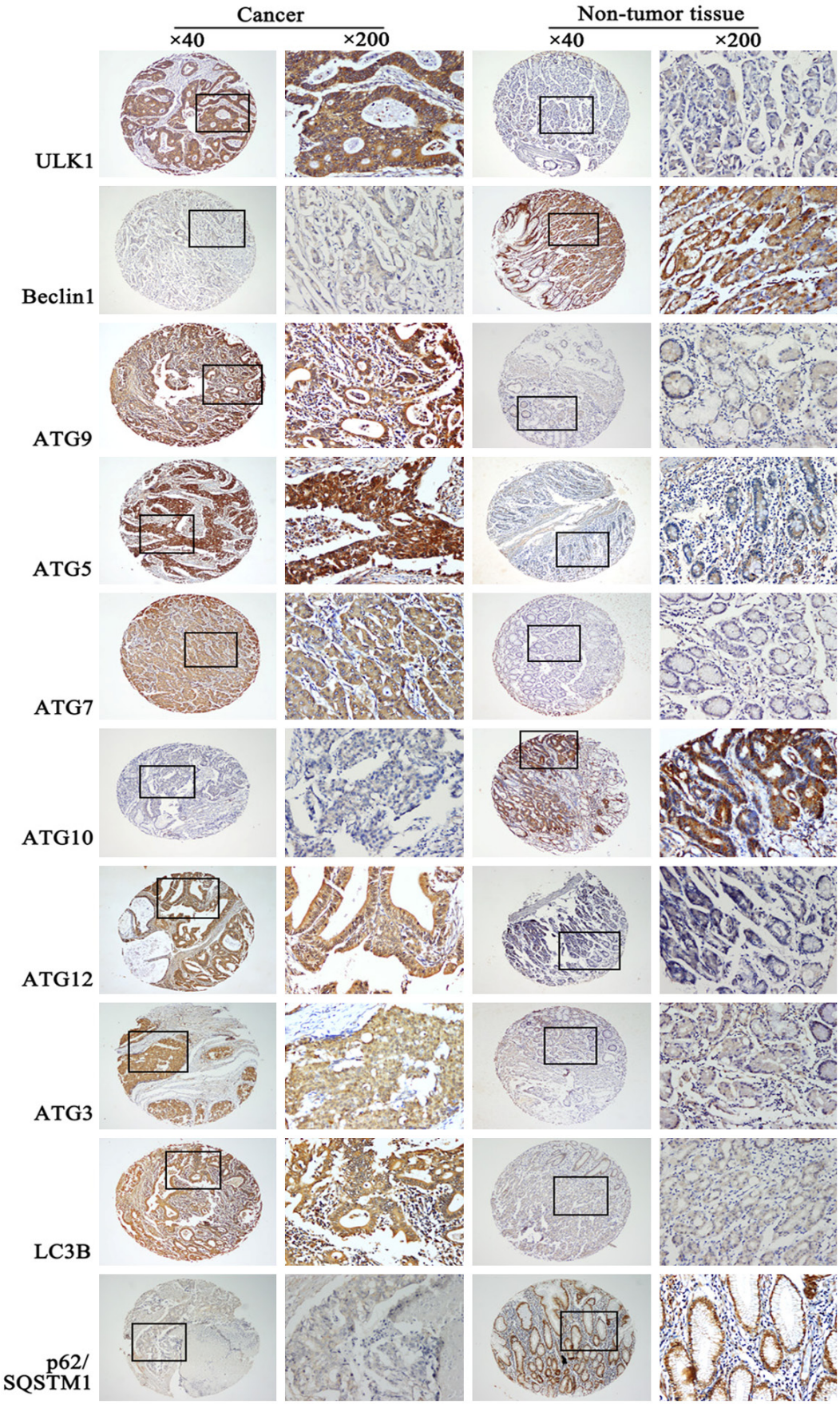
Cancer
Non-tumor tissue
×40
×200
×40
×200
ULK1
Beclin1
ATG9
ATG5
ATG7
ATG10
ATG12
ATG3
LC3B
p62/
SQSTM1

Figure S2. Immunohistochemical staining of ATG proteins and p62/SQSTM1 in gastric tumor tissues (left panels, ×40; right panels, ×200) and non-tumor tissues (left panels, ×40; right panels, ×200). In the tumor tissues, Beclin 1, ATG10 and p62/SQSTM1 were low expressed compared with the normal adjacent tissues. Conversely, ULK1, ATG3, ATG5, ATG7, ATG9, ATG12 and LC3B were highly expressed in the gastric cancer tissues compared with adjacent normal tissues.

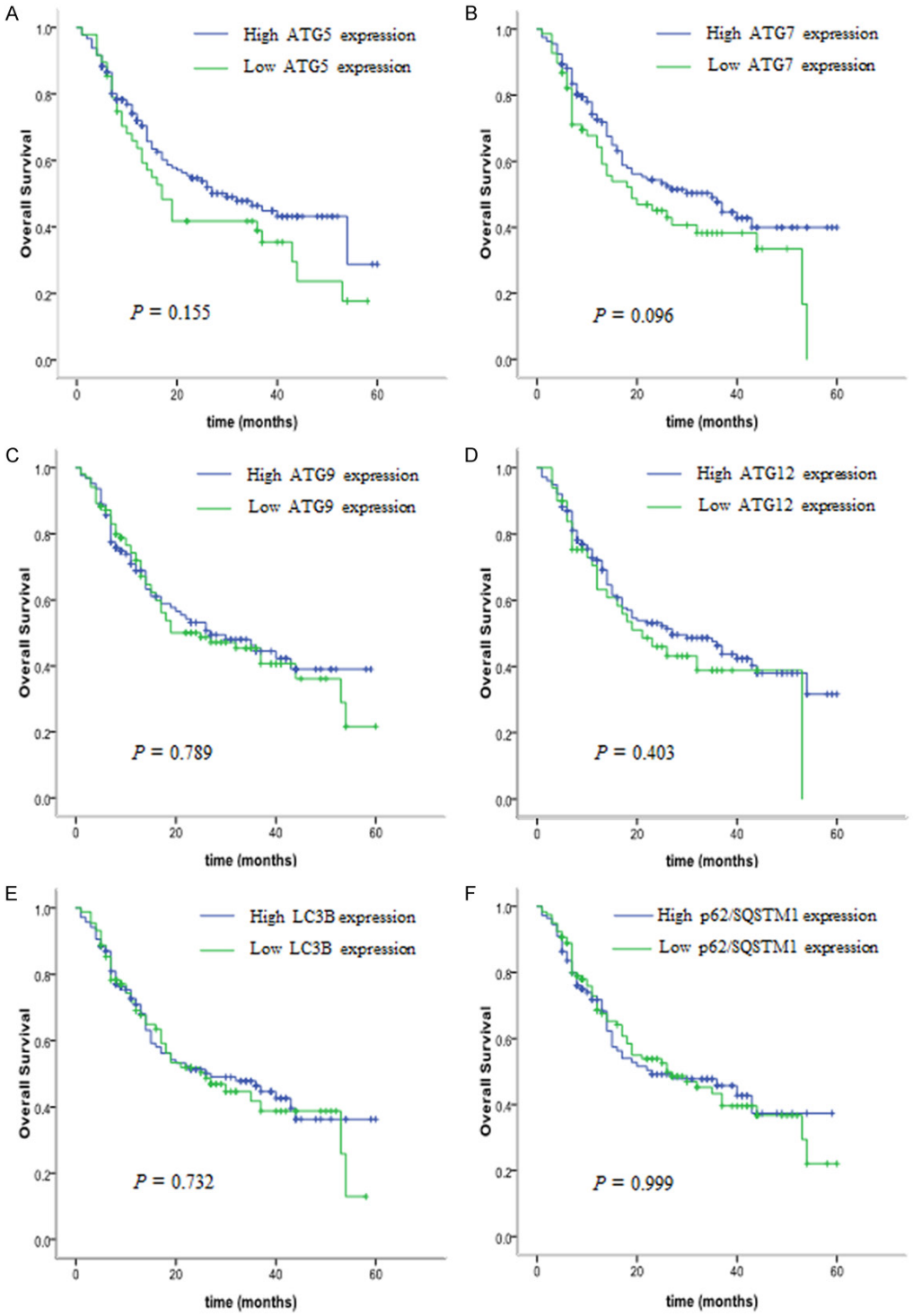

Figure S3. Kaplan-Meier estimated of overall survival according to ATGs proteins expression level in testing set. The statistically overall survival difference was not shown between the subgroups with high or low expression of ATG5 (A), ATG7 (B), ATG9 (C), ATG12 (D), LC3B (E) and p62/SQSTM1 (F).

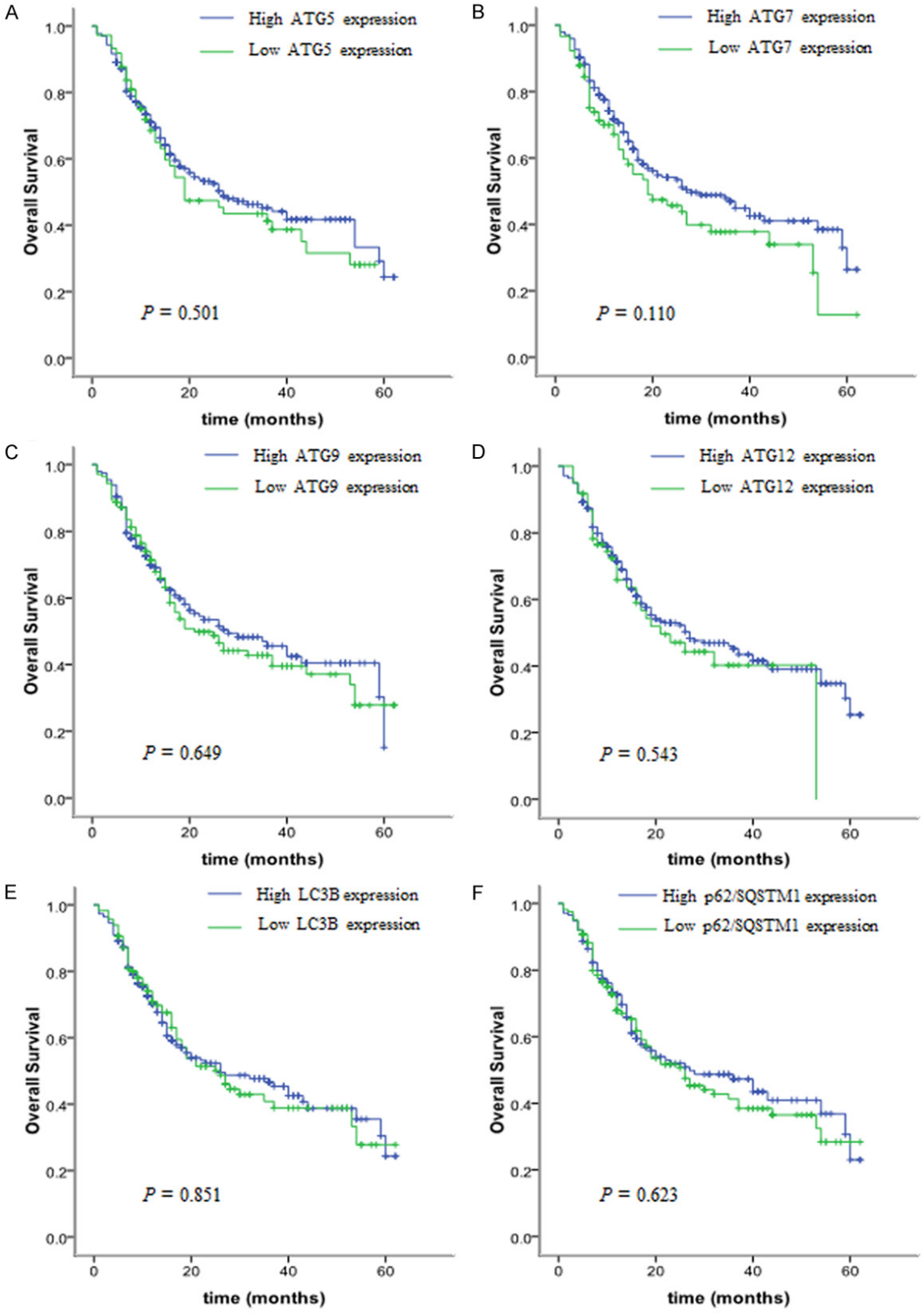

Figure S4. Kaplan-Meier estimated of overall survival according to ATGs proteins expression level in overall patients. The statistically overall survival difference was not shown between the subgroups with high or low expression of ATG5 (A), ATG7 (B), ATG9 (C), ATG12 (D), LC3B (E) and p62/SQSTM1 (F).