Clinicopathological Correlations of Autophagy-related Proteins LC3, Beclin 1 and p62 in Gastric Cancer

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Abstract. *Aim: This study evaluated the clinicopathological significance of autophagy, an intracellular degradation system, in gastric cancer. Materials and Methods: The expression levels of three autophagy-related proteins, namely light chain 3 (LC3), Beclin 1 and p62, were analyzed by immunohistochemistry using samples from 510 patients with primary gastric cancer. Results: LC3, Beclin 1, and p62 expression was positive in 79 (15.5%), 126 (24.7%) and 251 (49.2%) out of 510 carcinomas, respectively. Autophagy was defined when samples were positive for at least two out of the three proteins. Autophagy-positive cases were 113 (22.1%) out of the 510. Autophagy determined by LC3, Beclin 1, and p62 significantly correlated with lymph node metastasis, vessel invasion, and hepatic metastasis. A Kaplan–Meier survival curve showed that autophagy was significantly associated with poor survival of patients with gastric cancer, especially for those with disease at stage I. Multivariate analysis indicated that autophagy was an independent prognostic factor. Conclusion: Autophagy promotes the progression of gastric cancer at an early clinical stage.*

Autophagy is a conserved mechanism for the intracellular degradation system by which amino acids are recycled within cells, and plays various physiological roles such as in proliferation, differentiation and maintenance of cellular homeostasis (1, 2). This process is characterized by the formation of autophagosomes, double-membraned vesicles that sequester the cytoplasmic materials in the lysosome (3). While autophagy usually occurs at low levels in most cells, it is up-regulated in response to metabolic stresses such as starvation, hypoxia and growth factor deprivation, in order to generate intracellular nutrients and energy (1). In addition, autophagy is frequently involved in tumor progression and chemoresistance by promoting tumor cell survival in response to various stresses (4-7).

The activation of autophagy in response to various stresses leads to up-regulation of the expression levels of autophagy-related proteins including light chain 3 (LC3), Beclin 1, and p62 (8-10). LC3 is a specific marker of autophagosome formation and is widely monitored as a autophagy-related protein (11). Beclin 1 is an essential modifier of the autophagic process and has been implicated in tumor development (12). p62 is a multi-functional signaling molecule for cell survival and cell death (13) and localizes at the membranes of autophagosomes (14). It has been reported that these autophagy-related molecules are expressed in different manners in various types of cancers (15-17). Recent studies have reported that autophagy is associated with the malignant potential of cancer cells (18). However, the role of autophagy in cancer cells is complex and paradoxical (7, 19). The clinicopathological significance of autophagy in certain types of cancer is controversial (7, 20). This study aimed to investigate clinicopathological features of autophagy in gastric cancer in terms of the expression of the autophagy-related proteins, LC3, Beclin 1, and p62.

Materials and Methods

Clinical materials. A total of 510 patients who had undergone resection of primary tumor and were histologically confirmed to have gastric cancer were enrolled in the study (Table I). None of patients had undergone preoperative radiation or chemotherapy. The pathological diagnoses and classifications were made according to t the UICC TNM classification of malignant tumors (21) or the general rules for gastric cancer study of the Japanese Research Society for Gastric Cancer (22). This study was approved by Osaka

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City University Ethics Committee (Approval number: 924. Informed consent was obtained from all patients.

Immunohistochemical techniques. The immunohistochemical determination of LC3, Beclin 1 and p62 was performed as previously reported (23). In brief, paraffin-embedded sections were de-paraffinized in xylene and de-hydrated through graded ethanol. The sections were heated for 10 min at 105˚C by autoclave in Target Retrieval Solution (DAKO, Carpinteria, CA, USA). Then sections were incubated with 3% hydrogen peroxide to block endogenous peroxidase activity before immunohistochemistry using the following antibodies: anti-LC3 (NB100-2220, 1:200; Novus, Littleton, CO, USA), anti-Beclin 1 (NB500-249, 1:400; Novus) and anti p62 (PM045, 1:1000; MBL, Nagoya, Japan). The specimens were incubated with LC3, Beclin 1 and p62 antibody for 1 h at room temperature. The sections were incubated with an appropriate immunoglobulin G for 10 min, followed by three washes with phosphate-buffered saline (PBS). The slides were treated with streptavidin-peroxidase reagent, and were incubated in PBS with diaminobenzidine and 1% (vol/vol) hydrogen peroxide, followed by counterstaining with Mayer's hematoxylin and subsequently examined using light microscopy.

Immunohistochemical determination. Immunoreactivity of more than 20% of carcinoma cells was regarded as positivity for LC3, and p62, while immunoreactivity of more than 80% of cancer cells was defined as being Beclin 1-positive. Positive immunostaining was evaluated by two independent investigators who were blinded to patient outcomes and clinicopathological features. Autophagy was determined to be positive when samples were positive for expression of at least two out of the three proteins.

Statistical analysis. The χ^2 test was used to determine the significance of the differences between the covariates. The duration of survival from surgery to death was calculated using the Kaplan–Meier method and analyzed by the log-rank test to compare the cumulative survival durations in the patient groups. In addition, the Cox proportional hazards model was used to compute multivariate hazards ratios for the study parameters. In all of the tests, a *p*-value of less than 0.05 was defined as being statistically significant. The SPSS software program (SPSS Japan, Tokyo, Japan) was used for the analyses.

Results

Correlation between clinicopathological features and autophagy. Marked expressions of LC3, Beclin 1, and p62 were found in cancer cells but not in surrounding stromal cells (Figure 1). LC3 and Beclin 1 were mainly expressed in the cytoplasm of the cancer cells, while p62 was expressed in both the nucleus and cytoplasm. Of the 510 gastric carcinomas, LC3, Beclin 1, and p62 expression were positive in 79 (15.5%), 126 (24.7%), and 251 (49.2%), respectively. There was a statistically significant correlation among LC3-positive, Beclin 1-positive, and p62-positive expressions (Table II).

The relationships between the clinicopathological features of the tumors and LC3, Beclin 1 and p62 expression, as well as autophagy-positive status, are shown in Table III. LC3, Beclin 1, and p62 expression were significantly associated with age, macroscopic type, tumor size, histological type, T-stage (depth of tumor invasion), lymph node metastasis, tumor infiltration pattern (INF), lymphatic invasion, venous invasion, hepatic metastasis, and peritoneal metastasis. Autophagy was determined to be positive when two or three of the studied proteins were expressed. Autophagy was found in 113 cases (22.1%) of 510 gastric carcinomas. Autophagy was significantly positively associated with age ≥60 years (p=0.003), intestinal tumor type (p=0.006), lymph node metastasis (p<0.001), INF a/b (p<0.001), lymphatic invasion (p<0.001), venous invasion (p<0.001), and hepatic metastasis (p=0.004).

Table I. *Clinicopathological characteristics of 510 patients with gastric cancer.*

Clinicopathological feature		n=510
Gender	Female	220
	Male	290
Age (years)	Median	66
	Range	21-88
Macroscopic type[a]	Type-4	53
	Other types	457
Histological type	Intestinal type	253
	Diffuse type	257
T-Stage[b]	T1	223
	T2	57
	T3	43
	T4	187
Lymph node metastasis	Negative	273
	Positive	235
Clinical stage[c]	I	250
	II	76
	III	108
	IV	76

[a]Macroscopic type was classified as follows according to the general rules for gastric cancer study of the Japanese Research Society for Gastric Cancer (22). Type 1 is defined as a polypoid tumor, sharply demarcated from the surrounding mucosa and usually attached on a wide base. Type 2 is defined as a polypoid tumor with ulceration and sharply demarcated margins. Type 3 is defined as an ulcerated carcinoma with cancer infiltration into the surrounding wall. Type 4 is defined as a diffusely infiltrating flat carcinoma in which ulceration is usually not a marked feature. [b,c]T-Stage and clinical stage were determined according to the UICC TNM Classification of Malignant Tumors (21).

Survival. Figure 2 shows the Kaplan–Meier survival curve for the cohort of 510 patients with gastric cancer. The prognosis of the patients with LC3-positive, Beclin 1-positive, p62-positive, and autophagy-positive cancer was significantly worse (p=0.020, p=0.039, p<0.001, and p<0.001, respectively) than that of patients negative for the respective marker. Figure 3 demonstrates that the prognosis of autophagy-positive patients with clinical stage I disease was significantly worse (p=0.013) than that of autophagy-

Table II. *Correlation of autophagy and expression of autophagy-related proteins [light chain 3 (LC3), Beclin 1 and p62] with clinicopathological factors.*

	LC3			Beclin 1			p62			Autophagy		
Factor	Positive n=79	Negative n=431	*p*-Value	Positive n=126	Negative n=384	*p*-Value	Positive n=251	Negative n=259	*p*-Value	Positive n=113	Negative n=397	*p*-Value
Age												
<60 Years	18 (12.2%)	130 (87.8%)	0.225	26 (17.6%)	122 (82.4%)	0.018	53 (35.8%)	95 (64.2%)	<0.001	20 (13.5%)	128 (86.5%)	0.003
≥60 Years	61 (16.9%)	301 (83.1%)		100 (27.6%)	262 (72.4%)		198 (54.7%)	164 (45.3%)		93 (25.7%)	269 (74.3%)	
Gender												
Female	31 (14.4%)	189 (85.9%)	0.462	57 (25.9%)	163 (74.1%)	0.482	118 (53.6%)	102 (46.4%)	0.082	53 (24.1%)	167 (75.9%)	0.36
Male	48 (16.6%)	242 (83.4%)		69 (23.8%)	221 (76.2%)		133 (45.9%)	157 (54.1%)		60 (20.7%)	230 (79.3%)	
Macroscopic type[a]												
Type-4	5 (9.4%)	48 (90.6%)	0.233	10 (18.9%)	43 (81.1%)	0.4	38 (71.7%)	15 (28.3%)	0.001	8 (15.1%)	45 (84.9%)	0.224
Other	74 (16.2%)	383 (83.8%)		116 (81.1%)	341 (74.6%)		213 (46.6%)	244 (53.4%)		105 (23.0%)	352 (77.0%)	
Tumor size												
<30 mm	24 (13.4%)	155 (86.6%)	0.371	31 (17.3%)	148 (82.7%)	0.004	74 (41.3%)	105 (58.7%)	0.009	31 (17.3%)	148 (82.7%)	0.057
≥30 mm	55 (16.7%)	274 (83.3%)		95 (28.9%)	234 (71.1%)		176 (53.5%)	153 (46.5%)		82 (24.9%)	247 (75.1%)	
Histological type												
Intestinal	51 (20.2%)	202 (79.8%)	0.005	73 (28.9%)	180 (71.1%)	0.04	124 (49.0%)	129 (21.0%)	0.93	69 (27.3%)	184 (72.7%)	0.006
Diffuse	28 (15.5%)	229 (89.1%)		53 (20.6%)	204 (79.4%)		127 (49.4%)	130 (50.6%)		44 (17.1%)	213 (82.9%)	
T-Stage												
T1/T2	35 (12.5%)	245 (87.5%)	0.049	62 (22.1%)	218 (77.9%)	0.149	106 (37.9%)	174 (62.1%)	<0.001	53 (18.9%)	227 (81.1%)	0.055
T3/T4	44 (19.1%)	186 (80.9%)		64 (27.8%)	166 (72.2%)		145 (63.0%)	85 (62.1%)		60 (26.1%)	170 (73.9%)	
Lymph node metastasis												
Negative	37 (13.6%)	236 (86.4%)	0.219	52 (19.0%)	221 (81.0%)	0.001	97 (35.5%)	176 (64.5%)	<0.001	43 (15.8%)	230 (84.2%)	<0.001
Positive	42 (17.9%)	193 (82.1%)		74 (31.5%)	161 (68.5%)		153 (49.2%)	82 (34.9%)		70 (29.8%)	165 (70.2%)	
INF[b]												
a/b	70 (21.3%)	258 (78.7%)	<0.001	101 (30.8%)	227 (69.2%)	<0.001	166 (50.6%)	162 (49.4%)	0.181	99 (30.2%)	229 (69.8%)	<0.001
c	7 (5.3%)	125 (94.7%)		20 (15.2%)	112 (84.8%)		76 (57.6%)	56 (42.4%)		12 (9.1%)	120 (90.9%)	
Lymphatic invasion												
Negative	19 (8.9%)	194 (91.1%)	<0.001	36 (16.9%)	177 (83.1%)	<0.001	67 (31.5%)	146 (68.5%)	<0.001	25 (11.7%)	188 (88.3%)	<0.001
Positive	60 (20.3%)	236 (79.7%)		90 (30.4%)	206 (69.6%)		184 (62.2%)	112 (37.8%)		88 (29.7%)	208 (70.3%)	
Venous invasion												
Negative	56 (13.7%)	354 (86.3%)	0.03	91 (22.2%)	319 (77.8%)	0.01	179 (43.7%)	231 (56.3%)	<0.001	74 (18.0%)	336 (82.0%)	<0.001
Positive	23 (23.0%)	77 (77.0%)		35 (35.0%)	65 (65.0%)		72 (72.0%)	28 (28.0%)		39 (39.0%)	61 (61.0%)	
Hepatic metastasis												
Negative	73 (14.7%)	423 (85.3%)	0.012	120 (24.2%)	376 (75.8%)	0.121	240 (48.4%)	256 (51.6%)	0.03	105 (21.2%)	391 (78.8%)	0.004
Positive	6 (42.9%)	8 (57.1%)		6 (42.9%)	8 (57.1%)		11(78.6%)	3 (21.4%)		8 (57.1%)	6 (42.9%)	
Peritoneal metastasis												
Negative	72 (15.0%)	407 (85.0%)	0.302	117 (24.4%)	362 (75.6%)	0.527	230 (48.0%)	249 (52.0%)	0.041	103 (21.5%)	376 (78.5%)	0.181
Positive	7 (22.6%)	24 (77.4%)		9 (29.0%)	22 (71.0%)		21 (67.7%)	10 (32.3%)		10 (32.3%)	21 (67.7%)	

T-Stage: Depth of tumor invasion. a: Macroscopic type; Classification according to the general rules for gastric cancer study of the Japanese Research Society for Gastric Cancer (22). Type 1 is defined as a polypoid tumor, sharply demarcated from the surrounding mucosa and usually attached on a wide base. Type 2 is defined as a polypoid tumor with ulceration and sharply demarcated margins. Type 3 is defined as an ulcerated carcinoma with cancer infiltration into the surrounding wall. Type 4 is defined as a diffusely infiltrating flat carcinoma in which ulceration is usually not a marked feature. bINF; Pattern of tumor infiltration into the surrounding tissue. The predominant pattern of infiltrating growth into the surrounding tissue is classified as follows; INF a: The tumor shows expanding growth and a distinct border with the surrounding tissue. INF b: This category is between INF a and INF b. INF c: The tumor shows infiltrating growth and an indistinct border with the surrounding tissue.

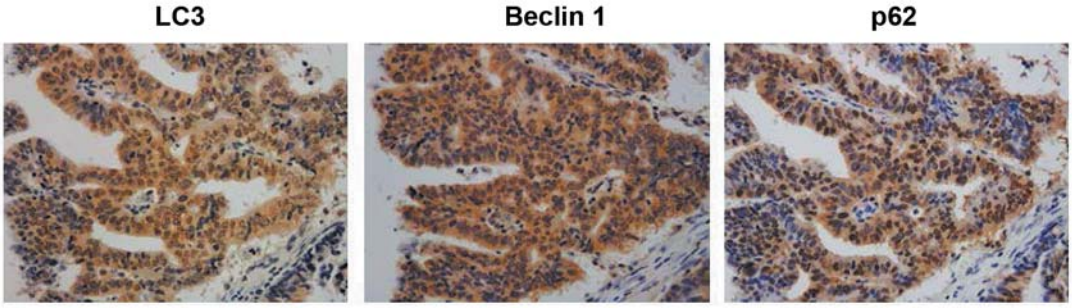

Figure 1. *Representative primary gastric carcinomas exhibiting immunostaining for light chain 3 (LC3), Beclin 1, and p62. LC3 and Beclin 1 were found to be mainly expressed in the cytoplasm of gastric cancer cells. p62 staining was found in both the nucleus and cytoplasm (×200).*

Table III. *Association of among autophagy-related proteins light chain 3 (LC3), Beclin 1 and p62.*

	Beclin 1			p62		
Factor	Negative (n=384)	Positive (n=126)	*p*-Value	Negative (n=259)	Positive (n=251)	*p*-Value
LC3 expression						
Negative (n=431)	343 (79.6%)	88 (20.4%)	<0.001	233 (54.1%)	198 (45.9%)	0.001
Positive (n=79)	41 (51.9%)	38 (48.1%)		26 (32.9%)	53 (67.1%)	
Beclin 1 expression						
Negative (n=384)				212 (55.2%)	172 (44.8%)	0.001
Positive (n=126)				47 (37.3%)	79 (62.7%)	

negative patients. At clinical stage II, the prognosis of autophagy-positive patients tended to be worse (p=0.099) than that of autophagy-negative patients. Univariate analysis revealed that poor survival was significantly correlated with LC3-positive (p=0.02), Beclin 1-positive (p=0.039), p62-positive (p<0.001), and autophagy-positive statuses (p<0.001), large tumor size (p<0.001), macroscopic tumor type 4 (p<0.001), diffuse tumor type (p=0.001), T3/4 stage (p<0.001), lymphatic invasion (p<0.001), venous invasion (p<0.001), hepatic metastasis (p<0.001), peritoneal metastasis (p<0.001) and lymph node metastasis (p<0.001). Multivariate analysis indicated that autophagy-positive status (p=0.027), macroscopic tumor type 4 (p=0.009), T3/4 stage (p=0.017), lymph node metastasis (p<0.001), hepatic metastasis (p=0.002), peritoneal metastasis (p=0.001), but not individual positivity for LC3, Beclin 1 and p62, were significantly associated with poor patient survival (Table IV).

Discussion

In the present study, we evaluated the expression of autophagy-related proteins in primary gastric cancer, and analyzed their relationships with clinicopathological parameters and clinical outcomes. Autophagy status was determined based on LC3, Beclin 1 and p62 expression. LC3 and p62 have been shown to be overexpressed under activation of autophagy due to their roles in the formation of autophagosomes (8-10, 14). Beclin 1 is a modifier of the autophagic process (24). Although most studies have reported that LC3, Beclin 1 and p62 are highly expressed in various types of cancer cell with autophagy-positive status (25-27), some studies have reported their low expression in cancer cells with autophagy-negative status (28, 29). Using a single marker to determine the autophagy status might prove unreliable; we, therefore, used three autophagy-related proteins, LC3, Beclin 1 and p62. Autophagy was then determined to be positive when samples were positive for expression of at least two of these proteins. Because the expression of LC3, Beclin 1, and p62 were closely associated with each other, these proteins may interact with each other and play a role in regulating autophagy activation.

Our data demonstrated that autophagy was associated with aggressive clinical behavior, such as vessel invasion, lymph node disease, and hepatic metastasis in gastric cancer. It has been reported that LC3 and Beclin 1 were associated with lymphatic invasion in pancreatic cancer (30) and oral cell

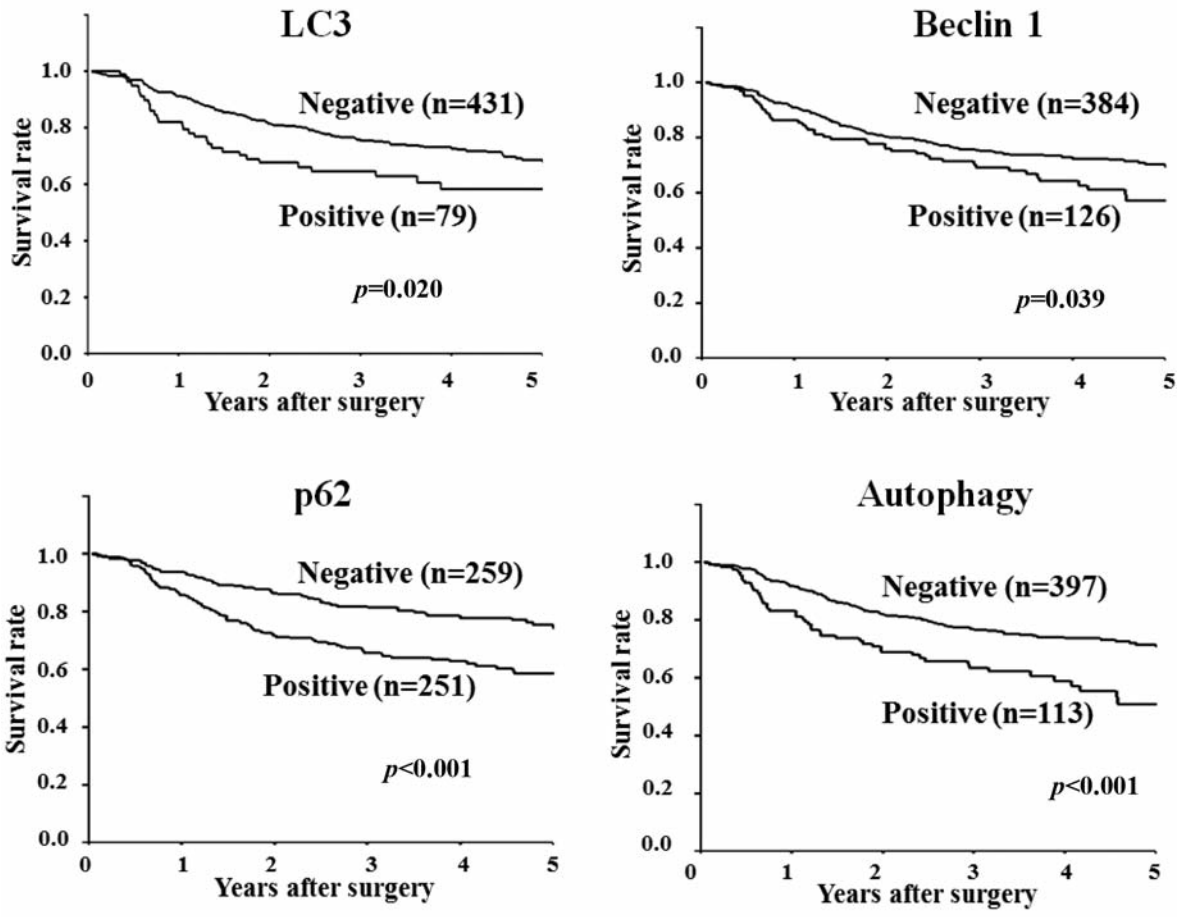

Figure 2. *Survival curves for the cohort of 510 patients according to light chain 3 (LC3), Beclin 1, p62, and autophagy status. The expressions of LC3, Beclin 1, and p62, and resultant autophagy status were significantly correlated with patient survival. The survival of patients with autophagy-positive tumors was significantly worse (log-rank p<0.001) than that of those with autophagy-negative tumors.*

carcinoma (31), and that p62 was correlated with vascular invasion (31). These results suggest that autophagy might play an important role in lymphovascular invasion and distant metastasis in gastric cancer.

Expressions of LC3, Beclin 1, and p62 were each associated with worse survival of patients with gastric cancer in univariate analysis. It has been reported that LC3 expression is advantageous to cancer development, especially in early-phase carcinogenesis of gastrointestinal cancer (11). These findings might suggest that autophagy is significantly associated with poor survival of patients with gastric cancer, especially for those with disease at an early stage. Multivariate analysis found that autophagy status was an independent prognostic factor, but that the individual markers LC3, Beclin 1 and p62 were not. These findings might suggest that autophagy promotes the progression of gastric cancer, especially at an early clinical stage, and that autophagy status determined by LC3, Beclin 1, and p62 expression might be a predictive prognostic factor in gastric cancer.

The expressions of LC3 and Beclin 1 have been reported to be prognostic factors in various human cancer types (32, 33). Furthermore, it was found that p62 expression was associated with poor prognosis in lung cancer and oral squamous cell carcinoma (15, 34). In contrast, certain studies have reported that aberrant expression of autophagy-related proteins correlates with poor prognosis (25, 28, 35). LC3 expression was correlated with a good prognosis in hepatocellular carcinoma (36). Reduced expression of Beclin 1 correlated with poor prognosis in ovarian cancer (37). Qi-Rong *et al*. showed that high Beclin 1 expression was associated with longer overall survival (38). Autophagy status determined by LC3, Beclin 1, and p62 expression might be a promising prognostic tool in gastric cancer. Autophagy has also been demonstrated as a protective mechanism against chemotherapy or radiation induced

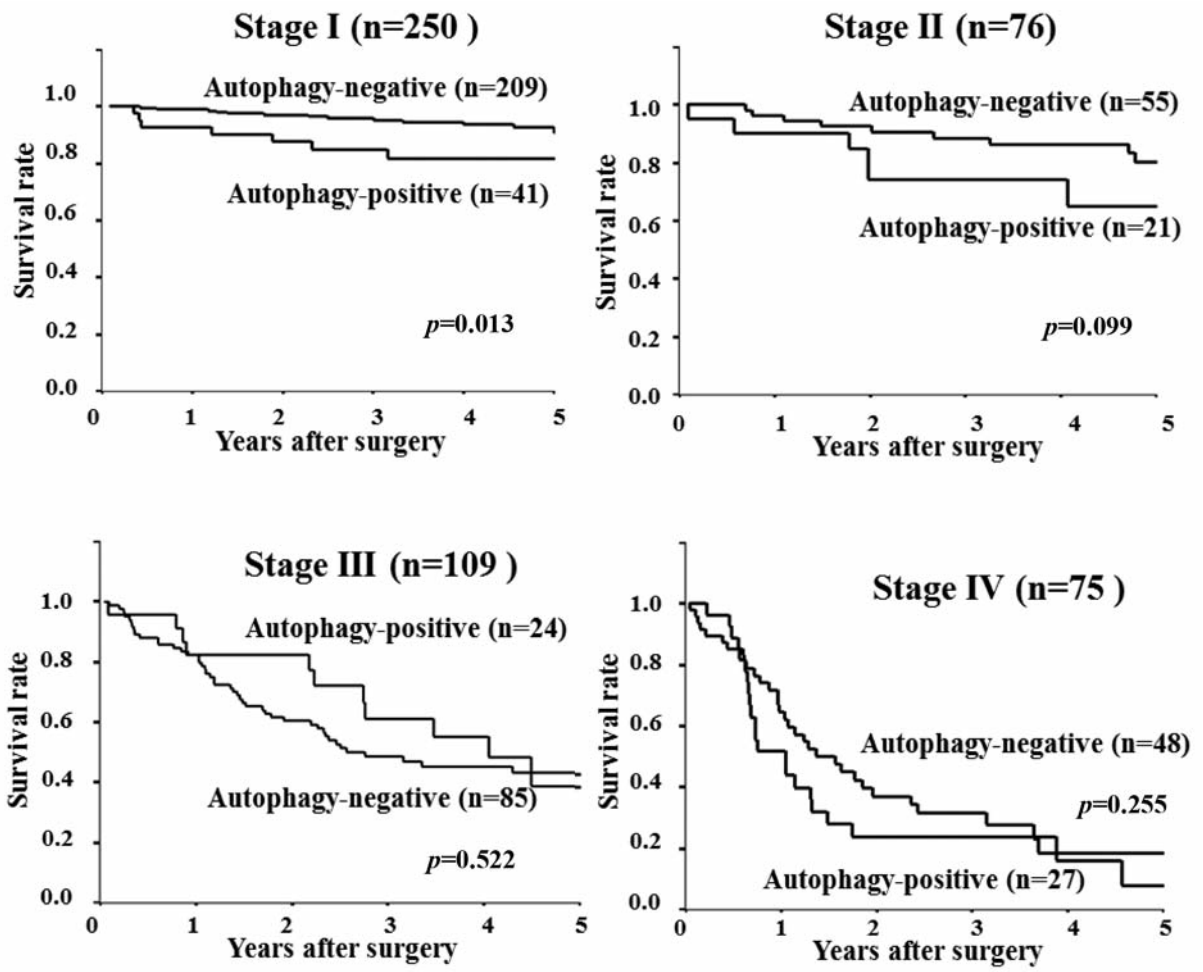

Figure 3. *Kaplan–Meier survival curves according to autophagy at each stage. At clinical stage I, the overall survival of patients with autophagy-positive gastric cancer was significantly worse (log-rank p=0.013) than that of patients with autophagy-negative cancer. At clinical stage II, III, and IV, the prognosis of patients was not significantly different between autophagy-positive cases and autophagy-negative cases.*

apoptosis (39). This process involves adapting cells to stress conditions, and its inhibition has been suggested as a promising strategy for cancer therapy (40). Autophagy might play an important role for cancer progression, and autophagy itself might therefore be an effective therapeutic target in gastric cancer. In summary, autophagy might promote the progression of gastric cancer, especially at an early stage. Autophagy status, as determined by combined LC3, Beclin 1 and p62 expression, might be independently associated with poor survival in patients with gastric cancer.

Conflicts of Interest

There exist no financial or other conflicts of interest with regard to this article.

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Table IV. *Univariate and multivariate analysis with respect to overall survival of patients with gastric cancer.*

	Univariate analysis			Multivariate analysis		
Variable	Hazard ratio	95% CI	*p*-Value	Hazard ratio	95% CI	*p*-Value
LC3						
Positive *vs*. negative	1.609	1.076-2.405	0.02	1.273	0.803-2.018	0.304
Beclin 1						
Positive *vs*. negative	1.452	1.019-2.069	0.039	1.024	0.692-1.517	0.905
p62						
Positive *vs*. negative	1.948	1.392-2.725	<0.001	1.172	0.811-1.694	0.399
Autophagy						
Positive *vs*. negative	1.907	1.343-2.707	<0.001	1.569	1.052-2.339	0.027
Age						
≥60 *vs*. <60 years	1.257	0.889-1.778	0.195			
Gender						
Female *vs*. male	1.256	0.913-1.728	0.16			
Macroscopic type[a]						
Type 4 *vs*. other	7.326	5.181-10.357	<0.001	1.839	1.167-2.898	0.009
Tumor size						
≥30 *vs*. <30 mm	5.535	3.427-8.940	<0.001	1.31	0.684-2.508	0.416
Histological type						
Diffuse *vs*. intestinal	1.761	1.280-2.423	0.001	1.152	0.756-1.756	0.511
T-Stage						
T3/T4 *vs*. T1/T2	6.39	4.422-9.235	<0.001	1.869	1.117-3.128	0.017
Lymph node metastasis						
Positive *vs*. negative	8.105	5.416-12.130	<0.001	3.275	1.837-5.841	<0.001
INF[b]						
c *vs*. a/b	1.824	1.320-2.520	<0.001	1.199	0.767-1.873	0.426
Lymphatic invasion						
Positive *vs*. negative	5.336	3.503-8.126	<0.001	1.335	0.716-2.490	0.364
Venous invasion						
Positive *vs*. negative	3.528	2.356-4.506	<0.001	1.231	0.852-1.780	0.269
Hepatic metastasis						
Positive *vs*. negative	6.582	3.711-11.675	<0.001	2.871	1.489-5.534	0.002
Peritoneal metastasis						
Positive *vs*. negative	9.147	6.041-13.852	<0.001	2.335	1.394-3.909	0.001

T-Stage: Depth of tumor invasion. a Macroscopic type; Classification according to the general rules for gastric cancer study of the Japanese Research Society for Gastric Cancer (22). Type 1 is defined as a polypoid tumor, sharply demarcated from the surrounding mucosa and usually attached on a wide base. Type 2 is defined as a polypoid tumor with ulceration and sharply demarcated margins. Type 3 is defined as an ulcerated carcinoma with cancer infiltration into the surrounding wall. Type 4 is defined as a diffusely infiltrating flat carcinoma in which ulceration is usually not a marked feature. bINF; Pattern of tumor infiltration into the surrounding tissue. The predominant pattern of infiltrating growth into the surrounding tissue is classified as follows: INF a: The tumor shows expanding growth and a distinct border with the surrounding tissue. INF b: This category is between INF a and INF b. INF c: The tumor shows infiltrating growth and an indistinct border with the surrounding tissue.

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