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Prognostic Significance of LC3B and p62/SQSTM1 Expression in Gastric Adenocarcinoma

JU SEOK KIM[1*], GO EUN BAE[2*], KYUNG-HEE KIM[2], SANG-IL LEE[3], CHAEUK CHUNG[4], DAHYE LEE[4], TAE HEE LEE[5], IN SUN KWON[6] and MIN-KYUNG YEO[2]

[1]*Department of Internal Medicine, Chungnam National University School of Medicine, Daejeon, Republic of Korea;*
[2]*Department of Pathology, Cancer Research Institute, Chungnam National University School of Medicine, Daejeon, Republic of Korea;*
[3]*Department of Surgery, Chungnam National University College of Medicine, Daejeon, Republic of Korea;*
[4]*Division of Pulmonology, Department of Internal Medicine, Chungnam National University College of Medicine, Daejeon, Republic of Korea;*
[5]*The Biobank of Chungnam National University Hospital, Daejeon, Republic of Korea;*
[6]*Clinical trials Center of Chungnam National University Hospital, Daejeon, Republic of Korea*

Abstract. *Background/Aim: Autophagy is a cellular mechanism that recycles cellular components to maintain homeostasis. To investigate the clinical implication of autophagy in gastric cancer, the autophagy markers with autophagosome formation, LC3B and selective autophagy substrate p62/SQSTM1 (P62) were validated. Materials and Methods: LC3B and p62 expression was examined using immunohistochemistry, western blot assays, and reverse-transcription polymerase chain reaction (RT-PCR). The relationship of LC3B and p62 expression in gastric adenocarcinomas with clinicopathological parameters, including patient survival, were analyzed. Results: Normal gastric mucosae exhibit no LC3B and p62 expression, while tubular adenoma and gastric adenocarcinomas exhibit variable nuclear or cytoplasmic p62 expression. High LC3B, high cytoplasmic p62, and low nuclear p62 protein expression in gastric adenocarcinomas is positively correlated with poor prognostic factors including survival. Conclusion: Dynamic LC3B and p62 changes are suggested to be involved in gastric tumorigenesis and cancer progression. LC3B and p62 could be used as prognostic biomarkers and potential therapeutic targets for gastric adenocarcinomas.*

*These Authors contributed equally to this study.

Correspondence to: Min-Kyung Yeo, MD, Ph.D., Department of Pathology, Cancer Research Institute, Chungnam National University School of Medicine, Munwha-ro 266, Jung-gu, Daejeon 35015, Republic of Korea. Tel: +82 422807196, Fax: +82 422808199, e-mail: mkyeo83@gmail.com

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Autophagy is a natural cell mechanism that removes dysfunctional cellular components. Autophagy is essential for maintaining homeostasis under stressful conditions like starvation, infection, degenerative disease, and cancer (1). Autophagy modulates extracellular matrix degradation and epithelial–mesenchymal transition, and is suggested to play a significant role in tumorigenesis and cancer progression (2, 3). Autophagy has become an attractive target for anti-cancer therapy, and clinical trials are in progress to demonstrate that the modulation of autophagy activity can enhance the efficacy of cancer therapies (4-11). However, autophagy has opposing and context-dependent roles, suggesting its dual properties in cancers (12). Thus, a better understanding of biology and the role of autophagy is required.

Gastric cancer is the fifth most common cancer and the third most common cause of cancer-related deaths worldwide (13). Despite a decline in the overall prevalence of gastric cancer, the treatment of stomach cancer remains challenging. Recurrence or metastasis often occurs even after successful gastrectomy, and the five-year survival rate is approximately 20% for patients with distant metastasis (14). Limited chemotherapeutic agents can only be applied to patients with gastric cancer recurrence or metastasis. Autophagy can be a promising candidate for the treatment of gastric cancer, as it may enhance chemotherapy or overcome drug resistance.

The autophagy-related proteins are known as beclin1, light chain 3 (LC3) A and B, p62/also called sequestosome 1 (SQSTM1), and sirtuin 1 (SIRT1) (15). Among these, LC3B and p62 are widely used autophagy markers for monitoring autophagy activity. LC3B is involved in autophagosome formation, and p62 serves as a selective autophagy substrate, which has multi-domains that interact with autophagy machinery as adaptors for the target cargo (16). The complex

regulation of LC3 and p62 is suggested to be related to autophagy activity in cancers (17). The role of p62 needs to be determined in order to interpret its function in relation to autophagy activity, and the up-regulation or down-regulation of p62 can play both tumorigenic or tumor-suppressive roles in cancers (16).

In this study, to investigate the roles of autophagy markers, LC3B and p62 expression in gastric adenocarcinomas, tubular adenomas with low-grade dysplasia, and non-neoplastic gastric mucosae was assessed. LC3B and p62 expression was analyzed in gastric adenocarcinomas as well.

Materials and Methods

Patients and tissue samples. This study was performed on 402 cases of gastric adenocarcinomas, operated at the Chungnam National University Hospital (Daejeon, Republic of Korea), from January 2011 to December 2012. Patients' clinical history, including disease-free and overall survivals, was reviewed. Patients who received preoperative chemo- or radiotherapy were excluded. Cancer stages were determined according to the American Joint Committee TNM criteria in the Cancer Staging System, eighth edition (18). Cases were classified according to the Lauren classification (17) and classified into Epstein Barr virus (EBV)-associated, microsatellite-unstable (MSI), and microsatellite-stable (MSS) types (19). Fifty gastric tubular adenoma and 10 non-neoplastic cases were also included for comparison analyses on gastric adenocarcinomas. Non-neoplastic tissue samples were acquired from tissues located more than 2 cm apart from the gastric adenocarcinomas.

Immunohistochemical staining analysis. Whole sections of gastric adenocarcinoma paraffin-embedded tissue samples were selected and validated in terms of the proper concentration, temperature, and time for immunohistochemistry. Tissue sections on the coated slides were de-paraffinized with xylene and hydrated in serial solutions of alcohol. The sections were heated in a pressure cooker [with 10 mmol/l sodium citrate (pH 6.0)] for 5 min for antigen retrieval. Endogenous peroxidase blocking (0.03% H_2O_2) was performed for 10 min. The sections were incubated overnight at 4˚C, with following antibodies (Table I); a mouse monoclonal anti-LC3B and p62 antibody and mismatch repair protein (MMR) for MLH1, MSH2, MSH6, and PMS2 antibody. Samples were incubated in a Dako REAL EnVision for 30 min at room temperature, followed by washing. Chromogen was developed for 2 min, after rinsing. Slides were counterstained with Meyer's haematoxylin. Epstein-Barr virus-encoded RNA *in situ* hybridization was performed to evaluate tumor EBV infection. Human nerve (LC3B), tonsil (p62), gastric mucosa (MMR), and lymph node (EBV) served as positive controls, and a primary antibody was omitted from the negative control.

A total of 402 gastric adenocarcinomas, 50 tubular adenomas, and 10 non-neoplastic gastric tissue samples were used to construct tissue microarrays using 3.0 mm thick sections in diameter where immunohistochemical staining of LC3B and p62 was performed. Staining of LC3B and p62 was scored using digitally scanned files in the ScanScope program (Aperio ScanScope CS System). The scoring system used the Allred *et al*. method (20). The immunohistochemical expression was categorized as "high" (expression at the median value or more) and "low" (expression at less than the medium value). The interpretation of the MMR expression followed the Rema *et al*. method (21). Based on the results concerning the MMR protein and the EBV *in situ* hybridization, 402 gastric adenocarcinomas were divided into EBV-associated, MSI, and MSS subtypes (19). Each sample was examined separately and scored by two pathologists (G.E.B. and M.K.Y.), who were blinded regarding the patients' details. Discrepancies in the scores were discussed to obtain a consensus.

Western blot analysis. Proteins were extracted from 67 pairs of gastric adenocarcinomas (obtainable among above 407 samples) and paired non-neoplastic frozen tissue samples were stored at −80˚C in liquid nitrogen using the PRO-PREP TM protein extraction solution (iNtRON Biotechnology, 17081, Kyungki-do, Republic of Korea). One vial (100 mg) from each set of paired samples was obtained and ground using TissueLyser (Qiagen, Hilden, Germany). Proteins were extracted, and a total 20 μg of protein were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (BIO-RAD®, Mini-PROTEAN TGXTM Gels, 456-1034) and then transferred to a polyvinylidene difluoride membrane (BIO-RAD, Immun-Blot® PVDF Membrane for Protein Blotting, 162-0177). The membrane was blocked with 2% dry skim milk and incubated with the anti-rabbit monoclonal LC3B (1:1000, L8918, Sigma-aldrich, Saint Louis, MO, USA) and anti-mouse monoclonal p62 (1:1000, clone2C11, Sigma-Aldrich) antibody. The membrane was incubated in the anti-rabbit IgG, anti-mouse IgG, and H&L chain-specific peroxidase conjugate secondary antibody (CALBIOCHEM, 401353, Darmstadt, Germany) at room temperature for 1.5 h. Protein bands were enhanced by Immobilon® western chemiluminescent HRP substrate (Millipore, WBKLS0500, Burlington, MA, USA). The images were digitalized using a UVITEC Cambridge alliance mini 4M system (UVItec Limited, Cambridge, UK). Mouse brain cell, NBP2-49688 (Novusbio, Centennial, CO, USA), was used as a positive control, and a tissue sample was omitted from the negative control.

Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). Thirty-six gastric adenocarcinomas (obtainable 67 samples) and paired non-neoplastic gastric tissue were obtained from the National Biobank of Korea. One vial (100 mg) of paired samples was obtained and the total RNA was extracted using a QIAGEN kit (Valencia, CA, USA). Reverse transcription was performed with RevertAid H Minus Reverse Transcriptase (Thermo Scientific, Waltham, MA, USA). Real-time PCR was performed in a Rotor-Genes Q cycler machine (Qiagen, Hilden, Germany) using a Rotor-Genes SYBR Green PCR kit (Qiagen), in a total volume of 20 μl. The LC3B, p62, and GAPDH primers used for PCR amplification (Table II). To correlate the threshold values, from the amplification plots to the copy number, a standard curve was generated, and a non-template control was run with every assay. All samples were run in duplicate, and the average value was used. The relative quantification values of LC3B and p62 in each tissue sample were categorized as high (greater than the paired non-neoplastic tissue value) and low (less than the paired non-neoplastic tissue value) for comparison analyses. Samples with insufficient RNA levels or failed PCR results were excluded.

Statistical analysis. Associations between the LC3B and p62 expression levels, as well as selected clinicopathological parameters for gastric adenocarcinomas, were examined using Spearman rank

Table I. *Antibodies for immunohistochemistry.*

Antibody	Clone, Company
LC3B	1:50, clone 5F10, Nanotool, Teningen, Germany
p62 (SQSTM1, D-3)	1:400, sc-28359, Santa Cruz Biotechnology, Santa Cruz, CA, USA
MLH1	Ready-to-Use (RTU), clone M1, VENTANA, Tucson, AZ, USA
MSH2	RTU, clone G219-1129, Cell Marque, Rocklin, CA, USA
MSH6	RTU, clone 44, VENTANA, Tucson, AZ, USA
PMS2	RTU, clone EPR3947, Cell Marque, Rocklin, CA, USA

Table II. *Primers for quantitative real-time reverse-transcription polymerase chain reaction.*

Primer	
LC3B	Forward 5'-GAG AAG CAG CTT CCT GTT CTG G-3', Reverse 5'-GTG TCC GTT CAC CAA CAG GAA G-3'
p62	Forward 5'-TGT GTA GCG TCT GCG AGG GAA A-3', Reverse 5'-AGT GTC CGT GGT TCA CCT TCC G-3'
GAPDH	Forward 5'-GAG TCA ACG GAT TTG GTC GT-3', Reverse 5'-TGG AAG ATG GTG ATG GGA TT-3'

correlation coefficients and Mann-Whitney *U*-tests. The Wilcoxon signed-rank test was used for group comparison. For univariate analysis, overall and disease-free survival curves, with log-rank tests, were generated using the Kaplan–Meier method. Multivariate survival analysis was performed using the Cox proportional hazard regression model. The statistical significance was set at $p<0.05$ (SPSS 24.0; SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics. A total of 402 gastric adenocarcinoma cases were evaluated. The patients' average age was 60.8 years (with a range of 21 to 86 years), and they were predominantly men (male/female=1.9:1). Patients received total gastrectomy (26%) or subtotal gastrectomy (74%). Patients were diagnosed with advanced (56%) or early gastric cancer (44%). Of the patients, 26.6% showed a lymph nodal metastasis, and 1.4% had a distant metastasis. Post-operative adjunctive or systemic chemotherapy was performed in 53% of patients for above-stage IIIB or at the time of recurrence. Gastric adenocarcinomas were classified into intestinal (59%), diffuse (30%), and mixed (11%) types, based on Lauren's classification, and classified into EBV-associated (7%), MSI (13%), and MSS (80%) types, based on Roh's classification (19).

Immunohistochemical expression of LC3B and P62. LC3B and p62 immunostaining showed protein presence in gastric epithelial cells, but not in stromal cells that LC3B exhibited a cytoplasmic punctate pattern (Figure 1A) and P62 exhibited both a nuclear and cytoplasmic pattern (Figure 1B). All non-neoplastic mucosae were found to be negative for LC3B and p62. Gastric adenomas were positive only for p62 in a cytoplasmic pattern, but they were negative for LC3B. Gastric adenocarcinomas showed positive for cytoplasmic LC3B puncta and showed both nuclear and cytoplasmic p62 expressions. Gastric adenocarcinomas were up-regulated LC3B expression, compared with the non-neoplastic gastric mucosae and tubular adenomas (Figure 1C). Gastric adenocarcinomas had up-regulated nuclear p62 expression, compared to non-neoplastic gastric mucosae and adenomas. Gastric adenomas showed significantly higher levels of cytoplasmic p62 than gastric adenocarcinomas. Cytoplasmic LC3B and cytoplasmic p62 levels were positively correlated. Cytoplasmic p62 and nuclear p62 levels were positively correlated.

Prognostic significance of LCB3 and p62 immunostaining. Immunohistochemical expression of LC3B, nuclear p62, and cytoplasmic p62, with the clinicopathological parameters of a total of 402 gastric adenocarcinomas, were assessed (Table III). LC3B expression was positively correlated with advanced cancer, a higher pathologic stage (I-II *vs*. III-IV), a higher T-stage, and a positive lymph node metastasis. The nuclear p62 expression was positively correlated with early gastric cancer, a lower pathologic stage, a lower T-stage, and a negative lymph node metastasis. The cytoplasmic p62 expression was positively correlated with advanced gastric cancer, a higher T-stage, and post-operative chemotherapy. Overall and disease-free survival analyses were performed with data from 402 gastric adenocarcinoma patients (Figure 2). The Kaplan–Meier survival curves and log-rank tests showed a significant association of high LC3B expressions, with a shortened overall and disease-free survival (Figure 2A and B). The Kaplan–Meier overall survival curves, with

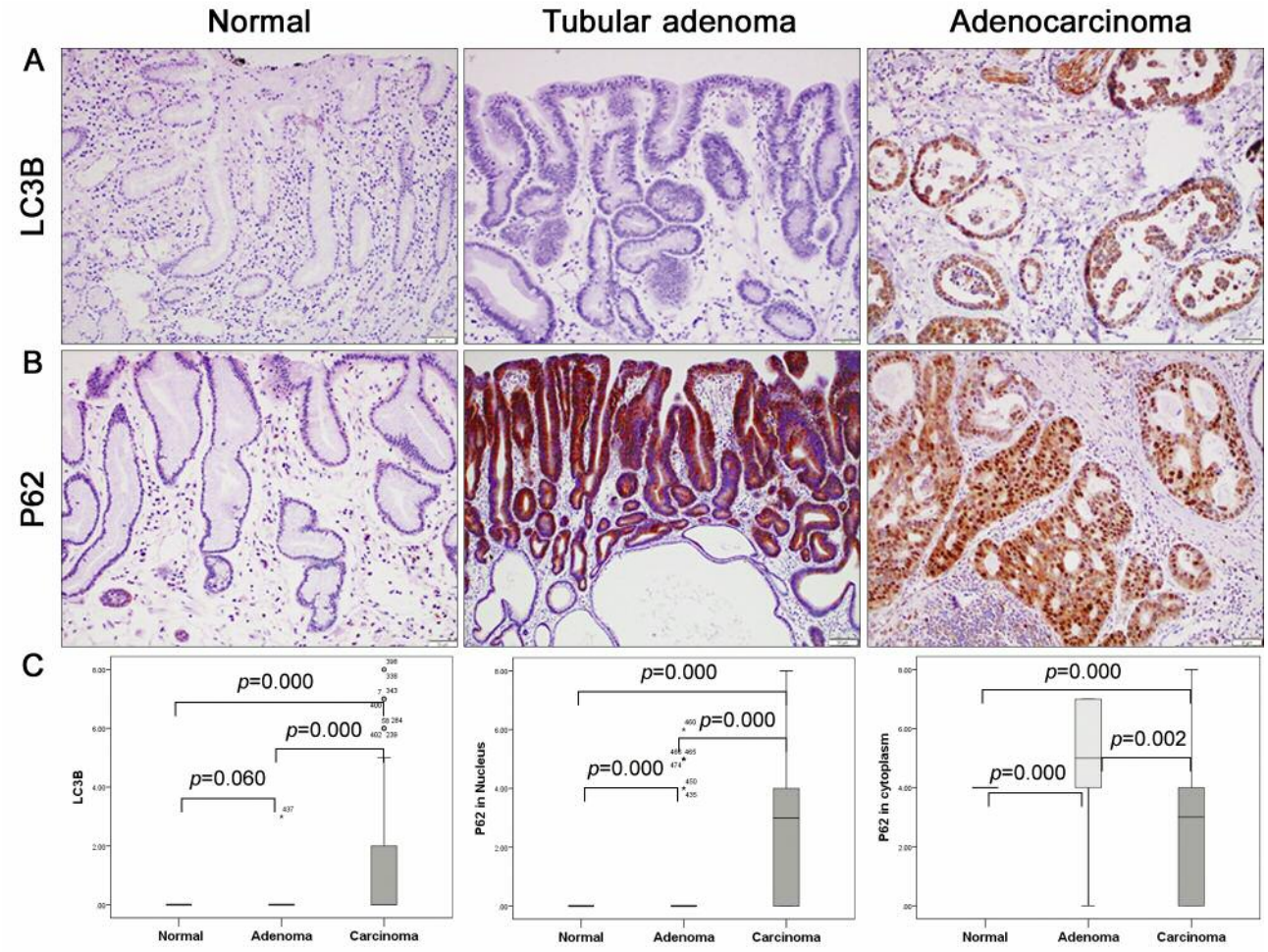


Figure 1. *Representative immunohistochemical expression levels of (A) LC3B (white arrow: peripheral nerve tissue – the internal control for LC3B) and (B) p62 in normal gastric mucosae, gastric tubular adenomas, and gastric adenocarcinomas. (C) Comparison analysis of the LC3B and p62 expression in the normal gastric mucosa, gastric tubular adenoma, and gastric adenocarcinoma groups.*

a low expression of nuclear P62, showed a shortened overall and disease-free survival (Figure 2C and D). The Kaplan–Meier overall survival curves, with a high cytoplasmic P62 expression, showed a tendency toward a shortened overall and disease-free survival, but this did not attain statistical significance (Figure 2E and F). In the intestinal subtype, high LC3B expressions showed a significant association with a shortened overall and disease-free survival (Figure 3A). In the diffuse and mixed subtypes, a high LC3B and low nuclear p62 were related with a shortened overall and disease-free survivals (Figure 3C and 3D). In the MSS subtype, high LC3B and low nuclear p62 expressions showed a significant association with a shortened disease-free survival (Figure 3E and F). Cytoplasmic p62 did not attain statistical significance in relation to disease-free survivals for any subtypes of gastric adenocarcinomas. Multivariate analyses, using the Cox's proportional hazard model, were performed and showed that LC3B, nuclear P62, and cytoplasmic p62 immunohistochemical expressions did not reach statistical significance in relation to the overall survival.

Western blot assay. To classify the autophagy activity of gastric cancer, western blot assays were performed using 67 pairs of gastric adenocarcinomas and non-neoplastic gastric tissue samples. Comparison expression patterns of cancer and paired non-neoplastic tissue were classified into 4 groups (Figure 4A). The overall and disease-free survivals for the four autophagy activity groups were compared. However, no statistic difference was observed (Figure 4B). Group C and D *vs.* group A and D were compared in terms of disease-free survival. A tendency toward poor survival in group C+D (autophagy activation) was shown, but this did not attain a prognostic significance.

Table III. *Correlation between the LC3B, nuclear P62, and cytoplasmic P62 immunohistochemical expressions and clinicopathologic factors in gastric adenocarcinomas. EGC, Early gastric cancer; AGC, advanced gastric cancer; EBV, Epstein Barr virus; MSI, microsatellite-unstable; MSS, microsatellite-stable.*

Characteristics	Patients	LC3B			Patients	Nuclear P62			Patients	Cytoplasmic P62		
	No. (%)	Low	High	*p*-Value	No. (%)	Low	High	*p*-Value	No. (%)	Low	High	*p*-Value
Gender				0.335				0.227				0.260
Male	261 (65)	160 (64)	101 (68)		263 (65)	109 (69)	154 (63)		263 (65)	91 (62)	172 (68)	
Female	139 (35)	92 (37)	47 (32)		139 (35)	49 (31)	90 (37)		139 (35)	56 (38)	83 (33)	
Age				0.001				0.061				0.067
≤60	180 (45)	129 (51)	51 (35)		181 (45)	62 (39)	119 (49)		181 (45)	75 (51)	106 (42)	
>60	220 (55)	123 (49)	97 (66)		221 (55)	96 (61)	125 (51)		221 (55)	72 (49)	149 (58)	
EGC *vs*. AGC				0.000				0.004				0.015
EGC	175 (44)	137 (54)	38 (26)		176 (44)	55 (35)	121 (50)		176 (44)	76 (52)	100 (39)	
AGC	225 (56)	115 (46)	110 (74)		226 (56)	103 (65)	123 (50)		226 (56)	71 (48)	155 (61)	
Pathologic stage				0.000				0.001				0.185
I-II	271 (68)	189 (75)	82 (55)		272 (68)	91 (58)	181 (74)		272 (68)	105 (72)	167 (66)	
III-IV	128 (32)	62 (25)	66 (45)		129 (32)	66 (42)	63 (26)		129 (32)	41 (28)	88 (35)	
T-stage				0.000				0.010				0.017
T1&2	240 (60)	172 (69)	68 (46)		241 (60)	82 (52)	159 (65)		241 (60)	99 (68)	142 (56)	
T3&T4	159 (40)	79 (32)	80 (54)		160 (40)	75 (48)	85 (35)		160 (40)	47 (32)	113 (44)	
LN metastasis				0.005				0.049				0.552
Absent	296 (74)	198 (79)	98 (66)		298 (74)	109 (69)	189 (78)		298 (74)	111 (76)	187 (73)	
Present	103 (26)	53 (21)	50 (34)		103 (26)	49 (31)	54 (22)		103 (26)	35 (24)	68 (27)	
Lauren				0.000				0.039				0.002
Intestinal	235 (59)	128 (51)	107 (73)		237 (59)	100 (64)	137 (56)		237 (59)	70 (48)	167 (66)	
Diffuse	121 (30)	94 (38)	27 (18)		121 (30)	48 (31)	73 (30)		121 (30)	57 (39)	64 (25)	
Mixed	42 (11)	29 (12)	13 (9)		42 (11)	9 (6)	33 (14)		42 (11)	19 (13)	23 (9)	
Molecular				0.000				0.507				0.014
EBV	29 (7)	11 (4)	18 (12)		29 (7)	13 (8)	16 (7)		29 (7)	6 (4)	23 (9)	
MSI	50 (13)	21 (8)	29 (20)		51 (13)	23 (15)	28 (12)		51 (13)	12 (8)	39 (15)	
MSS	321 (80)	220 (87)	101 (68)		322 (80)	122 (77)	200 (82)		322 (80)	129 (88)	193 (76)	
Chemotherapy				0.002				0.069				0.033
Not done	187 (47)	133 (53)	54 (37)		188 (47)	65 (41)	123 (50)		188 (47)	79 (54)	109 (43)	
Done	213 (53)	119 (47)	94 (64)		214 (53)	93 (59)	121 (50)		214 (53)	68 (46)	146 (57)	

Prognostic implication of LC3B and P62 mRNA levels. LC3B and p62 mRNA levels were examined, and the relative quantitation level was determined for 36 pairs of gastric tissues (Figure 5). The LC3 mRNA expression was elevated in 19 cases (53%), and the p62 mRNA expression was elevated in 25 (69%) cases of gastric adenocarcinomas, compared to non-neoplastic gastric tissue samples (Figure 5A). The associations between the LC3B and p62 mRNA levels, with prognostic implications, were evaluated (Figure 5B). The Kaplan–Meier survival curves and log-rank tests showed that a high LC3B mRNA level was correlated with a tendency toward a worse survival rate, but this did not attain statistical significance. The P62 mRNA levels were not related with disease-free survivals. The LC3B and P62 mRNA expressions were assessed using clinicopathologic parameters (Table IV) and the LC3B mRNA level was related with the diffuse- and mixed-type gastric cancers, but was not related to other factors.

Discussion

Autophagy plays opposing or context-dependent roles in cancers and gastric adenocarcinoma has been evaluated using LC3B and p62 markers (22). In this study, protein expression of LC3B and p62 was found to differ during gastric tumorigenesis. LC3B was not expressed in normal and tubular adenomas, while p62 was expressed in the cytoplasm of tubular adenomas but not in normal gastric mucosae. LC3B was significantly elevated in the cytoplasm of gastric adenocarcinomas with a punctate pattern, and p62 showed both nucleic and cytoplasmic expression patterns in gastric adenocarcinomas. Previous studies showed similar results, *i.e.*, an elevated cytoplasmic punctate pattern for LC3B and elevated nuclear and cytoplasmic p62 expression for p62 in gastric adenocarcinomas (23, 24). The up-regulation of LC3B during tumorigenesis indicates that the LC3B protein expression is elevated in precancerous and cancerous lesions

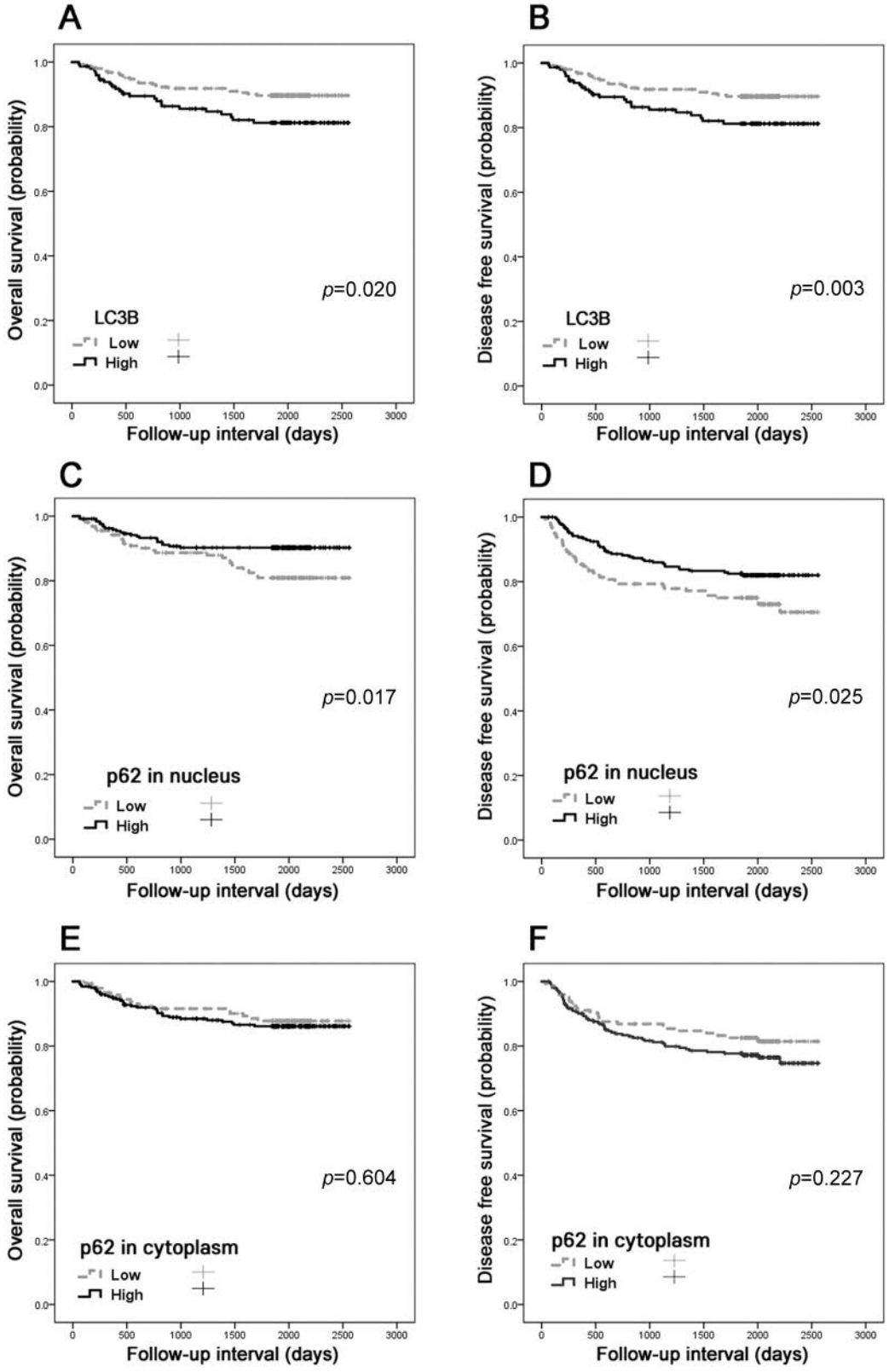


Figure 2. *Kaplan–Meier curves according to the immunohistochemical status of (A) LC3B (B) nuclear p62 (C) cytoplasmic P62 expressions in gastric adenocarcinomas with respect to overall and disease-free survivals.*

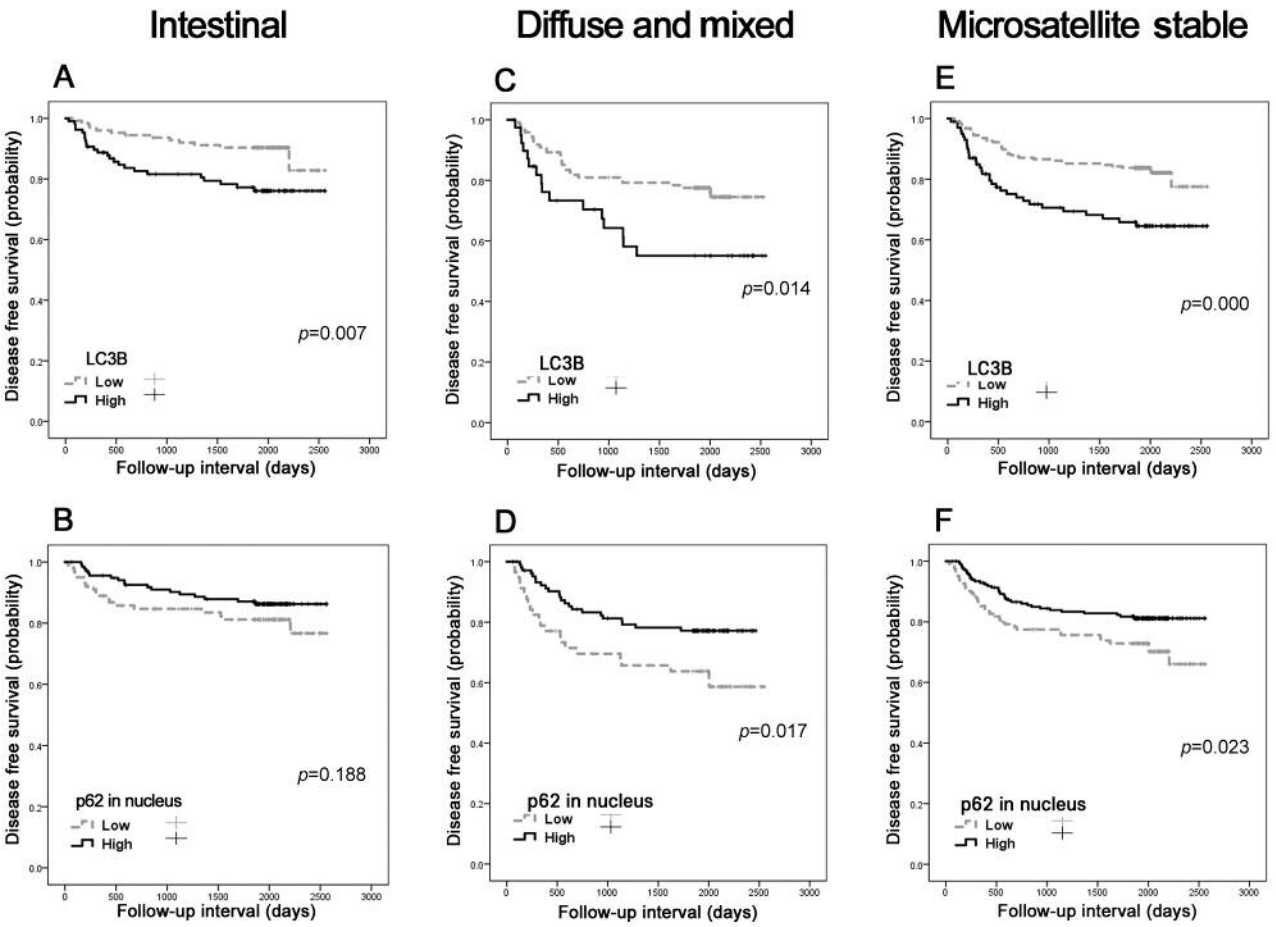
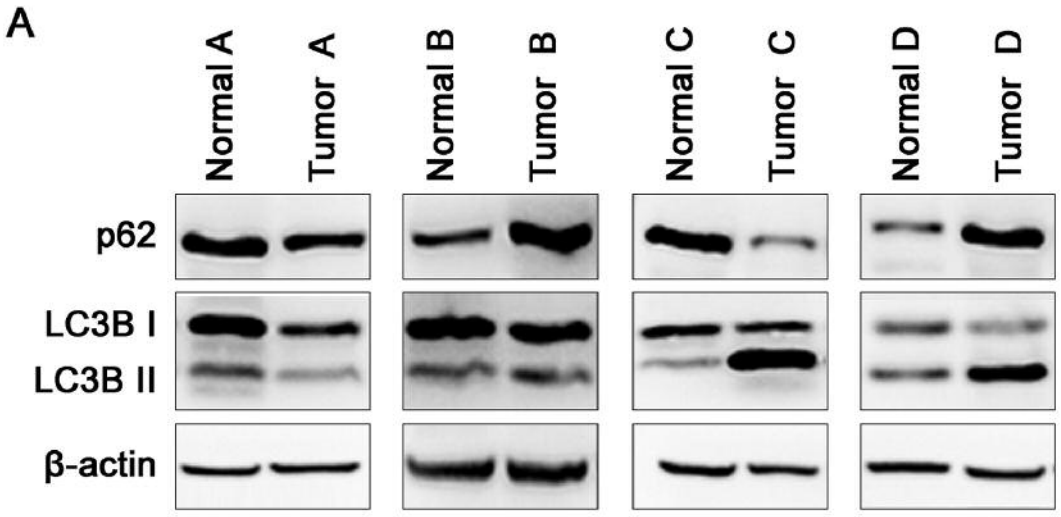


Figure 3. *Kaplan–Meier curves according to (A, C, E) LC3B and (B, D, F) nuclear p62 immunohistochemical expressions in the subtypes of gastric adenocarcinomas: (A, D) intestinal type; (B, E) diffuse and mixed types; (C, F) microsatellite-stable types.*

(25, 26). A sequential change in p62 expression was observed that high nuclear and low cytoplasmic p62 expression was found in normal oral epithelia, while low nuclear and high cytoplasmic p62 expression was found in oral cavity cancer (26). p62 expression has been shown to be elevated in many cancers (25 out of 29 different types of cancer), mostly showing a cytoplasmic pattern (27).

In this study, the LC3B and p62 mRNA levels were elevated in more than half of the cases of gastric adenocarcinomas, compared to paired gastric normal gastric mucosae. In previous studies, a higher level of LC3B mRNA has been correlated with the development gastric cancer (28), while lower expression of LC3B mRNA has been correlated with the development of lung and pharyngeal cancers (29, 30). According to data generated from gene expression profiling interactive analysis, p62 mRNA level has significantly higher expression in many cancers, including gastric adenocarcinomas, compared with paired normal tissues (27). The LC3B transcriptional level of cancers showed conflicting results, *i.e.*, upregulation or downregulation was shown to depend on the type of cancer, and the p62 transcriptional level of cancers, including gastric adenocarcinomas, was generally increased. The aberrant cytoplasmic protein expression of LC3B and alterations of the p62 protein localization was found to be involved in carcinogenesis and also related to the type of cancer.

The expression levels of LC3B, nuclear p62, and cytoplasmic p62 were separately evaluated using clinicopathologic factors, including patient survival. High LC3B and cytoplasmic p62 expression was positively correlated with unfavorable clinicopathologic parameters. Conversely, nuclear p62 expression was negatively correlated with unfavorable clinicopathologic parameters. High LC3 and low nuclear p62 protein expression were significantly related to shortened overall and disease-free survival times. Previous studies showed a significant prognostic implication of LC3B



Group	N=67	Autophagic activity
A	34	Basal level of autophagy
B	8	Basal level of autophagy but impairment later steps
C	12	Autophagy activation
D	13	Autophagy activation but impairment at later steps

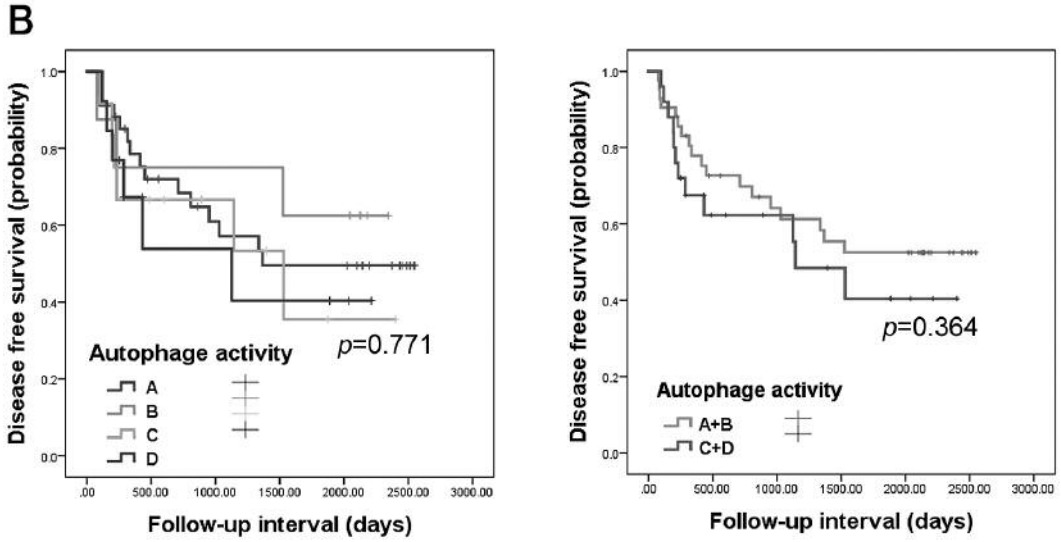


Figure 4. *Classification of autophagic activity followed by (A) a western blot assay (representative image) and (B) Kaplan–Meier curves, according to the four subgroups of autophagic activity relating to disease-free survival.*

and p62 in gastric adenocarcinomas, *i.e.*, that high expression of LC3B and high cytoplasmic p62 is associated with shortened gastric cancer patient survival; however, the nuclear p62 expression was not validated in these studies (24, 28, 31). The pooled results indicate that the prognostic significance of LC3 and p62 is different depending on the type of cancer. Low LC3B expression is related to unfavorable clinicopathological parameters in breast and lung

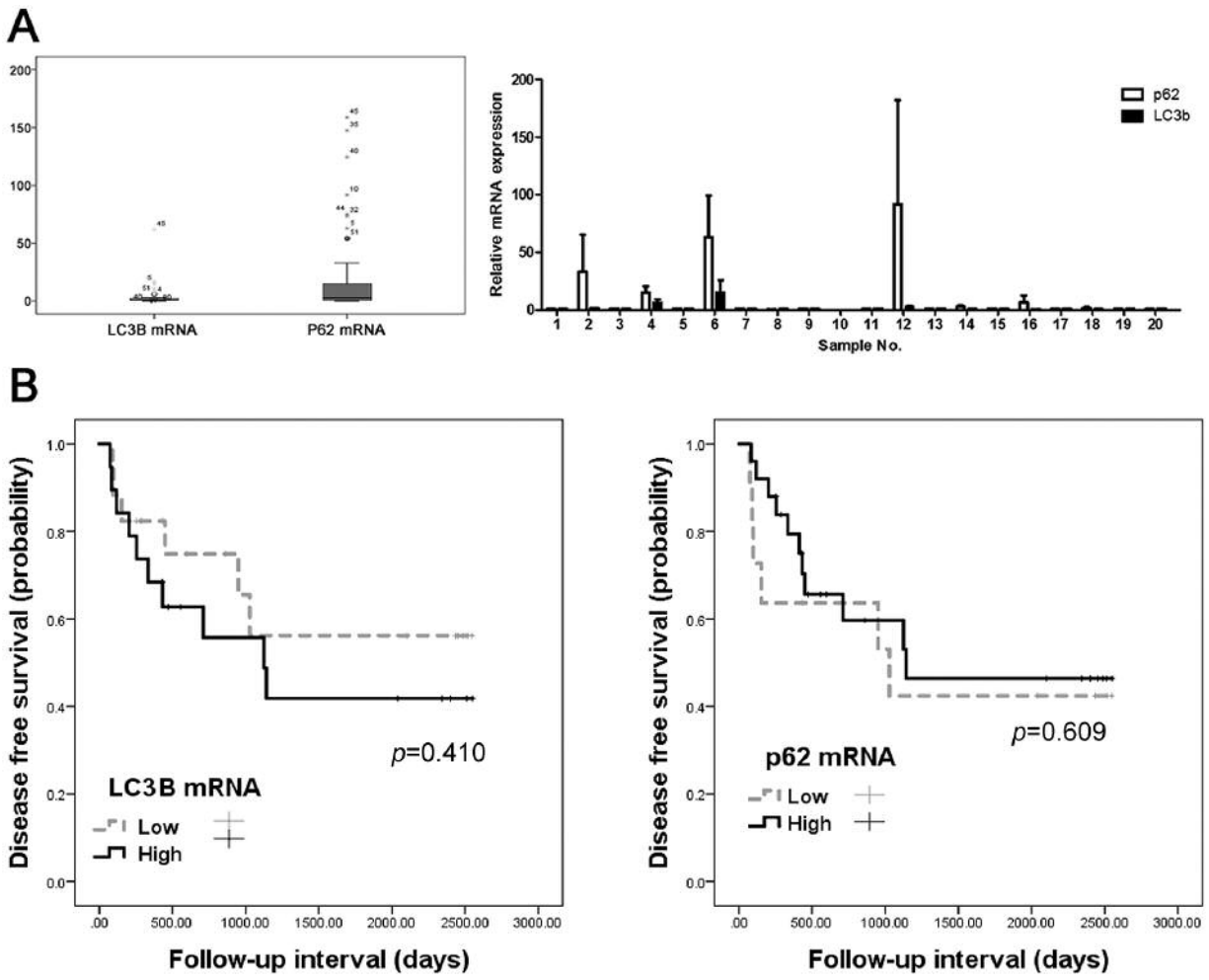


Figure 5. *(A) LC3B and p62 mRNA levels detected by qRT-PCR. The relative quantification values of LC3B and p62 gastric adenocarcinomas, compared to paired normal gastric mucosae. (B) Kaplan–Meier curves, according to the LC3B and p62 mRNA levels in relation to disease-free survivals of patients with total- and intestinal-type gastric adenocarcinomas.*

cancers (32-34), whereas high LC3B expression is associated with lower patient survival rates in gastric and hepatocellular carcinomas (35). p62 overexpression is mostly associated with worse prognosis in gastric adenocarcinomas, but not hepatocellular carcinomas (27).

Functional autophagy activity (active autophagosome-LC3B formation with substrate-P62 loss) in cancer was suggested to be more related to poor patient prognosis than impaired autophagy function, especially in early carcinogenesis (26). In this study, a western blot assay was performed to evaluate the autophagy activity in gastric adenocarcinomas. Statistical significance was not attained from the four different autophagy activity groups in relation to their prognostic implications for gastric adenocarcinomas. Autophagy is a tightly-regulated multi-step process (36). Autophagy activity depends on the loss of a substrate-p62 and the formation of an autophagosome-LC3B; thus, the relative balance of LC3B and p62 is important. High LC3B expression in gastric adenocarcinomas is suggested to elevate autophagy activity, thereby supporting tumor survival and leading to poor prognosis. The intracellular p62 level is dependent on both the transcription level and autophagy activity, and a low level of autophagy activity can cause the accumulation of intracellular p62. Elevated cytoplasmic p62 can act as a tumorigenic factor through multiple signaling pathways (16). The nuclear–cytoplasmic movement of p62 is also suggested to play a key role in the regulation of p62, and the function of p62 not only relies on the transcriptional level, but also on distribution of p62 (37). The p62 mRNA level is elevated in gastric adenocarcinomas, and high cytoplasmic p62 and low nuclear p62 expression are positively correlated with an unfavorable prognosis. Even

Table IV. *Correlation between the LC3B and P62 mRNA level and clinicopathologic factors in gastric adenocarcinomas. EGC, Early gastric cancer; AGC, advanced gastric cancer; EBV, Epstein Barr virus; MSI, microsatellite-unstable; MSS, microsatellite-stable.*

Characteristics	Patients	LC3BmRNA			Patients	P62 mRNA		
	No. (%)	Low	High	*p*-Value	No. (%)	Low	High	*p*-Value
Gender				0.74				0.176
Male	28 (78)	11 (65)	17 (90)		28 (78)	7 (64)	21 (84)	
Female	8 (22)	6 (35)	2 (11)		8 (22)	4 (36)	4 (16)	
Age				0.013				0.464
≤60	14 (39)	3 (18)	11 (58)		13 (27)	3 (40)	10 (36)	
>60	22 (61)	14 (82)	8 (42)		23 (73)	8 (60)	15 (64)	
EGC *vs*. AGC				NA				NA
EGC	0 (0)	0 (0)	0 (0)		0 (0)	0 (0)	0 (0)	
AGC	36 (100)	17 (100)	19 (100)		36 (100)	17 (100)	19 (100)	
Pathologic stage				0.595				0.350
I-II	8 (23)	3 (19)	5 (26)		7 (30)	3 (30)	4 (16)	
III-IV	27 (77)	13 (81)	14 (74)		28 (70)	7 (70)	21 (84)	
T-stage				0.446				0.490
T1&2	3 (9)	2 (13)	1 (5)		2 (6)	1 (10)	1 (4)	
T3&T4	32 (91)	14 (88)	18 (95)		33 (94)	9 (90)	24 (96)	
LN metastasis				0.709				0.124
Absent	16 (44)	7 (41)	9 (47)		16 (44)	7 (64)	9 (36)	
Present	20 (56)	10 (59)	10 (53)		20 (56)	4 (36)	16 (64)	
Lauren				0.048				0.298
Intestinal	16 (46)	10 (63)	6 (32)		17 (49)	3 (30)	14 (56)	
Diffuse	14 (40)	6 (38)	8 (42)		14 (40)	6 (60)	8 (32)	
Mixed	5 (14)	0 (0)	5 (26)		4 (11)	1 (10)	3 (12)	
Molecular				0.052				0.290
EBV	5 (14_	0 (0)	5 (14)		5 (14)	2 (18)	3 (12)	
MSI	9 (25)	6 (35)	3 (25)		8 (22)	4 (36)	4 (16)	
MSS	22 (61)	11 (65)	11 (61)		23 (64)	5 (46)	18 (72)	
Chemotherapy				0.114				0.871
Not done	5 (14)	4 (24)	1 (5)		6 (17)	2 (18)	4 (16)	
Done	31 (86)	13 (44)	18 (95)		30 (83)	9 (82)	21 (84)	

though the function of p62 in the nucleus is largely unknown, the movement of p62 indicates a method to interact with many signaling pathways with their specific domains. Therefore, the simple inhibition of autophagosome formation can be a double-edged sword in inducing oncogenic p62 function (16, 38). Strategies involving the inhibition of LC3B and p62 have been suggested as potential treatments for gastric adenocarcinomas.

Conclusion

Sequential change (elevated or translocation) of LC3B and p62 protein expression in normal, dysplasia, and gastric carcinoma suggests that the autophagic process is dynamically related to tumorigenesis. Accumulated cytoplasmic LC3B protein can reflect activated autophagic activity and lower accumulation of nuclear p62 protein can lead to higher cytoplasmic autophagy substrate with activated autophagy. Both LC3B and p62 showed sequential expression changes during gastric carcinogenesis and have also an impact on cancer progression related to patient survival. Therefore, LC3B and p62 can be prognostic biomarkers and potential therapeutic targets for gastric adenocarcinomas. Further investigation into underlying mechanism of LC3B and p62 regulation is necessary to introduce autophagy modulation as an anti-cancer therapy in gastric adenocarcinoma.

Ethics Approval

This study protocol was approved by the Institutional Review Board of Chungnam National University Hospital and complied with the tenets of the Declaration of Helsinki (CNUH 2018-03-015). The study was retrospective, and a waiver of consent was approved by the Institutional Review Board. All bio-specimens and data used for this study were provided by the Biobank of Chungnam National University Hospital, a member of the Korea Biobank Network.

Conflicts of Interest

The Authors declare that there are no known conflicts of interest associated with the work presented in this manuscript. Furthermore, the Authors confirm that the funding provided for these studies did not influence the results in anyway.

Authors' Contributions

Conceptualization, M.-K. Y.; Funding acquisition, M.-K. Y.; Investigation, G.E.B., K.-H.K., C.C., and D.L.; Methodology, J.S. K., C.C., D.L.; Project administration, M.-K. Y.; Resources, K.-H. K, S.-L.L., C.C., D.L., and T.H.L.; Supervision, M.-K. Y.; Validation, J.S.K., G.E.B, and M.-K. Y.; Statistical consult, I.S.K.; Writing–original draft, J.S. K., M.-K. Y.; Writing–review & editing, J.S. K., G.E.B., K.-H. K, C.C., D.L., and M.-K. Y.

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