

Beclin 1 expression is an independent prognostic factor for gastric carcinomas

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Abstract Beclin 1, an important autophagy-related protein in human cells, is involved in autophagy, differentiation, anti-apoptosis, and cancer progression, which is increased during periods of cell stress and extinguished during the cell cycle. In order to clarify the role of *Beclin 1* in gastric carcinogenesis and subsequent progression, its expression was examined by immunohistochemistry and in situ hybridization (ISH) on tissue microarrays containing gastric carcinomas, adjacent non-neoplastic mucosa, and metastatic lymph node. Gastric carcinoma tissue and cell lines were studied for *Beclin 1* expression by Western blot or RT-PCR, respectively. The results demonstrated that *Beclin 1* was distinctively expressed in GES-1, AGS, BGC-823, GT-3 TKB, HGC-27, KATO-III, MGC-803, MKN28, MKN45, SCH, SGC-7901, or STKM-2 at both mRNA and protein levels. However, *Beclin 1* mRNA was highly expressed in gastric carcinoma than matched mucosa by real-time PCR and ISH ($P<0.05$). Beclin 1 expression was negatively related to distant metastasis and poor prognosis of gastric carcinoma ($P<0.05$). Beclin 1 was highly expressed in male than female patients with gastric carcinoma

($P<0.05$). The 65-year-elder patients with gastric carcinoma had higher Beclin 1 expression than the younger ones ($P<0.05$). The diffuse-type carcinomas showed less Beclin 1 expression than intestinal- and mixed-type ones ($P<0.05$). In intestinal-type gastric carcinoma, Beclin 1 expression was inversely associated with venous invasion, lymph node metastasis, and tumor–node–metastasis (TNM) staging ($P<0.05$). Kaplan–Meier analysis indicated that Beclin 1 expression was positively linked to favorable prognosis of the patients with overall and intestinal-type carcinoma ($P<0.05$). Cox’s proportional hazard model indicated that venous invasion, lymph node metastasis, distant metastasis, TNM staging, and Beclin 1 expression were independent prognostic factors for gastric carcinomas ($P<0.05$). It was suggested that aberrant Beclin 1 expression is closely linked to pathogenesis, metastasis, and differentiation of gastric carcinoma. Beclin 1 expression might be employed to indicate the favorable prognosis of gastric carcinomas as an independent factor.

Keywords Gastric carcinoma · Beclin 1 · Pathogenesis · Progression · Prognosis

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Introduction

Despite a worldwide decline in incidence and mortality since the second half of the twentieth century, gastric cancer is still ranked as the fourth most common and the second most frequent cause of death from cancer, accounting for 10.4 % of cancer deaths worldwide. It continues to be a major health concern because of the slow decrease in incidence in Asia and high mortality from diagnosed gastric carcinomas in the West, even though advanced diagnostic and operative techniques are widely applied in clinical practice [13]. Tumorigenesis and progression of gastric carcinoma is a multistage process involving a multifactorial

etiology, which mainly result from gene–environmental interactions. Increased understanding on the changes that occur in gene expression during carcinogenesis, particularly identification of novel biomarkers for cancer diagnosis and novel targets for treatment, may result in the improvement of diagnosis, treatment, and prevention.

Autophagy is a protein degradation system characterized by the formation of double-membrane vacuoles, termed as autophagosomes. Beclin 1, the mammalian orthologue of the yeast Apg6/Vps30 gene, functions as a scaffold for the formation of autophagosomes and may be a haploinsufficient tumor suppressor gene. *Beclin 1* maps to a 150-kb centromeric region of breast cancer 1 on chromosome 17q21 [20]. Its complete cDNA sequence encodes a 2,098-bp transcript and then 60-kDa protein. Beclin 1 is predicted to consist of at least three domains within the evolutionarily conserved C-terminal like coiled coil and leucine zipper domain. The first ten residues of the leucine zipper also constitute a nuclear export signal essential for the cytoplasmic localization of Beclin 1 [21]. Additionally, it interacts with several cofactors (Atg14L, UV radiation resistance-associated gene, Bax-interacting factor-1 Bim Bcl-2-like protein 11, Rubicon, activating molecule in Beclin 1-regulated autophagy, high-mobility group protein B1, nPIST, vacuole membrane protein 1, signaling lymphocytic activation molecule, IP(3)R, PINK, and survivin) to regulate the lipid kinase Vps-34 protein and promote the formation of Beclin 1-Vps34-Vps15 core complexes, thereby resulting in autophagy activation, inhibition of cell proliferation, and tumorigenicity. Beclin 1 possesses a putative Bcl-2-homology-3 (BH3) amphipathic α -helix (amino acids 112–123), which can interact with BH3 receptor domain (hydrophobic groove) of the anti-apoptotic proteins (Bcl-2, Bcl-xL, and Bcl-w). The binding of endogenous Bcl-2 anti-apoptotic homologs to Beclin 1 may regulate basal autophagy levels and cell survival [11, 12]. This interaction can be disrupted by phosphorylation of Bcl-2 and Beclin 1, or ubiquitination of Beclin 1. Beclin 1-mediated macroautophagy involves regulation of caspase-9 expression in cervical cancer HeLa cells [18]. However, caspase 8- or caspase 3-mediated cleavage of Beclin 1 reduces autophagy or promotes apoptosis [34, 41].

Ectopic Beclin 1 expression restores full autophagy potential in tetraploid MCF-7 cells [10]. Mice with biallelic loss of *Beclin 1* are early embryonically lethal, and mice with monoallelic loss of *Beclin 1* have an increased incidence of spontaneous tumorigenesis like lymphomas, liver, and lung cancers, display abnormal proliferation of mammary epithelial cells and germinal center B lymphocytes, and have increased susceptibility to neurodegeneration and desmin-related cardiomyopathy [24, 26, 30, 37]. In humans, monoallelic deletions of *Beclin 1* are frequently observed in sporadic breast, ovarian, and prostate carcinoma [2]. Down-regulated Beclin 1 protein expression was confirmed in a

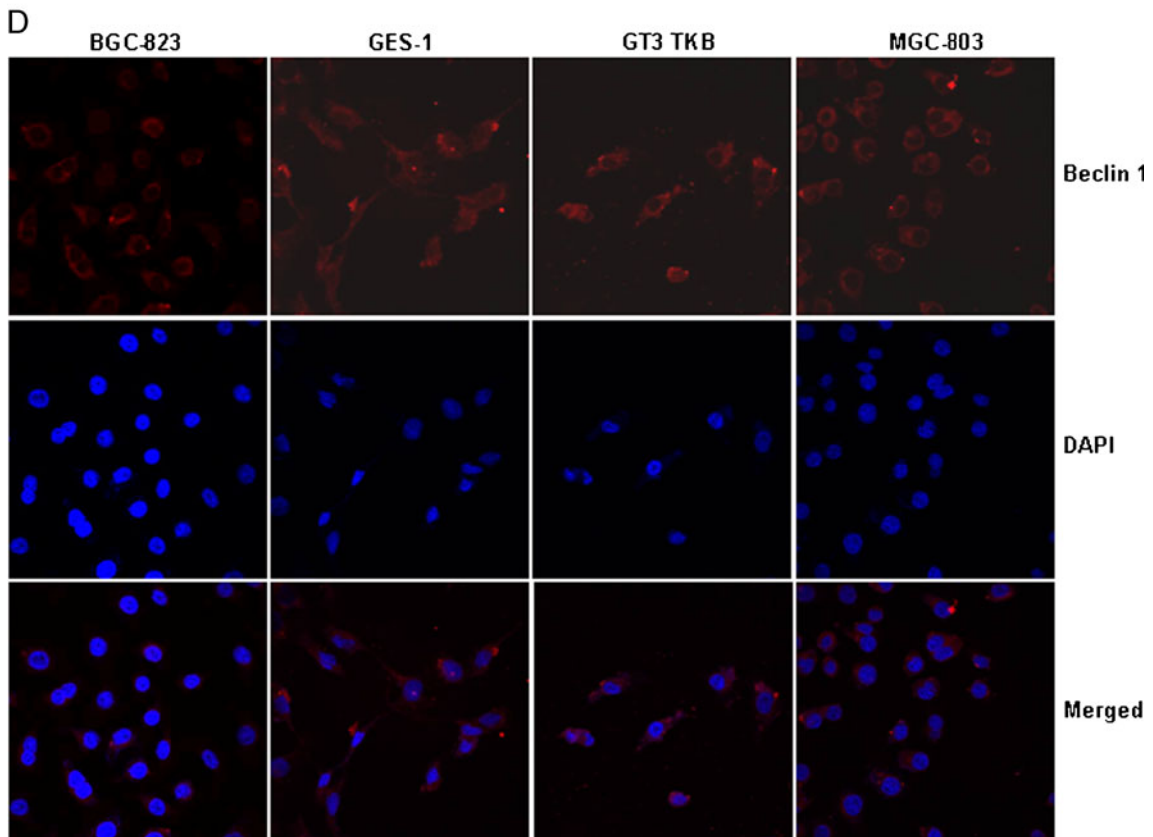
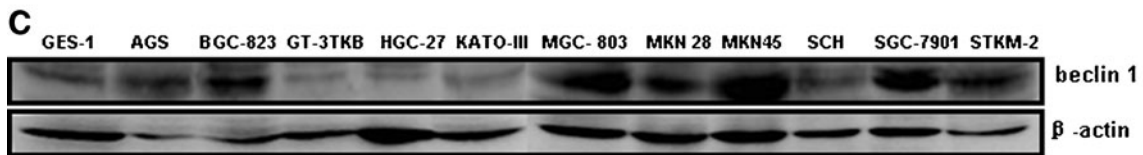
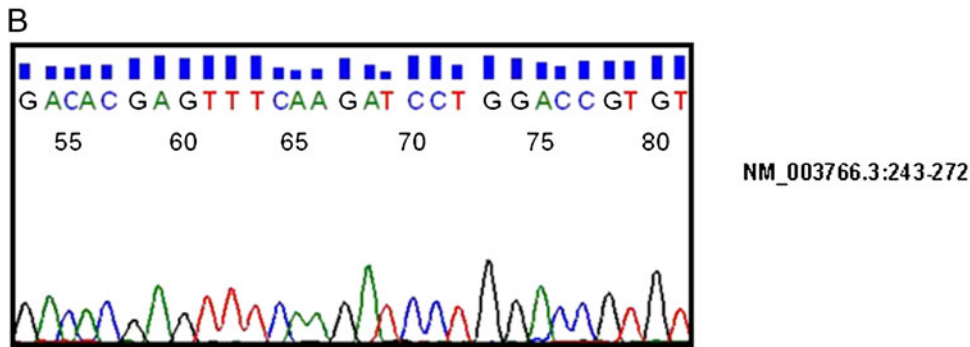
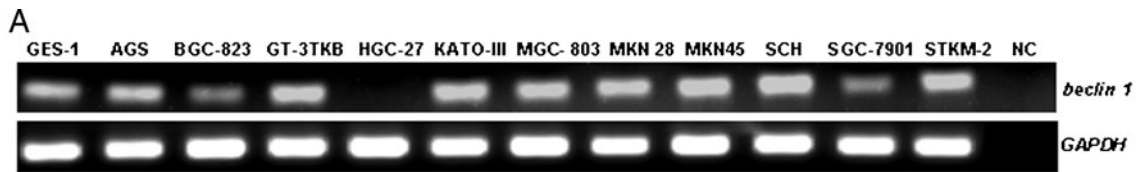
Fig. 1 *Beclin 1* expression in gastric carcinoma or epithelial cell lines. **a** Distinct expression of *Beclin 1* mRNA (160 bp) was detected and showed consistent density in all gastric carcinoma cell lines with an internal control of GAPDH (135 bp). Lane 1, GES-1; lane 2, AGS; lane 3, BGC-823; lane 4, GT-3; Lane 5, HGC-27; lane 6, KATO-III; lane 7, MGC-803; lane 8, MKN28; lane 9, MKN45; lane 10, SCH; lane 11, SGC-9701; and lane 12, STKM-2. **b** DNA sequence of Beclin 1 amplicons (NM_174953.1: 2842–2872). **c** Cell lysate was loaded and probed with anti-Beclin 1 antibody (60 kDa) with β -actin (42 kDa) as an internal control. Lane 1, GES-1; lane 2, AGS; lane 3, BGC-823; lane 4, GT-3; lane 5, HGC-27; lane 6, KATO-III; lane 7, MGC-803; lane 8, MKN28; lane 9, MKN45; lane 10, SCH; lane 11, SGC-9701; and lane 12: STKM-2. **d** Gastric carcinoma and epithelial carcinoma cells were subjected to immunofluorescence staining of Beclin 1 protein (red, Beclin 1; blue, DAPI)

small series of human tumors such as cervical [33], hepatocellular [27], and ovarian carcinoma [6]. Decreased *Beclin 1* mRNA expression was also demonstrated in glioblastoma multiforme, high-grade brain tumors [7], and lung cancer [9]. In contrast, higher Beclin 1 expression was detected in colorectal, gastric carcinomas [1], cutaneous squamous cell carcinoma [22], and pancreatic ductal adenocarcinoma [14]. It was also found that *Beclin 1* mRNA expression was markedly increased in intrahepatic cholangiocellular carcinoma [5] and hepatic cell carcinoma [29]. Thus, taken together, evidence is emerging that Beclin 1 plays an essential role in tumor suppression, development, aging prevention, innate immunity, neuroprotection, and cardioprotection. To the roles of *Beclin 1* expression in gastric carcinogenesis and subsequent progression, we examined the expression of *Beclin 1* mRNA and its encoding product in gastric carcinoma and compared Beclin 1 expression with clinicopathological parameters of carcinomas.

Material and methods

Cell culture

Gastric carcinoma cell lines, MKN28 (well-differentiated adenocarcinoma), AGS (moderately-differentiated adenocarcinoma), BGC-823, MGC-803, MNK45, and SGC-7901 (poorly-differentiated adenocarcinoma), KATO-III (signet ring cell carcinoma), HGC-27, GT-3 TKB and STKM-2 (undifferentiated adenocarcinoma), SCH (chorionic carcinoma) and gastric epithelial cell line, GES-1 come from the Japanese Physical and Chemical Institute, Tokyo, Japan and Beijing Institute for Cancer Research, Beijing, China, and Cell bank of Chinese Academy of Sciences, Shanghai, China, respectively. They were maintained in RPMI 1640 (BGC-823, MGC-803, MKN28, MKN45, KATO-III, SCH, and STKM-2), MEM (HGC-27), DMEM (GES-1, GT-3 TKB, and SGC-7901), and Ham F12 (AGS) medium supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin, in a humidified atmosphere of 5 % CO₂ at 37 °C. All



cells were harvested by centrifugation, rinsed with phosphate-buffered saline (PBS), and subjected to total protein extraction in radioimmunoprecipitation assay buffer lysis buffer

(50 mmol/L Tris-HCl (pH7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5 % Nonidet P-40, 5 mmol/L dithiothreitol, 10 mmol/L NaF, and protease inhibitor cocktail [Sigma]).

Subjects

Gastric carcinomas ($n=565$), adjacent non-neoplastic mucosa (NNM, $n=586$, ≥ 4 cm far from the margin of the carcinomas) and lymph node with metastases ($n=167$) were collected from surgical resection in The Affiliated Hospital of Kanagawa Cancer Center. The patients with gastric carcinoma were 400 men and 165 women (24–87 years old, mean=62.1 years). We did not choose the interface of carcinoma and normal mucosa because of the difficulty for the identical sampling for tissue microarray. Intestinal and diffuse components were subjected to establishment of tissue microarray in 114 cases of mixed-type carcinomas. Among them, 296 cases have tumors accompanied with lymph node metastasis and 33 with distant metastasis. Fresh gastric carcinoma and adjacent non-neoplastic mucosa were collected from the First Affiliated Hospital of China Medical University and frozen in -80 °C until RNA and protein extraction by homogenization. None of the patients underwent chemotherapy, radiotherapy, or adjuvant treatment before surgery. They all provided consent for use of tumor tissue for clinical research, and our University Ethical Committee approved the research protocol. We followed up the patients by consulting their case documents and through telephone.

Pathology

All tissues were fixed in 10 % neutral formalin, embedded in paraffin, and sections cut at 4 μ m. These sections were stained by haematoxylin and eosin (HE) to confirm their histological diagnosis and other microscopic characteristics. The tumor–node–metastasis (TNM) staging for each gastric carcinoma was evaluated according to the Union Internationale Contre le Cancer system for the extent of tumor spread [28]. Histological architecture of gastric carcinoma was expressed in terms of Lauren's classification [38, 39]. Furthermore, tumor size, depth of invasion, and lymphatic and venous invasion were determined.

Reverse transcriptase-polymerase chain reaction

Total RNA was extracted from gastric carcinoma cell lines or tissues using QIAGEN RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol. Two micrograms of total RNA was subjected to cDNA synthesis using AMV transcriptase and random primer (Takara, Otsu, Japan). Oligonucleotide primers for polymerase chain reaction (PCR) were 5'-GATGGAAGGGTCTAAGACGTCCAA-3' and 5'-TTTCGCCTGGGCTGTGGTAAG-3' for *Beclin 1* (160 bp, 162–321, NM_003766.3), and sense 5'-CAATGACCCCTTCATTGACC-3' and anti-sense 5'-TGGAA GATGGTGATGGGATT-3' for *GAPDH* (135 bp, 201–335,

Fig. 2 Beclin 1 expression in gastric non-neoplastic mucosa and carcinoma by immunohistochemistry. Beclin 1 protein is distributed to the cytoplasm. Beclin 1 was expressed in the proliferative and intestinal superficial mucosa (a, b), propria glands (c, d), infiltrating inflammatory cells (a–d), immature cells around germinal center (e), well-differentiated carcinoma (f–h), or moderately-differentiated carcinoma (i, j), mucinous carcinoma (k), poorly-differentiated carcinoma (l, m), and metastatic carcinoma in lymph node (n, o)

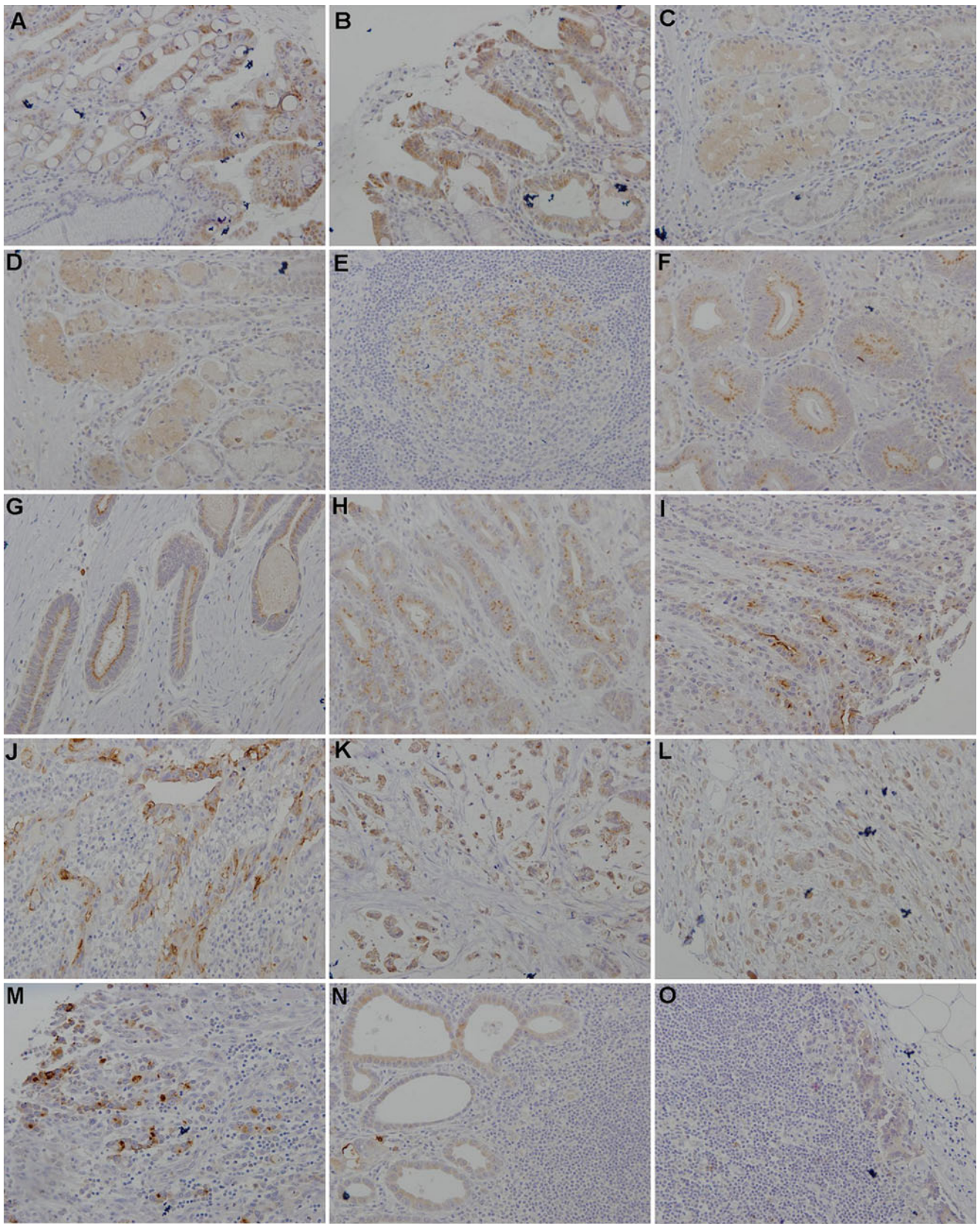
NM_002046.3). PCR amplification of cDNA was performed in 25- μ L mixtures containing 0.125 μ L Pfu (Stratagene, West Cedar Creek, USA) with 2.0 mmol/L $MgCl_2$, 2.5 μ L $\times 10$ PCR buffer, 2 μ L dNTP mixture, 1 μ mol/L of each primer set, and 100 ng of template cDNA. PCR condition was denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing for 30 s, and extension at 72 °C for 50 s. As a termination step, the extension time of the last cycle was increased to 7 min. The amplicons were electrophoresed in 2 % agarose gel for 30 min. Real-time PCR was performed according to the protocol of SYBR Premix Ex TaqTM II Kit (Takara).

Direct DNA sequencing

Amplicons were subjected to electrophoresis in 2 % agarose gel and purified with QIAquick gel extraction kit (QIAGEN). After extraction, the DNA was quantified by Nanodrop ND-1000 Spectrophotometer (Laboratory & Medical Supplies, Tokyo, Japan) and then sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster, USA) and *Beclin 1* primers as the recommendation's protocol. The sequence data were compared with the human *Beclin 1* cDNA sequence (NM_003766.3) using BLAST.

Western blot

Denatured protein was separated on an SDS-polyacrylamide gel (10 % acrylamide) and transferred to Hybond membrane (Amersham, Amersham, Germany), which was then blocked overnight in 5 % skim milk in Tris-buffered saline and Tween 20 (TBST) (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1 % Tween 20). For immunoblotting, the membrane was incubated for 15 min with the mouse antibody against Beclin 1 (1:250, Sigma). Then, it was rinsed by TBST and incubated with anti-rabbit or anti-mouse IgG-conjugated to horseradish peroxidase (1:1,000; DAKO, Carpinteria, CA 93013, USA) for 15 min. All the incubations were performed in a microwave oven to allow intermittent irradiation as recommended by Li et al. [19]. Bands were visualized with X film (Fuji, Japan) by ECL-Plus detection reagents (Santa Cruz, USA). After that, membrane was washed with WB Stripping Solution (pH2–3; Nacalai, Tokyo, Japan) for 1 h and treated as described above except anti- β -actin antibody (1:1,000, sc-47778; Santa Cruz) as internal control.



Tissue microarray (TMA)

Representative areas of solid tumors were identified in HE-stained sections of the selected tumor cases, and a tissue core of 2 mm in diameter per donor block was punched out and transferred to a recipient block with a maximum of 48 cores using a Tissue Microarrayer (Azumaya KIN-1, Japan). Four-micrometer-thick sections were consecutively incised from the recipient block and transferred to polylysine-coated glass slides. HE staining was performed on TMA for confirmation of tumor tissue.

Immunofluorescence

Cells were grown on glass coverslips, were washed twice with PBS, fixed with 4 % formaldehyde for 10 min at room temperature, and permeabilized with 0.2 % Triton X-100 for 10 min at room temperature. After having been washed with PBS, cells were incubated overnight at 4 °C with the mouse antibody against Beclin 1 (1:50, Sigma). They were then washed with TBST and incubated with anti-mouse Alexa Fluor 568 IgG (1:2,000, Invitrogen). Nuclei were stained with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI, Sigma) for 30 min at 37 °C. Finally, coverslips were mounted with SlowFade® Gold anti-fade reagent (Invitrogen) and observed under a laser confocal scanning microscope (Leica, Germany).

Immunohistochemistry (IHC)

Consecutive sections were deparaffinized with xylene, rehydrated with alcohol, and subjected to antigen retrieval by irradiating in target retrieval solution (DAKO, USA) for 15 min in a microwave oven (Oriental Rotor Lmt. Co., Tokyo, Japan). The sections were quenched with 3 % hydrogen peroxide in absolute methanol for 20 min to block endogenous peroxidase activity. Five percent bovine serum albumin was then applied for 5 min to prevent non-specific binding. The sections were incubated with anti-Beclin 1 antibody (1:50, Sigma) for 15 min and then treated with the anti-rabbit conjugated to horseradish peroxidase (DAKO, USA) antibodies for 15 min. All the incubations were performed in a microwave oven to allow intermittent irradiation as described previously [17]. After each treatment, the slides were washed with TBST three times for 1 min. Binding sites were visualized with 3, 3'-diaminobenzidine. After having been counterstained with Mayer's hematoxylin, the sections were dehydrated, cleared, and mounted. Omission of the primary antibody was used as a negative control.

As indicated in Fig. 3, Beclin 1 protein was positively localized in the cytoplasm. One hundred cells were randomly selected and counted from five representative fields of each section blindly by two independent

observers (Yu M and Zheng HC). The inconsistent data were confirmed by both persons until final agreements were reached. The positive percentage of counted cells was graded semi-quantitatively according to a four-tier scoring system [40]: negative (–), 0–5 %; weakly positive (+), 6–25 %; moderately positive (++), 26–50 %; and strongly positive (+++).

In situ hybridization

To perform RNA-DNA in situ hybridization (ISH) for *Beclin 1*, a digoxigenin-labeled *Beclin 1* probe was made in 35-cycle PCR using the above-mentioned primers and 15-ng template DNA of 30-cycle products from the SGC7901 cDNA by using Pfu polymerase (Stratagene, USA). Four-micrometer-thick sections were deparaffinized and digested with 20 µg/mL proteinase K in 50 mmol/L Tris–HCl at 37 °C for 10 min. Then 20 µL of a 1:20 probe dilution in hybridization buffer (22 mmol/L Tris–HCl, pH 7.4, 2.75 mmol/L ethylenediaminetetraacetic acid, 660 mmol/L NaCl, 1x Denhardt solution, 5.5 % dextran sulfate, 0.33 % dimethyl sulfoxide, 0.55 % ethoquad 18/25, and 44 % deionized formamide) was added to each slide. After coverslipping, heating at 95 °C for 5 min, and incubation overnight in a humidified chamber at 37 °C, sections were rinsed for 10 min in TBST and incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche Diagnostics, Germany) for 20 min at 37 °C. The slides were then washed for 5 min and immersed in solution II (100 mmol/L Tris–HCl pH 9.5, 100 mmol/L NaCl, and 50 mmol/L MgCl₂) for 30 min and followed by exposure to nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolylphosphatase *p*-toluidine salt as a chromogen. Finally, counterstaining was performed using methyl green for 2 min.

As indicated in Fig. 3, the positive signal of *Beclin 1* mRNA was localized in the cytoplasm. One hundred cells were randomly selected and counted blindly from five representative fields of each section by two independent observers (Yu M and Zheng HC). The inconsistent data were confirmed by both persons until final agreements were reached. The scoring system was referred to the section of immunohistochemistry.

Statistical analysis

Statistical evaluation was performed using Spearman correlation test to analyze the rank data and Wilcoxon test to differentiate the means of different groups. Kaplan–Meier survival plots were generated and comparisons were made with the log-rank statistic. Cox's proportional hazards model was employed for multivariate analysis. $P < 0.05$ was considered as statistically significant. SPSS 10.0 software was employed to analyze all data.

Table 1 Beclin 1 protein expression in gastric non-neoplastic mucosa, primary carcinoma, and lymph node metastases

Groups	n	Beclin 1 protein expression				
		–	+	++	+++	PR (%)
NNMs	586	234	171	143	38	60.1
Primary carcinoma	565	245	142	110	68	56.6
Metastatic carcinoma in lymph node	167	79	44	32	12	52.7

PR positive rate, NNM non-neoplastic mucosa

Results

Beclin 1 expression in gastric carcinoma cell lines or tissue samples

To check *Beclin 1* mRNA expression, we designed the primer of reverse transcriptase (RT)-PCR and found that *Beclin 1* was strongly detectable in GES-1, AGS, BGC-823, GT-3 TKB, KATO-III, MKN28, MKN45, SCH, and STKM-2, weakly in MGC-803 and SGC-7901, but not in HGC-27 (Fig. 1a). Additionally, the amplicons were subjected to direct DNA sequencing with the confirmation of *Beclin 1* (Fig. 1b). Beclin 1 protein was expressed in gastric carcinoma or epithelial cells at comparatively weak level (Fig. 1c). The immunofluorescence staining showed that Beclin 1 was distributed in the cytoplasm of BGC-823, GES-1, GT-3 TKB, and MGC-803 (Fig. 1d).

As indicated in Fig. 2, Beclin 1 was expressed in the proliferative superficial and intestinal mucosa, propria

glands, infiltrating inflammatory cells, immature cells in the middle of germinal center, and primary and metastatic carcinoma by IHC. Statistically, Beclin 1 expression was detectable in gastric NNM (60.1 %, 352/586), primary carcinoma (56.6 %, 320/565), and metastatic carcinoma in lymph node (52.7 %, 88/167), respectively. Statistically, there was no difference in Beclin 1 expression between gastric NNM and primary and metastatic carcinomas ($P>0.05$, Table 1).

To confirm *Beclin 1* mRNA expression, ISH was also employed on the tissue microarray of the gastric carcinoma and adjacent mucosa (Fig. 3). *Beclin 1* mRNA was detected in gastric NNM (64.6 %, 31/48), primary carcinoma (81.6 %, 31/38), and metastatic carcinoma in lymph node (93.3 %, 28/30). Statistically, its expression was lower in NNM than carcinoma ($P<0.05$, Table 2). There was no significant difference in *Beclin 1* mRNA expression between primary carcinoma and metastatic carcinoma in lymph node ($P>0.05$, Table 2).

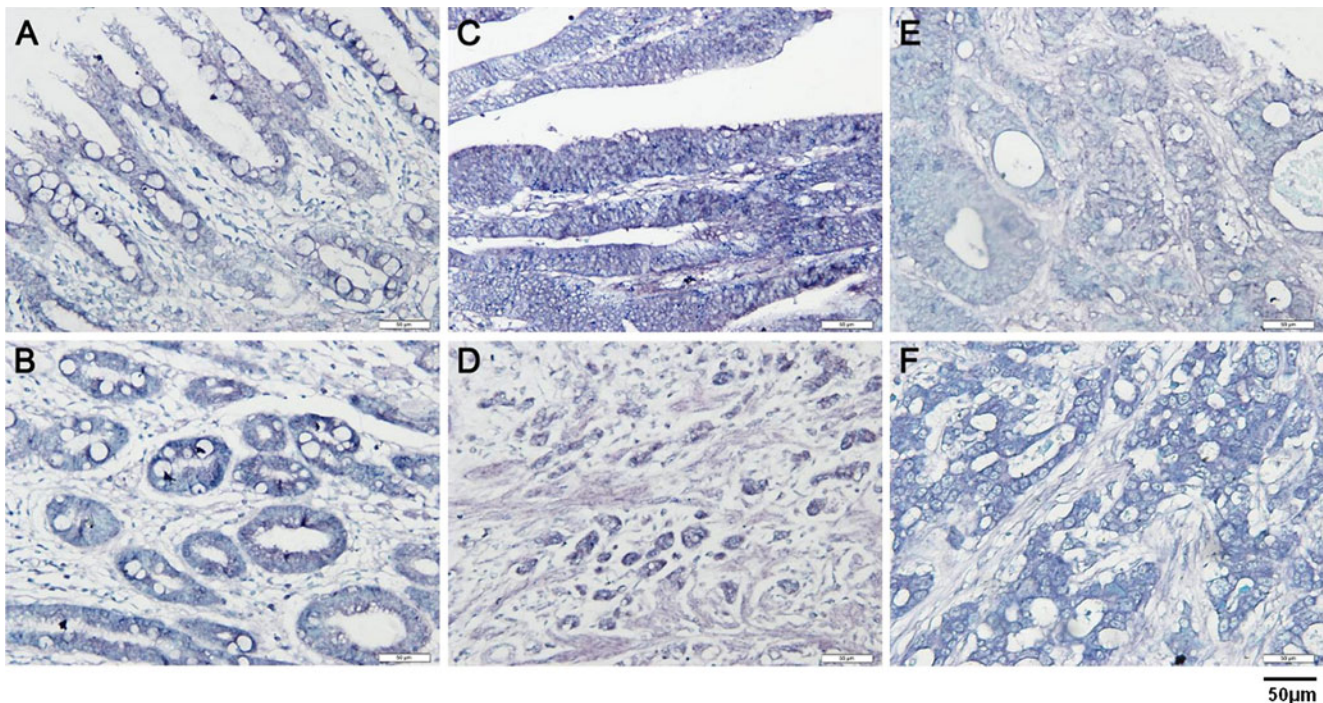


Fig. 3 *Beclin 1* mRNA expression in gastric carcinogenesis by in situ hybridization. *Beclin 1* mRNA was observed in the gastric non-neoplastic mucosa (a, b), primary carcinomas (c, d), and metastatic carcinoma in lymph node (e, f)

Table 2 *Beclin 1* mRNA expression in gastric non-neoplastic mucosa, primary carcinoma, and lymph node metastases

Groups	<i>n</i>	<i>Beclin 1</i> mRNA expression				
		–	+	++	+++	PR (%)
NNMs	48	17	9	19	3	64.6
Primary carcinoma	38	7	5	9	17	81.6*
Metastatic carcinoma in lymph node	30	2	2	8	18	93.3

PR positive rate, NNM non-neoplastic mucosa

* $P < 0.05$, compared with NNM

Among 19 gastric carcinomas, 14 cases showed higher *Beclin 1* expression in carcinoma than matched non-neoplastic mucosa (NNM) by real-time PCR. Its mRNA expression was significantly greater in carcinoma than in

adjacent NNM ($P < 0.05$, Fig. 4a). Among 30 cases of frozen gastric samples, *Beclin 1* was detected in all the samples, including gastric carcinoma and the adjacent mucosa with no difference in bands' intensity ($P > 0.05$, Fig. 4b).

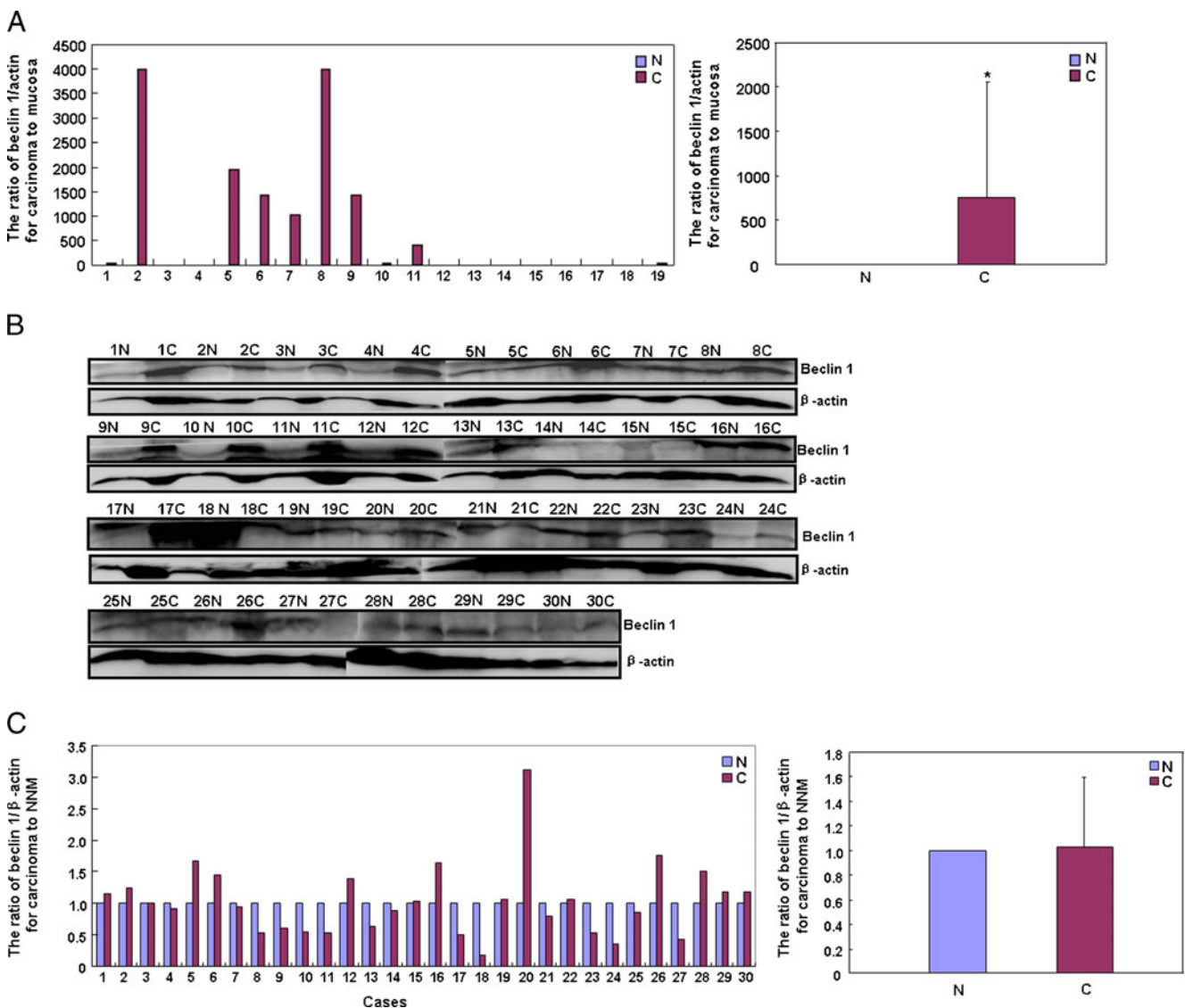


Fig. 4 *Beclin 1* expression in gastric carcinoma and matched non-neoplastic mucosa. **a** *Beclin 1* mRNA was quantified in gastric carcinoma and non-neoplastic mucosa by real-time PCR. *Beclin 1* mRNA expression level was significantly higher in gastric carcinoma than paired mucosa by real-time RT-PCR (asterisk indicates $P < 0.05$). **b**

Tissue lysate was loaded and probed with anti-*Beclin 1* antibody (60 kDa) with β -actin (42 kDa) as an internal control. **c** The densitometric analysis showed no difference in *Beclin 1* expression density between carcinoma and matched mucosa ($P > 0.05$). N, non-neoplastic mucosa; C, carcinoma

The relationship between Beclin 1 protein expression and clinicopathological and prognostic parameters of gastric carcinoma

As summarized in Table 3, Beclin 1 expression was negatively related to distant metastasis ($P < 0.05$), but not correlated with age, sex, depth of invasion, lymphatic or venous invasion, lymph node metastasis, or TNM staging ($P > 0.05$). Beclin 1 was more expressed in male than female patients with gastric carcinoma ($P < 0.05$). The 65-year-elder patients with gastric carcinoma had higher Beclin 1 expression than the younger ones ($P < 0.05$). The diffuse-type carcinomas showed less Beclin 1 expression than intestinal- and mixed-type ones ($P < 0.05$). In intestinal-type gastric carcinoma, Beclin 1 expression was inversely associated with venous invasion, lymph node metastasis, and TNM staging ($P < 0.05$, data not shown).

Follow-up information was available on 524 gastric carcinoma patients for periods ranging from 2 months to 10.8 years (median=68.5 months). Survival curves for gastric carcinomas were stratified according to Beclin 1 protein expression (Fig. 5). Univariate analysis using Kaplan–Meier method indicated that the cumulative survival rate of patients with weak, moderate, or strong expression of Beclin 1 was obviously higher than those without its expression in overall (Fig. 5a) and intestinal-type (Fig. 5b) carcinomas ($P < 0.05$). Multivariate analysis using Cox’s proportional hazard model indicated that venous invasion, lymph node metastasis, distant metastasis, TNM staging, and Beclin 1 expression ($P < 0.05$), but not age, sex, depth of invasion, lymphatic invasion, or Lauren’s classification were independent prognostic factors for overall gastric carcinomas ($P > 0.05$, Table 4). Beclin 1 expression was also an independent factor for intestinal-type gastric carcinomas ($P < 0.05$, data not shown).

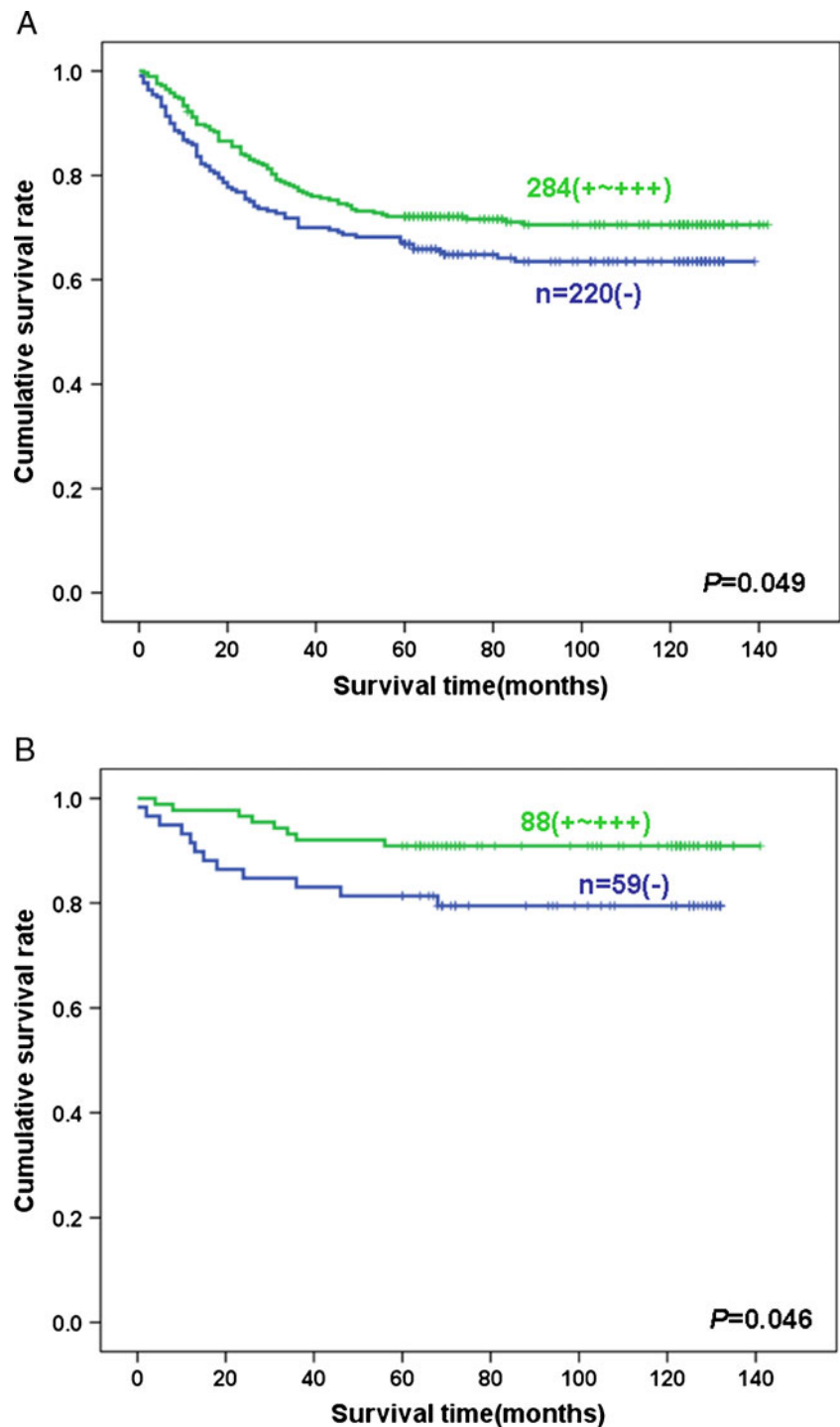
Table 3 Relationship between Beclin 1 protein expression and clinicopathological features of gastric carcinomas

Clinicopathological features	n	Beclin 1 protein expression				PR (%)	P value
		–	+	++	+++		
Age (year)							0.020
<65	321	153	78	53	37	52.3	
≥65	244	92	64	57	31	62.3	
Sex							0.047
Male	400	166	98	81	55	58.5	
Female	165	79	44	29	13	52.1	
Depth of invasion							0.271
T _{is-1}	268	112	66	51	39	58.2	
T ₂₋₄	291	129	76	58	28	55.7	
Lymphatic invasion							0.900
–	311	136	77	63	35	56.3	
+	244	105	64	44	31	57.0	
Venous invasion							0.360
–	305	128	76	64	37	58.0	
+	251	113	66	43	29	55.0	
Lymph node metastasis							0.877
–	313	138	74	59	42	55.9	
+	296	105	66	49	76	64.5	
Distant metastasis							0.013
–	532	225	134	105	68	57.7	
+	33	20	8	5	0	39.4	
TNM staging							0.161
I–II	324	134	82	62	46	58.6	
III–IV	230	105	59	45	21	54.3	
Lauren’s classification							–
Intestinal-type	176	70	49	35	22	60.2	
Diffuse-type	201	110	41	33	17	45.3 ^a	
Mixed-type	185	63	52	42	28	65.9	

PR positive rate, T_{is} carcinoma in situ, T₁ lamina propria and submucosa, T₂ muscularis propria and subserosa, T₃ exposure to serosa, T₄ invasion into serosa, TNM tumor–node–metastasis

^aCompared with intestinal- and mixed-type carcinoma

Fig. 5 Correlation between Beclin 1 status and prognosis of the gastric carcinoma patients. Kaplan–Meier curves for cumulative survival rate of patients with overall (a) and intestinal-type (b) gastric carcinomas according to Beclin 1 protein expression status



Discussion

Autophagic cell death is differentiated from apoptosis by the presence of double or multiple-membrane enclosed vesicles and involved in bulk degradation through an autophagosome–lysosomal pathway. Beclin 1 is an autophagy-essential protein, was initially isolated as a Bcl-2-interacting protein 1, and essential for the formation of these autophagic vesicles and in normal growth control. Functionally, Beclin

1 might delay cell cycle progression and induce autophagy as a tumor suppressor in mammalian systems [3]. Here, we examined in situ Beclin 1 expression in gastric mucosa, carcinoma samples, carcinoma, and epithelial cell lines. It was found that Beclin 1 protein was mainly localized in the cytoplasm of all cell lines, the proliferative and intestinal superficial mucosa, propria glands, infiltrating inflammatory cells, immature cells in the middle of germinal center, and primary and metastatic carcinoma. It was suggested that

Table 4 Multivariate analysis of clinicopathological variables for the survival of the patients with gastric carcinomas

Clinicopathological parameters	Relative risk (95 % CI)	P value
Age (<65 years/≥65 years)	1.351 (0.979–1.863)	0.067
Sex (male/female)	0.713 (0.493–1.031)	0.072
Depth of invasion (T _{is-1} /T ₂₋₄)	2.149 (0.705–6.550)	0.179
Lymphatic invasion (-/+)	1.368 (0.871–2.148)	0.174
Venous invasion (-/+)	1.606 (1.002–2.575)	0.049
Lymph node metastasis (-/+)	2.206 (1.185–4.108)	0.013
Distant metastasis (-/+)	3.673 (2.325–5.802)	<0.001
TNM staging (I–II/III–IV)	5.317 (2.429–11.634)	<0.001
Lauren's classification (IT/DT/MT)	1.172 (0.933–1.472)	0.172
Beclin 1 protein expression(-/+~+++)	0.692 (0.501–0.954)	0.025

CI confidence interval, TNM tumor–node–metastasis, IT intestinal-type, DT diffuse-type, MT mixed-type

Beclin 1 expression pattern has cellular specificity, which determines its biological functions. However, the mechanisms of its cell-specific characteristics should be further investigated.

To clarify the clinicopathological significance of Beclin 1 protein, IHC was performed on TMA of gastric lesions. Different from the other findings [1, 6, 7, 9, 14, 22, 27, 33], there was no difference in Beclin 1 expression between gastric carcinoma and adjacent NNM in line with the result of Western blot, although Beclin 1 expression in stromal cells from gastric samples could be excluded from the immunohistochemical data by virtue of their specific histomorphological features and topographic location in tissue sections. These data indicated that Beclin 1 expression might be not linked to the malignant transformation of gastric epithelial cells. To check transcriptional regulation of *Beclin 1*, RT-PCR and ISH were employed, and they demonstrated *Beclin 1* mRNA overexpression in gastric carcinoma, compared with paired NNM, consistent with the other reports [5, 29]. The paradoxical phenomena could be explained by the different sensitivities of both approaches and the differences in mRNA stability, translation, and protein degradation of Beclin 1 between gastric carcinoma and NNM.

In the present study, Beclin 1 expression was negatively linked to distant metastasis, indicating that Beclin 1 might be involved in the metastasis of gastric cancer. Dong et al. [5] found that low Beclin 1 expression was significantly associated with lymph node metastasis of intrahepatic cholangiocellular carcinoma. It was documented that Beclin 1 expression was inversely correlated with the tumor size and the primary tumor stage in squamous cell carcinoma and adenocarcinoma of the lung [36]. The decreased expression of Beclin 1 was found to associate with pelvic lymph node

metastasis and histological grade of cervical [35] and ovarian [6] cancer. There were significant associations between increased Beclin 1 expression and the absence of lymphatic invasion and low rate of distant metastasis of pancreatic ductal adenocarcinoma [14]. Chen et al. [4] found that Beclin 1 expression was significantly correlated with depth of invasion, lymph node metastasis, and clinical stage of esophageal squamous cell carcinoma. Pirtoli et al. [25] found that high cytoplasmic expression of Beclin 1 protein score was positively correlated with apoptosis and negatively with cell proliferation in high-grade glioma. Reportedly, autophagic trigger upregulates Beclin 1, which in turn binds to PI3KC3 or Bcl-X(L) to form complexes, which is necessary for the autophagy and differentiation [32]. Additionally, Beclin 1 overexpression and phosphorylation of Bcl-2 and p53 are involved in the JNK-mediated autophagic cell death [23]. Transfection of the low beclin 1 gene-expressing colon cancer cell line with *Beclin 1* gene resulted in cell growth inhibition and G1 arrest [15]. Taken together, reduced expression of Beclin 1 in gastric carcinoma with distant metastasis might cause the imbalance between cellular proliferation and cell death.

Although all gastric cancers are malignant tumors that originate from the same gastric epithelium, the morphological features of the cancers vary substantially in individual patients. Here, a higher Beclin 1 expression was found in intestinal- than diffuse-type gastric carcinoma. According to Lauren's classification, intestinal-type carcinomas are characterized by cohesive carcinoma cells that form gland-like tubular structures with expanding or infiltrative growth patterns. Cell cohesion is less apparent or absent in diffuse-type carcinoma, and the cancer cells diffusely spread in gastric wall lesions [38, 39]. Therefore, we hypothesized that differential Beclin 1 expression possibly underlay the molecular mechanisms of both carcinomas, might be a good differentiation indicator of gastric carcinoma, and could be employed to separate the intestinal- and diffuse-type carcinomas. In intestinal-type gastric carcinoma, Beclin 1 expression was inversely associated with venous invasion, lymph node metastasis, and TNM staging, suggesting that Beclin 1 expression might play an important role in invasion and metastasis of intestinal-type ones. Moreover, Beclin 1 was significantly more expressed in older men patients than younger women ones, possibly due to its stronger expression in intestinal-type carcinoma commonly seen in the former population [38, 39].

The prognostic relevance of Beclin 1 expression was discussed in various malignancies, but the results were controversial. Here, a positive link between Beclin 1 expression level and favorable survival was revealed in overall and intestinal-type gastric carcinomas as described as pancreatic ductal adenocarcinoma [14], intrahepatic cholangiocellular carcinoma [5], esophageal squamous cell carcinoma [4], stage IIIB colon carcinoma [16], and nasal-type extranodal

natural killer T cell lymphoma [8]. Multivariate analysis demonstrated that venous invasion, lymph node metastasis, distant metastasis, TNM staging, and Beclin 1 expression were independent factors for overall gastric carcinoma, and the prognostic significance of Beclin 1 was also independent of other clinicopathological features in intestinal-type carcinomas. These findings suggested that higher Beclin 1 was an indicator for the favorable prognosis of overall and intestinal-type gastric carcinoma patients, independent of other parameters. In contrast, Wan et al. [31] found that the patients with lower Beclin 1 expression displayed a significant overall survival advantage than those with higher expression in nasopharyngeal carcinoma.

In summary, our study indicated that aberrant *Beclin 1* mRNA expression might impact the malignant transformation of gastric epithelial cells. Lower Beclin 1 expression is closely linked to metastasis and dedifferentiation. Beclin 1 expression might be employed to indicate the favorable prognosis of gastric carcinomas as an independent factor. Nevertheless, the biological functions of Beclin 1 in gastric carcinomas need further investigation.

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Conflicts of interest None

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