ORIGINAL RESEARCH

Prognostic Value of Autophagy-related Proteins in Human Gastric Cancer

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Purpose: Autophagy-related proteins (ATG) play a crucial role in autophagy. Recently, the functions of autophagy in cancer have been gathering attention. However, the prognostic value of ATGs in gastric cancer (GC) has not been explored.

Methods: The Kaplan–Meier plotter (KM plotter) online database was used to examine the value of ATGs gene expression levels in overall survival (OS) prediction in GC patients with different clinical stage, differentiation, gender, HER2 status, and therapeutic strategy. In vitro experiments applied VE-822, an effective GC treatment, to assess cell migration and proliferation in gastric mucosa epithelial cells, and real-time PCR was used to measure alterations of autophagy-related gene expression.

Results: High *ATG3*, *ATG4C*, *ATG5*, and *ATG10* mRNA levels were associated with good OS, while increased *ATG4B*, *ATG7*, *ATG12*, *ATG16L1*, and *TECPR1* mRNA levels related to unfavorable OS in patients with GC. *ATG12* overexpression had different effects on OS due to high levels of heterogeneity. High *ATG12* expression was correlated with good OS in female patients with GC and with bad OS for male patients. Additionally, the increased *ATG12* expression was more likely to get a satisfactory OS in patients who underwent surgery alone but was associated with poor OS for patients treated with 5-FU adjuvant. In addition, elevated *TECPR1* expression was related to favorable OS for patients with poorly differentiated type, while for patients with moderate differentiation, it was relevant to poor OS. The in vitro experiments showed that berzosertib could significantly inhibit the migration and proliferation of human gastric mucosa epithelial cells, and further real-time PCR assessment of ATG expressions partially coincided with the bioinformation analysis above.

Conclusion: These results indicate that individual ATGs have unique prognostic significance interpreted using Kaplan–Meier plotter analysis and in vitro experiments, and this may help guide clinical therapeutic strategy and promote OS by individualizing therapy for GC patients.

Keywords: autophagy-related proteins, prognostic values, gastric cancer, overall survival

Introduction

According to statistics from World Health Organization, gastric cancer (GC) is the fifth most common cancer and greatly impacts human health.[1] Although the mortality rate for patients with GC has declined over the past three decades, it remains high and GC remains the third highest cause of cancer-related death globally.[2] In China approximately 679,000 new cases of GC were diagnosed in 2015, and about 490,000 patients with GC died.[3,4] Therefore, it is necessary to identify the biomolecules to improve its diagnosis and overall survival (OS).

Autophagy is a metabolic and cell protective process that sustains nutrient supply when following starvation or harm. Autophagy achieves this by digesting impaired proteins and organelles, or some pathogens, to recycle nutrients to

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generate ATP or other proteins and organelles.[5] It is important for organismal maturation, saves resources, and regulates pathophysiology.[6,7] However, this protective characteristic seems suspicious in gastric cancer. One recent study revealed that miR-5100 was able to inhibit tumorigenesis through propelling apoptosis and suppressing autophagy by targeting *CAAP1* the apoptosis inhibitor and autophagy promotor, and when applying the inhibitor of miR-5100, autophagy was then upregulated, and the tumor grew faster.[8] Therefore, in fact, autophagy plays dual roles in cancer based on the cancer type, stage, and genetic background of the patient. Initially, autophagy acts as a tumor suppressor by reducing oncogenic protein p62, and assists in eliminating impaired organelles or DNA to stop further cellular injury and cancer development.[9,10] Later, autophagy can protect tumors from injuries caused by nutrient shortage, radiation, and chemotherapeutics, and strengthen tumor metabolism and growth, leading to drug resistance.[10,11]

Autophagy-related proteins (ATG) are crucial components in the autophagy system. In total there are 38 kinds of ATGs[12,13] that participate in almost every step of autophagy, including initiation, phagophore expansion, autophagosome assembly and formation, fusion with lysosomes, and digestion.[14] Therefore, aberrant autophagic gene expression might result in the digestion of important endogenic cytokines or regulatory proteins that function as tumor promotors. One of previous study reported that autophagy may be involved in the progression of AQP3-mediated cisplatin chemotherapeutic resistance in GC cells, while, the autophagy inhibitor, chloroquine, can intensify the effects of, and sensitivity to, cisplatin in GC cells.[15,16] High Beclin1 expression is an independent predictor of poor prognosis and poor OS in patients with GC.[17] Furthermore, LC3, which is related to autophagosome biosynthesis, is expressed at higher levels in the center of GC tumors than it is at the edges and can be considered an advanced indicator in the early stage of GC.[18] Biomarkers play significant roles in cancer diagnosis, determination of therapeutic strategy, and prognosis. Accumulating data in animal models or from clinical studies indicate expression of ATGs, including *ATG2B*, *ATG5*, *ATG9B*, *ATG12*, and *ATG16L1* are closely related to GC and may influence autophagy in the development and progression of GC.[19–22] However, systematic clinical evidence and analysis is required for the application of treatments with improved efficacy to achieve better survival outcomes.

In this study, the autophagic gene expression was examined to determine its prognostic significance for GC patients. Specifically, *ATG3*, *ATG4B*, *ATG4C*, *ATG5*, *ATG7*, *ATG10*, *ATG12*, *ATG16L1*, and *TECPR1* expressions were analyzed using a database Kaplan–Meier plotter (KM plotter) and the corresponding in vitro experiment was conducted as a verification for the theory obtained from the bioinformatic analysis. This research is hopefully to provide a new clinical treatment direction and significantly contribute to reducing mortality in patients with GC.

Materials and Methods
Prognostic Value Analysis of ATGs
The recently developed Kaplan–Meier plotter (http://kmplot.com/analysis) was used to assess the association between expression of ATG genes and overall survival (OS) in patients with GC. The candidate genes examined in this study were *ATG3*, *ATG4B*, *ATG4C*, *ATG7*, *ATG10, ATG12, ATG16L1*, and *TECPR1*. Clinical characteristics, including tumor pathological stage, *HER2* expression status, treatment strategy, gender, and differentiation were included and considered crucial factors for OS in combination with the expression of different ATGs. Gene expression levels in the Kaplan–Meier plot surpassing or below the median were classified as high and low expression, respectively, and the test value was ranked as risk by referring under main plot. Hazard ratios, 95% confidence intervals, and log rank *P* were calculated. $P<0.05$ was considered statistically significant.

Cell Culture and Regents
Human gastric mucosa epithelial cell lines (GES 1) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM (GENOM, Hangzhou, China), containing 10% FBS (Thermo Fisher Scientific, Waltham, MA, USA), and 1% penicillin-streptomycin (Gibco/Thermo Fisher Scientific) at 37°C in a humidified atmosphere with 5% CO_2. The ATR inhibitor, VE-822, was purchased from Selleck Chemicals (Houston, TX, USA).

Colony Formation Assay
Cells were seeded into six-well plates at 500–1000 cells/well. When cells formed colonies visible to the naked eye, VE-822 was added at the indicated doses for two days.

The results were visualized by crystal violet staining and counted for statistical analysis.

Migration Assay

Cells were equally distributed into six-well plates and crystal pipette tips were applied to scratched in the middle of the well to make a blank linear gap when cells grew to a proper density. The cells were washed with PBS to remove nonadherent cells. VE-822 was added and incubated with the cells for 24 h. During this period, cells could grow into blank area and filled the gap. The width of the liner gap in each well was observed under a microscope and images were captured.

Real-time Polymerase Chain Reaction (Real-time PCR)

Real-time PCR was performed to measure ATG gene expression levels. Gene specific primers were designed and are listed in in Table 1. Gene expression in cells treated with 0, 4, and 6 μM VE-822 were assessed. RNA was isolated from cells using a total RNA Kit (Tiangen),

Table 1 Primers Used in RT-qPCR

Gene	Primer	Sequence
GAPDH	Forward Reverse	TGACTTCAACAGCGACACCCA CACCCTGTTGCTGTAGCCAAA
ATG3	Forward Reverse	GACCCCGGTCCTCAAGGAA TGTAGCCCATTGCCATGTTGG
ATG4B	Forward Reverse	ATGGACGCAGCTACTCTGAC TTTTCTACCCAGTATCCAAACGG
ATG4C	Forward Reverse	ACCCCAACAATTTCTCTGAAGG GTCCATACCAATCTCCTGCTTTT
ATG5	Forward Reverse	AGAAGCTGTTTCGTCCTGTGG AGGTGTTTCCAACATTGGCTC
ATG7	Forward Reverse	CAGTTTGCCCCTTTTAGTAGTGC CCAGCCGATACTCGTTCAGC
ATG10	Forward Reverse	AGACCATCAAAGGACTGTTCTGA GGGTAGATGCTCCTAGATGTGAC
ATG12	Forward Reverse	CTGCTGGCGACACCAAGAAA CGTGTTCGCTCTACTGCCC
ATG16L1	Forward Reverse	CTTAACCCTGCGGTCCAT CTCTTCCTTCCCAGTCCC
TECPR1	Forward Reverse	GGGCTCGCTCACGAAGATG CCTGAATGAGGTGGTGGCG

Abbreviation: ATG, autophagy-related gene. A

following the manufacturer's instructions. The High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.) was used to generate cDNA. An ABI PRISM 7500FAST PCR Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was also used.

Statistical Analysis

All the assays were repeated more than three times and the corresponding data were analyzed using GraphPad Prism 8.2.0 (GraphPad Software lnc., San Diego, USA). One-way ANOVA test was used to compare means and $P<0.05$ was considered significant.

Results
GC Prognostic Values of Autophagy-related Proteins

ATGs were all successfully retrieve from http://www.kmplot.com/. *ATG3* was detected with the Affymetrix ID: 221492_s_at. OS curves were plotted for all patients with gastric cancer (n=876) (Figure 1A), intestinal cancer (n=320) (Figure 1B), diffuse cancer (n=241) (Figure 1C), and mixed cancer (n=32) (Figure 1D). Increased *ATG3* mRNA expression was related to satisfactory OS in all patients with GC (HR=0.51, 95%CI: 0.41−0.63, P=2.2e−10), including patients with intestinal cancer (HR=0.43, 95%CI: 0.31−0.59, P=1.5e−07), diffuse cancer (HR=0.45, 95%CI: 0.31−0.65, P=1.8e−05), and mixed cancer (HR=0.32, 95%CI: 0.11−0.92, P=0.026).

The prognostic significance of *ATG4B* mRNA expression was evaluated in the database (Affymetrix ID is 204902_s_at). OS curves revealed a correlation between *ATG4B* mRNA expression level and a poor OS for all patients with gastric cancer (HR=1.8, 95%CI 1.52−2.13, P=8.2e−12; Figure 2A), including those with intestinal cancer (HR=2.16, 95%CI: 1.57−2.99, P=1.6e−06; Figure 2B), diffuse cancer patients (HR=1.49, 95%CI: 1.06−2.09, P=0.022; Figure 2C), and in mixed cancer patients (HR=3.04, 95%CI: 0.97−9.54, P=0.046; Figure 2D).

High expression of *ATG4C* (Affymetrix ID is 228190_at) mRNA was related to good OS in all patients with GC (HR=0.63, 95%CI: 0.5−0.8, P=0.00014; Figure 3A). Histological subtype results showed that increased *ATG4C* mRNA levels were related to good OS in patients with intestinal cancer (HR=0.45, 95%CI: 0.31−0.65, P=1e−05; Figure 3B). However, ATG4C mRNA expression level was not related to OS in patients with diffuse cancer (HR=0.75, 95% CI: 0.53−1.08, P=0.12; Figure 3C) or mixed cancer (HR=3.29, 95%CI: 0.73−14.91, P=0.1; Figure 3D).
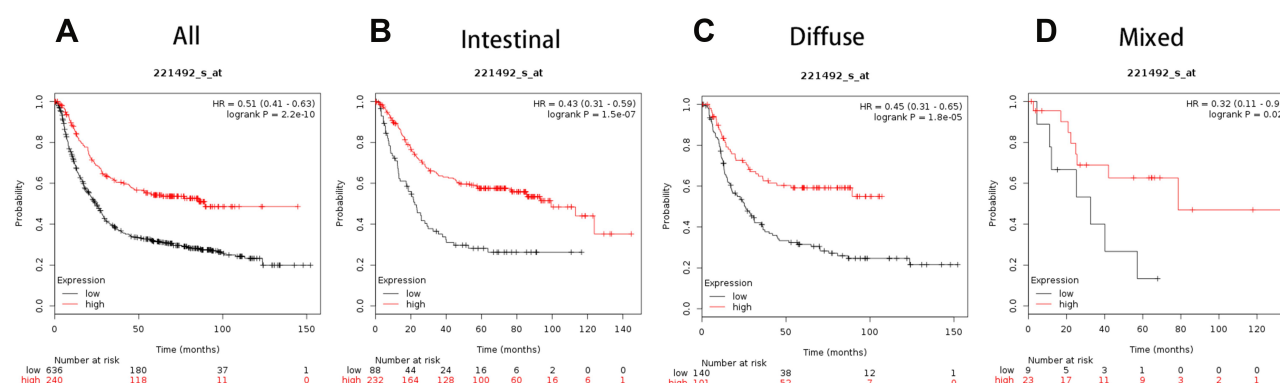
Figure 1 The prognostic significance of *ATG3* expression in gastric cancer. The prognostic value of autophagy-related protein 3 expression in www.kmplot.com. Affymetrix ID for *ATG3* is 221492_s_at, overall survival curves were plotted for all patients (**A**), and for those with intestinal type (**B**), diffuse type (**C**), and mixed type cancers (**D**).
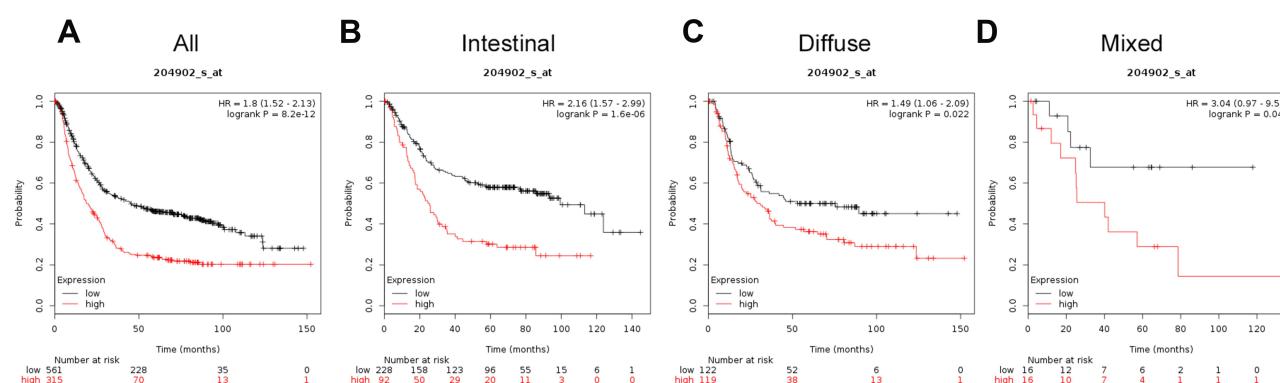

Figure 2 The prognostic significance of *ATG4B* expression in gastric cancer. The prognostic value of autophagy-related protein 4B expression in www.kmplot.com. Affymetrix ID for *ATG4B* is 204902_s_at, overall survival curves were plotted for all patients (**A**), and for those with intestinal type (**B**), diffuse type (**C**), and mixed type cancers (**D**).
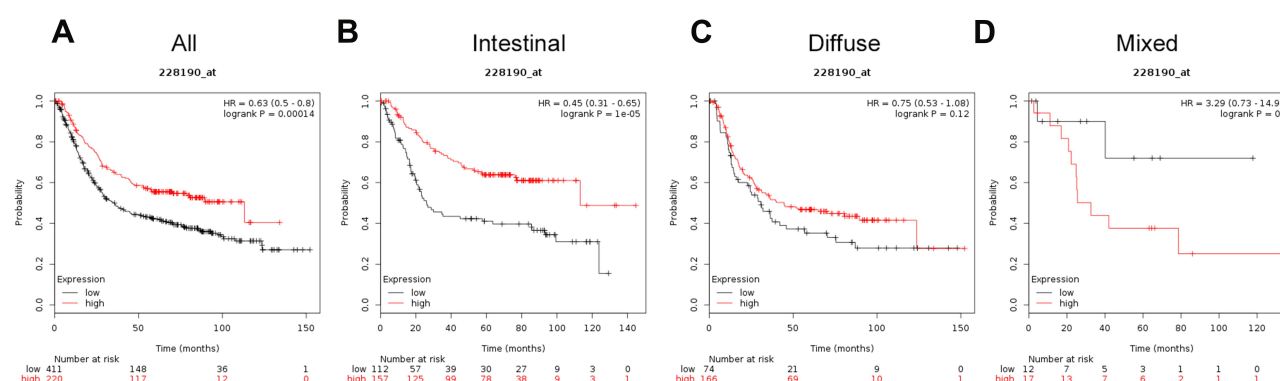

Figure 3 The prognostic significance of *ATG4C* expression in gastric cancer. The prognostic value of autophagy-related protein 4C expression in www.kmplot.com. Affymetrix ID for *ATG4C* is 228190_s_at, overall survival curves were plotted for all patients (**A**), and for those with intestinal type (**B**), diffuse type (**C**), and mixed type cancer (**D**).

Similar results were observed for *ATG5* (Affymetrix ID is 202512_s_at). Increased *ATG5* mRNA expression was significantly correlated with favorable OS for all patients with GC (HR=0.61, 95%CI: 0.51−0.73, *P*=7.7e−08; Figure 4A) and intestinal cancer (HR=0.42, 95%CI: 0.3 −0.59, *P*=3.9e−07; Figure 4B). However, *ATG5* mRNA expression was not correlated with OS in patients with diffuse cancer patients (HR=0.78, 95%CI: 0.56−1.1, *P*=0.16 Figure 4C) or mixed cancer (HR=2.89, 95%CI: 0.65−12.9, *P*=0.15; Figure 4D).
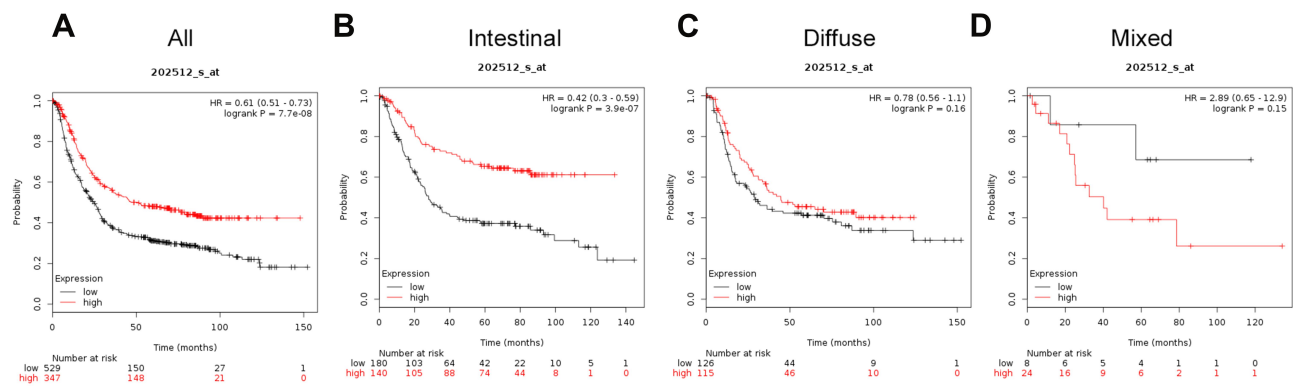
Figure 4 The prognostic significance of ATG5 expression in gastric cancer. The prognostic value of autophagy-related protein 5 expression in www.kmplot.com. Affymetrix ID for *ATG5* is 202512_s_at, overall survival curves were plotted for all patients (**A**), and for those with intestinal type (**B**), diffuse type (**C**), and mixed type cancers (**D**).

The prognostic value of *ATG7* (Affymetrix ID is 218673_s_at) was assessed using information in the database (Figure 5). *ATG7* mRNA expression levels were associated with inferior OS for all patients with GC (HR=1.47, 95%CI: 1.24−1.74, *P*=1.1e−05), intestinal cancer (HR=1.82, 95%CI: 1.33−2.5, *P*=0.00018), and diffuse cancer (HR=1.86, 95%CI: 1.29−2.67, *P*=0.00076), but not in those with mixed cancer (HR=1.94, 95%CI: 0.69 −5.44, *P*=0.2).

The prognostic value of *ATG10* (Affymetrix ID is 218214_at) was assessed using the database (Figure 6). The OS curves revealed a connection between *ATG10*
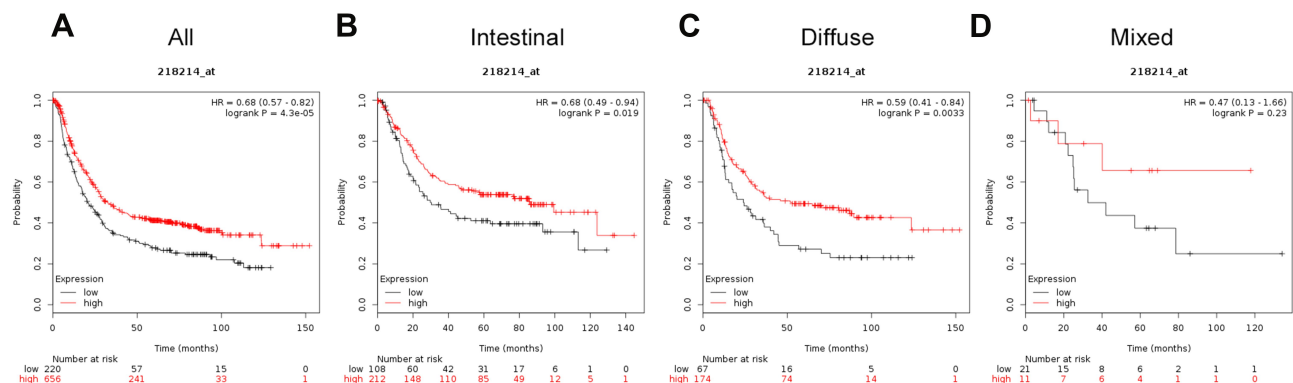

Figure 5 The prognostic significance of *ATG7* expression in gastric cancer. The prognostic value of autophagy-related protein 7 expression in www.kmplot.com. Affymetrix ID for *ATG7* is 218673_s_at, overall survival curves were plotted for all patients (**A**), and for those with intestinal type (**B**), diffuse type (**C**), and mixed type cancers (**D**).
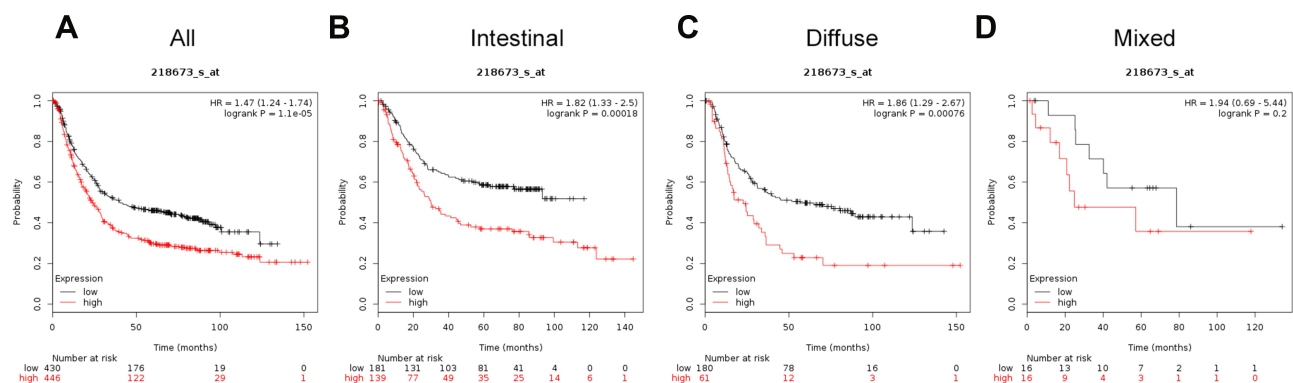

Figure 6 The prognostic significance of *ATG10* expression in gastric cancer. The prognostic value of autophagy-related protein 10 expression in www.kmplot.com. Affymetrix ID for *ATG10* is 218214_s_at, OS curves were plotted for all patients (**A**), and for those with intestinal type (**B**), diffuse type (**C**), and mixed type cancers (**D**).

mRNA expression and good OS for all patients with GC (HR=0.68, 95%CI: 0.57−0.82, *P*=4.3e−05), intestinal cancer (HR=0.68, 95%CI: 0.49−0.94, *P*=0.019), diffuse cancer (HR=0.59, 95%CI: 0.41−0.84, *P*=0.0033) but for those with mixed cancer (HR=0.47, 95%CI: 0.13−1.66, *P*=0.23).

The prognostic value of *ATG12* (Affymetrix ID for ATG12 is 213026_at) was assessed using the database (Figure 7). High *ATG12* mRNA expression was associated with poor OS in all patients with GC (HR=1.31, 95%CI: 1.09−1.56, *P*=0.0031) and patients with diffuse subtype GC (HR=1.78, 95%CI: 1.26−2.5, *P*=0.00087) but was not associated with OS in patients with intestinal cancer (HR=1.42, 95%CI: 1−2.01, *P*=0.051), or mixed cancer (HR=0.58, 95%CI: 0.18–1.85, *P*=0.35).

The prognostic value of *ATG16L1* mRNA (Affymetrix ID: 232612_s_at) was assessed by plotting OS curves for all patients with GC (Figure 8A), intestinal cancer (Figure 8B), diffuse cancer (Figure 8C), and mixed cancer

(Figure 8D). High *ATG16L1* mRNA expression was related to unfavorable OS for all GC patients (HR=1.43, 95%CI: 1.15−1.77, *P*=0.0012) and intestinal cancer patients (HR=1.83, 95%CI: 1.25−2.69, *P*=0.0016). For patients with diffuse and mixed cancer types, OS was independent of expression of *ATG16L1* mRNA levels (for patients with diffuse cancer (HR=0.83, 95%CI: 0.58 −1.19, *P*=0.3), and for patients with mixed cancer types (HR=1.92, 95%CI: 0.59−6.25, *P*=0.27).

The Affymetrix ID for *TECPR1* is 227580_s_at. Increased *TECPR1* mRNA expression is related to poor OS for all patients with GC patients (HR=1.49, 95%CI: 1.14−1.95, *P*=0.0031; Figure 9A) and intestinal cancer (HR=1.86, 95%CI: 1.2−2.86, *P*=0.0044; Figure 9B). However, *TECPR1* mRNA overexpression in patients with diffuse (HR=1.23, 95%CI: 0.82−1.84, *P*=0.32; Figure 9C) and mixed cancer (HR=5.43, 95%CI: 0.7 −42.33, *P*=0.071; Figure 9D) was not correlated with OS.
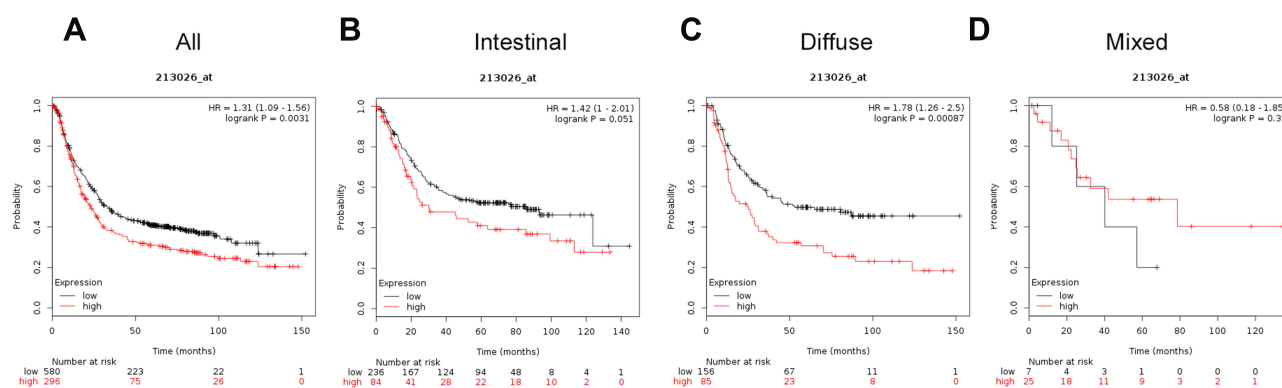

Figure 7 The prognostic significance of *ATG12* expression in gastric cancer. The prognostic value of autophagy-related protein 12 expression in www.kmplot.com. Affymetrix ID for *ATG12* is 213026_s_at, overall survival curves were plotted for all patients (**A**), and for those with intestinal type (**B**), diffuse type (**C**), and mixed type cancers (**D**).
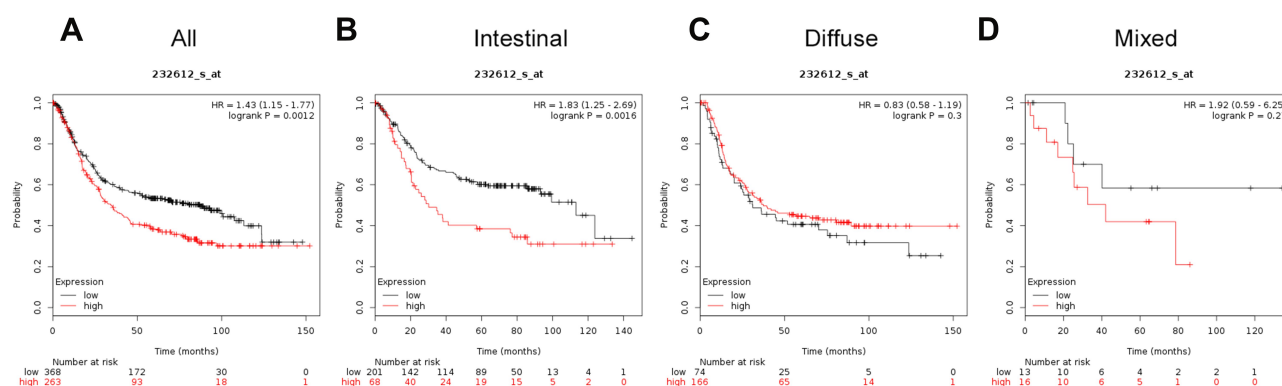

Figure 8 The prognostic significance of *ATG16L1* expression in gastric cancer. The prognostic value of autophagy-related protein 16L1 expression in www.kmplot.com. Affymetrix ID for *ATG 16L1* is 232612_s_at, overall survival curves were plotted for all patients (**A**), and for those with intestinal type (**B**), diffuse type (**C**), and mixed type cancers (**D**).
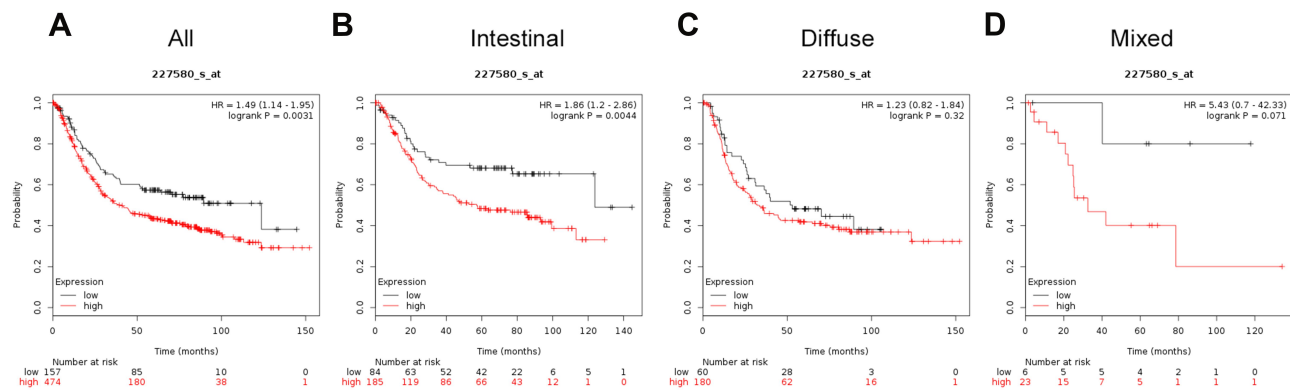
Figure 9 The prognostic significance of *TECPR1* expression in gastric cancer. The prognostic value of tectonin b-propeller repeat containing 1 expression in www.kmplot. com. Affymetrix ID for *TECPR1* is 227580_s_at, overall survival curves were plotted for all patients (**A**), and for those with intestinal type (**B**), diffuse type (**C**), and mixed type cancers (**D**).

We also assessed the correlation between the expression of UBL conjugation system genes and GC patient other clinicopathological features, including pathological stage (Table 2), HER2 status (Table 3), treatment strategy (Table 4), gender (Table 5), and differentiation (Table 6). Increased expression of *ATG3* is correlated with satisfactory OS in patients with GC in stages I, III, and IV (Table 2). Increased *ATG4C* and *ATG10* mRNA expression correlates with improved OS in patients with stages I and III GC. Furthermore, high *ATG5* and *ATG12* expressions correlate with good OS only in patients with stage III GC. Patients with poor OS exhibit overexpression of *ATG4B* (stage I and stage III), *ATG7* (stage III), *ATG12* (stage IV), *ATG16L1* (stage II and stage III), and *TECPR1* (stage III).

The relationship between the expression of *ATGs* and *HER2* gene status and prognosis in patients with GC was assessed (Table 3). Patients with negative *HER2* gene status and high levels of *ATG3* and *ATG4C* expressions have favorable OS. Conversely, negative *HER2* status and increased *TECPR1* mRNA levels are associated with poor OS in patients with GC. *HER2* gene status does not appear to influence OS in patients with GC. Increased *ATG5* and *ATG10* expression always correlates with improved OS, while increased *ATG4B*, *ATG7*, *ATG12*, and *ATG16L1* expression is generally correlated with an unfavorable OS.

We examined the effects of treatment in patients with GC with different UBL conjugation system gene expression levels (Table 4). When patients received surgery alone, *ATG3*, *ATG4C*, *ARG5*, and *ATG10* overexpression was usually related to favorable OS, and *ATG12* and *ATG16L1* overexpression generally resulted in

unfavorable OS. When patients were treated with adjuvant, those with high levels of *ATG3* (other adjuvant), *ATG5* (5-FU), and *ATG12* (5-FU) expression were associated with good OS, but patients with high *ATG4C* expression were unable to achieve good OS. Additionally, increased *ATG4B* expression was always associated with poor OS, irrespective of the treatment received.

The correlation between the expression of UBL conjugation system genes and OS in patients with GC of different genders was also assessed (Table 5). High levels of *ATG3*, *ATG5*, and *ATG10* expression was usually associated with good OS in both male and female patients with GC, and high *ATG4B* expression was associated with poor OS. Overexpression of *ATG7*, *ATG12*, *ATG16L1*, and *TECPR1* in male patients was associated with poor OS, and increased *ATG4C* and *ATG12* expression correlates with favorable OS only in male and female patients, respectively.

The correlation between ATG gene expression and OS in patients with gastric cancer and different differentiation was analyzed (Table 6). Obvious heterogeneity in ATG gene expression levels was observed. For patients with poorly differentiated gastric cancer, high *ATG12* or *ATG16L1* expression was associated with poor OS, while high *TECPR1* expression correlated with good OS. For patients with moderately differentiated GC, increased *ATG4C* expression always correlated with better OS, while increased *TECPR1* expression was related to poorer OS. For patients with well differentiated GC, *ATG4B* overexpression was generally correlated with very poor OS, and *ATG5* overexpression was associated with good OS.

Table 2 Correlation of ATG mRNA Expression Levels with OS in Different Pathological Stages in Gastric Cancer Patients

ATGs	Stages	Cases	HR (95%CI)	P-value
ATG3	I	67	0.16 (0.02–1.21)	0.042*
	II	140	0.5 (0.23–1.09)	0.075
	III	305	0.59 (0.44–0.8)	0.00042*
	IV	148	0.57 (0.37–0.88)	0.0097*
ATG4B	I	67	3.8 (1.37–10.54)	0.0058*
	II	140	1.48 (0.8–2.71)	0.21
	III	305	1.89 (1.4–2.53)	1.8e−05*
	IV	148	1.26 (0.84–1.9)	0.26
ATG4C	I	62	0.35 (0.11–1.08)	0.056
	II	135	1.58 (0.81–3.09)	0.18
	III	197	0.42 (0.28–0.63)	1.8e−05*
	IV	140	0.65 (0.41–1.02)	0.061
ATG5	I	67	0.47 (0.18–1.28)	0.13
	II	140	0.55 (0.29–1.04)	0.063
	III	305	0.58 (0.42–0.8)	0.00076*
	IV	148	1.35 (0.85–2.13)	0.2
ATG7	I	67	1.53 (0.56–4.17)	0.4
	II	140	1.81 (0.94–3.48)	0.072
	III	305	1.85 (1.37–2.51)	4.8e−05*
	IV	148	1.38 (0.94–2.03)	0.096
ATG10	I	67	0.27 (0.09–0.83)	0.014*
	II	140	0.67 (0.37–1.23)	0.19
	III	305	0.73 (0.55–0.98)	0.034*
	IV	148	0.73 (0.5–1.07)	0.1
ATG12	I	67	0.49 (0.16–1.52)	0.21
	II	140	1.43 (0.75–2.71)	0.27
	III	305	0.63 (0.46–0.86)	0.0035*
	IV	148	1.96 (1.3–2.95)	0.001*
ATG16L1	I	62	0.47 (0.15–1.45)	0.18
	II	135	2.28 (1.19–4.37)	0.011*
	III	197	1.69 (1.14–2.49)	0.008*
	IV	140	0.76 (0.48–1.2)	0.24
TECPR1	I	62	2.08 (0.63–6.86)	0.22
	II	135	0.62 (0.33–1.17)	0.14
	III	197	1.64 (1.02–2.64)	0.04*
	IV	140	1.41 (0.89–2.25)	0.14

Note: *$P<0.05$.
Abbreviation: ATG, autophagy-related gene.

Table 3 Correlation of ATG mRNA Expression with OS in Gastric Cancer Patients with HER2 Expression Status

ATGs	HER2 Status	Cases	HR (95%CI)	P-value
ATG3	Negative	532	0.46 (0.36–0.59)	1.6e−10*
	Positive	344	0.77 (0.6–1)	0.052
ATG4B	Negative	532	1.85 (1.48–2.32)	5.9e−08*
	Positive	344	1.67 (1.28–2.18)	0.00013*
ATG4C	Negative	429	0.62 (0.48–0.82)	0.00059*
	Positive	202	0.68 (0.45–1.01)	0.055
ATG5	Negative	532	0.67 (0.53–0.86)	0.0016*
	Positive	344	0.52 (0.39–0.68)	2.3e−06*
ATG7	Negative	532	1.39 (1.11–1.74)	0.0037*
	Positive	344	1.43 (1.08–1.89)	0.013*
ATG10	Negative	532	0.68 (0.54–0.85)	0.00084*
	Positive	344	0.71 (0.54–0.93)	0.011*
ATG12	Negative	532	1.35 (1.05–1.72)	0.018*
	Positive	344	1.42 (1.1–1.84)	0.0077*
ATG16L1	Negative	429	1.57 (1.2–2.05)	8e−04*
	Positive	202	1.74 (1.18–2.58)	0.0051*
TECPR1	Negative	429	1.53 (1.17–2)	0.0016*
	Positive	202	0.68 (0.44–1.06)	0.084

Note: *$P<0.05$.
Abbreviation: ATG, autophagy-related gene.

dependent manner in cells treated with VE-822 (Figures 10 and 11). These results indicate that GC cell proliferation could be inhibited by VE-822. Cell migratory capacity was also suppressed by VE-822 as determined by the narrowed scratch in wells treated with VE-822 (Figure 11).

VE-822 Inhibitory Effects on GC Cells Involves Changes in Autophagy-related Gene Expression

To explore the influence of autophagy on the observed inhibitory effects of VE-822, ATG gene expression levels were assessed using real-time PCR (Figure 12). Of the nine targets, expression of ATG3, ATG4B, ATG4C, ATG5, ATG7, and TCEPR1 showed a declining trend in a VE-822 dose-dependent manner and ATG10, ATG12, and ATG16L1 showed no significance in this process. Partially consistent with the Kaplan–Meier plotter database results, ATG4C, ATG7, and TCEPR1 expression correlated with poor OS in patients with GC patients. However, high ATG3, ATG4B, and ATG5 expression, which were associated with good OS, was also reduced

VE-822 Suppresses GES 1 Cell Proliferation and Migration

Colony formation and cell migration assays were performed to assess the effects of VE-822 treatment on gastric cancer cell proliferation and cell migration. Compared with the control group, cell colony sizes were reduced in a dose-

Table 4 Correlation of ATGs mRNA Expression with OS in Gastric Cancer Patients with Different Treatment Strategy

ATGs	Treatment	Cases	HR (95%CI)	P-value
ATG3	Surgery alone	380	0.75 (0.56−1)	0.047*
	5-FU adjuvant	153	0.7 (0.48−1.02)	0.064
	Other adjuvant	76	0.26 (0.1−0.65)	0.0019*
ATG4B	Surgery alone	380	1.37 (1.03−1.82)	0.031*
	5-FU adjuvant	153	1.88 (1.32−2.69)	4e−04*
	Other adjuvant	76	3.28 (0.96−11.19)	0.045*
ATG4C	Surgery alone	380	0.72 (0.54−0.98	0.033*
	5-FU adjuvant	34	1.61 (0.64−4.04)	0.31
	Other adjuvant	76	2.9 (1.2−7.01)	0.013*
ATG5	Surgery alone	380	0.64 (0.48−0.86)	0.0025*
	5-FU adjuvant	153	0.54 (0.37−0.79)	0.0011*
	Other adjuvant	76	3.32 (0.77−14.32)	0.088
ATG7	Surgery alone	380	0.83 (0.62−1.11)	0.21
	5-FU adjuvant	153	1.41 (0.99−2.01)	0.055
	Other adjuvant	76	2.32 (0.95−5.69)	0.058
ATG10	Surgery alone	380	0.67 (0.49−0.92)	0.013*
	5-FU adjuvant	153	1.37 (0.93−2.03)	0.11
	Other adjuvant	76	0.4 (0.12−1.36)	0.13
ATG12	Surgery alone	380	1.49 (1.1−2.02)	0.01*
	5-FU adjuvant	153	0.54 (0.38−0.78)	7e−04*
	Other adjuvant	76	0.73 (0.3−1.77)	0.49
ATG16L1	Surgery alone	380	1.44 (1.07−1.92)	0.014*
	5-FU adjuvant	34	1.97 (0.76−5.16)	0.16
	Other adjuvant	76	1.62 (0.67−3.92)	0.28
TECPR1	Surgery alone	380	1.82 (1.25−2.65)	0.0016*
	5-FU adjuvant	34	0.43 (0.16−1.13)	0.079
	Other adjuvant	76	0.55 (0.23−1.34)	0.18

Note: *P<0.05.
Abbreviations: 5-FU, 5-fluorouracil; ATG, autophagy-related gene.

Table 5 Correlation of ATGs mRNA Expression with OS in Gastric Cancer Patients in Different Gender

ATGs	Gender	Cases	HR (95%CI)	P-value
ATG3	Female	236	0.48 (0.31−0.72)	0.00034*
	Male	545	0.54 (0.43−0.68)	9.3e−08*
ATG4B	Female	236	2.26 (1.57−3.24)	6.7e−06*
	Male	545	1.84 (1.49−2.28)	1.4e−08*
ATG4C	Female	187	0.66 (0.43−1.02)	0.062
	Male	349	0.67 (0.5−0.9)	0.0076*
ATG5	Female	236	0.62 (0.44−0.88)	0.0068*
	Male	545	0.55 (0.44−0.69)	2.1e−07*
ATG7	Female	236	1.39 (0.98−1.97)	0.061
	Male	545	1.68 (1.36−2.09)	1.7e−06*
ATG10	Female	236	0.51 (0.36−0.72)	9.7e−05*
	Male	545	0.72 (0.57−0.9)	0.0037*
ATG12	Female	236	0.56 (0.38−0.8)	0.0015*
	Male	545	1.64 (1.29−2.07)	3.1e−05*
ATG16L1	Female	187	1.4 (0.9−2.19)	0.14
	Male	349	1.36 (1.02−1.83)	0.038*
TECPR1	Female	187	1.71 (0.98−2.99)	0.056
	Male	349	1.75 (1.22−2.51)	0.002*

Note: *P<0.05.
Abbreviation: ATG, autophagy-related gene.

after VE-822 treatment. Therefore, the underlining that the mechanism needs to be studied further.

Discussion

Unique biomarkers express in different cancer stages and cancer type could help with the diagnosis, accurate therapy and evaluation of prognosis. The research of Kim et al found that rich LC3B and p62 normally occurred in GC patients, especially, for tubular adenoma and gastric adenocarcinoma types,[23] and in another study, the researchers reported that LC3A/B and Beclin-1 generally distributed in whole cells for gastric cancer patients with high-risk grade, while, for *AMBRA-21* (Beclin-1-regulated autophagy protein-1), it probably only appeared in the nucleus.[24] Therefore, more biomarkers should be discovered, and the underlying mechanisms involved in the development and progression of GC should be explored to help gastric cancer patients with their diagnosis, therapy, and prognosis.

Autophagy plays a dual role in cancer. On the one hand, autophagy can eliminate the damaged cellular components or entire cells, while on the other hand, the energy or nutrients that come from degradation can provide tumor cells nutrition or be recycled for apoptosis-related protein synthesis of apoptosis relative proteins. Therefore, the ultimate decision of autophagy pathway that closely correlated to OS of gastric cancer patients need to be further studied.

In this study, the prognostic value of autophagy associated gene expression was preliminarily explored by online retrieval from the Kaplan–Meier plotter. GC patients with high *ATG3*, *ATG4C*, *ATG5*, and *ATG10* mRNA expression had better OS, while those with high *ATG4B*, *ATG7*, *ATG12*, *ATG16L1*, and *TECPR1* mRNA expression had poorer OS. Then, VE-822 that has previously been testified as effective on gastric cancer[25] was

Table 6 Correlation of ATG mRNA Expressions with OS in Gastric Cancer Patients with Different Differentiation

ATGs	Differentiation	Cases	HR (95%CI)	P-value
ATG3	Poor Moderate Well	165 67 32	0.81 (0.52–1.26) 0.59 (0.3–1.17) 0.4 (0.13–1.18)	0.35 0.13 0.086
ATG4B	Poor Moderate Well	165 67 32	1.36 (0.84–2.18) 1.75 (0.82–3.76) 3.33 (1.37–8.11)	0.2 0.15 0.0052*
ATG4C	Poor Moderate Well	126 67 32	1.41 (0.83–2.39) 0.49 (0.26–0.95) Not available	0.2 0.03* Not available
ATG5	Poor Moderate Well	165 67 32	1.25 (0.84–1.87) 1.94 (0.79–4.78) 0.24 (0.07–0.83)	0.27 0.14 0.015*
ATG7	Poor Moderate Well	165 67 32	1.32 (0.83–2.1) 0.79 (0.41–1.52) 1.53 (0.61–3.82)	0.24 0.47 0.36
ATG10	Poor Moderate Well	165 67 32	0.71 (0.43–1.16) 1.65 (0.85–3.18) 0.58 (0.23–1.51)	0.17 0.13 0.26
ATG12	Poor Moderate Well	165 67 32	1.53 (1.03–2.29) 0.77 (0.4–1.49) 0.58 (0.25–1.39)	0.036* 0.44 0.22
ATG16L1	Poor Moderate Well	121 67 32	1.75 (1.06–2.88) 1.77 (0.89–3.54) Not available	0.027* 0.1 Not available
TECPR1	Poor Moderate Well	121 67 32	0.49 (0.26–0.89) 2.83 (1.09–7.33) Not available	0.017* 0.025* Not available

Note: *P<0.05.
Abbreviation: ATG, autophagy-related gene.

employed for the mechanism study. Following VE-822 treatment, expression of *ATG4C*, *ATG7*, and *TCEPR1*, all of which are associated with poor OS were decreased. We would like to analyze the potential reasons for these results.

ATG3, the crucial component of the E2-like enzyme in LC3 lipidation, assists *ATG8* in phagophore expansion and autocatalyzes itself to form the *ATG12-ATG3* complex to maintain mitochondrial homeostasis.[26,27] In colon cancer, *ATG3* gene expression is distinctively elevated by down-regulation of miR-431-5p and *ATG3* knockdown significantly reduces colon cancer proliferation and invasion.[28] Additionally, in non-small-cell lung cancer, *ATG3* is targeted by lncRNA *KCNQ1OT1* and can suppress miR-204-5p, while *ATG3* upregulation resulted in increased

expression of apoptosis-related proteins Bax, cleaved caspase-3, and cleaved caspase-9.[29] In GC, *ATG3* expression was elevated and was related to favorable OS, especially in combination with negative HER2 status. However, the function of *ATG3* differs depending in the type of cancer being examined, indicating that the underlying mechanism needs further study.

ATG5 covalently conjugates with *ATG12* and interacts with *ATG16* to form a complex involved in autophagosome formation and elongation.[30] Our results show that high *ATG5* expression is associated with better OS in patients with GC. A previous study reported that *ATG5* might be cleaved into a 24K fragment during apoptosis, and that this fragment functions in the release of cytochrome c into the cytosol and caspase-3 cleavage, a crucial
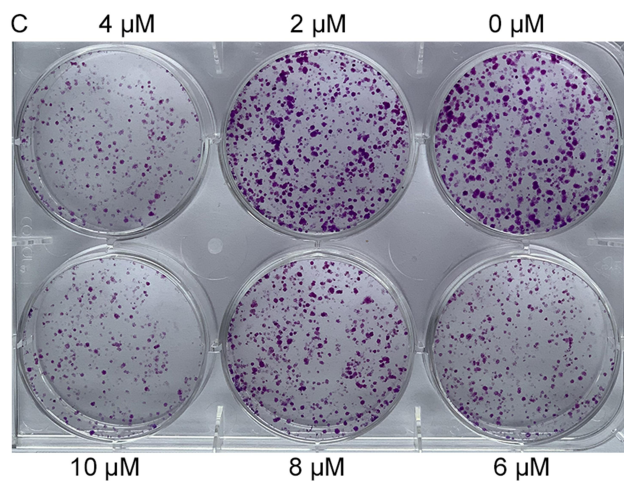
Figure 10 Inhibitory effects of VE-822 on GES 1 proliferation. Cell colonies was reduced in a dose-dependent manner.

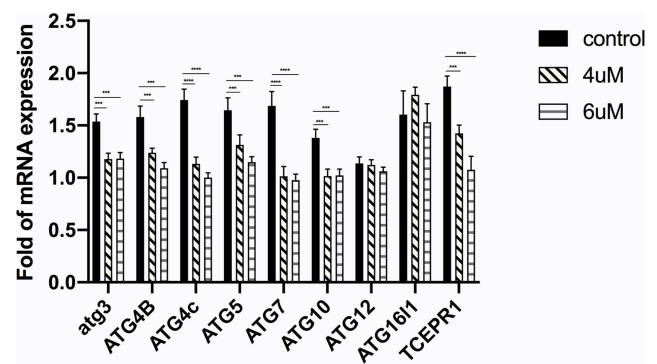**Figure 12** The detection of mRNA expression changes of ATGs by RT-PCR in GES 1 when treated with VE-822. ATG3, ATG4B, ATG4C,ATG5, ATG7, ATG10 and TECPR1 mRNA levels were downregulated when treated with VE-822. Each data was compared with the control group, ***P < 0.001, ****P < 0.0001.

step in apoptosis.[31] Therefore, increased *ATG5* expression may increase susceptibility towards apoptosis stimuli and chemotherapy efficiency. This is consistent with our result that better OS was observed in patients with higher *ATG5* expression and who were also treated with 5-FU adjuvant. Additionally, increased *ATG5* expression positively correlated with collagen V gene expression in the airway of asthma pathogenesis, which could promote pulmonary fibrosis development.[28] Another study found that when suffering injury, quiescent stellate cells of the liver are activated, ultimately leading to liver fibrosis, and that this process was accelerated by *ATG5* and *ATG7* taking part in lipid metabolism and energy production.[32] Taken together, these data imply that *ATG5* plays an important role in fibrosis by blocking tumor blood supply and preventing tumor invasion, ultimately leading to good OS in patients with GC.

ATG10 is the E2-like enzyme and, when conjugated with *ATG12* and *ATG5*, takes charge of phagophore expansion. In SW480 colorectal cancer cells, *ATG10* overexpression is correlated with upregulated cell proliferation, lymph vascular invasion, and chemoresistance, all of which is reversed by the *ATG10* downregulator, miR-
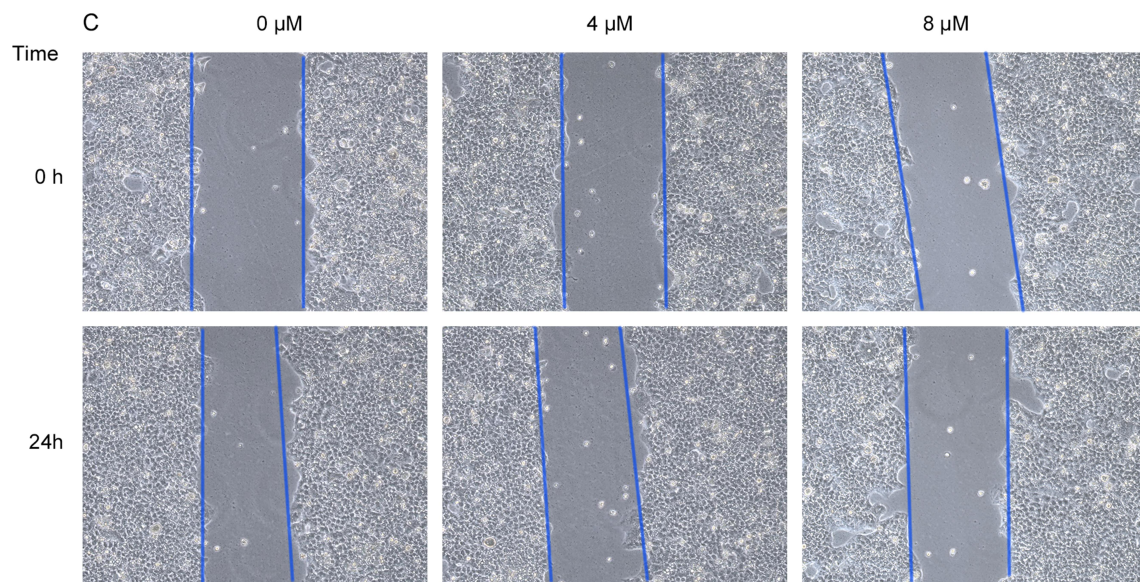

Figure 11 Inhibitory effects of VE-822 on GES 1 migration. The scratch width was reduced in a dose-dependent manner.

27b-3p.[33] In GC, high levels of *ATG10* expression is associated with good OS (*P*=4.3e-0.5 and HR=0.68, 95% CI: 0.57–0.82). *ATG10* variants, including rs10514231, rs1864182, and rs1864183, are correlated with shorter OS in patients with non-small-cell lung cancer.[34] However, Yuan reported that *ATG10* rs1864182 and rs10514231 are associated with a lower risk of breast cancer.[35] The distinct mechanism of *ATG10* in GC should be further explored because taking into account the poor nutrition status of GC patients and the role of autophagy in host energy supply.

The ATG4s family, includes four isoforms: *ATG4A*, B, C, and D. The *ATG4* family completes its role in autophagy by regulating LC3 activity in autophagosome maturation. During this process, *ATG4* (mainly the *ATG4B*) cleaves the C-terminus of LC3/*GABARAP* leading to lipid conjugation. After autophagosome expansion and maturation, *ATG4B* is reactivated to release LC3 from the membrane junction which leads to fusion of the engulfed contents and lysosomes.[36] However, *ATG4B* actually promotes tumor cell migration or proliferation among several cancer types, including breast cancer,[37] pancreatic ductal adenocarcinoma,[38] colorectal cancer,[39] and prostate cancer.[40] When blocked using an inhibitor or miRNA of *ATG4B*, tumor cell proliferation was controlled and susceptibility towards chemotherapy was enhanced due to the repressed autophagy, in this process, pro-LC3 cannot be cleaved, and the fusion of the autophagosomes with lysosomes would also be obstructed, which means that downregulation of *ATG4B* is helpful to cancer cell apoptosis. Well, this trend was also witnessed in the present study in Figure 11, in our study, the high *ATG4B* expression correlated with inferior OS, however, when treated with VE-822, the mRNA level of *ATG4B* was significantly declined. *ATG4C* is responsible for LC3 lipidation and delipidation during starvation, and its function in tumors differs slightly. In breast cancer, *ATG4C* is regulated in an ATM-dependent manner, drives mammosphere formation, and promotes breast cancer development.[41] Well, in another study, the deficiency of *ATG4C* promotes the development of fibrosarcomas, which coincided with the outcomes of our study that the highlight expression of *ATG4C* seems suppress the development of GC.

ATG7 is another crucial protein for autophagosome formation through the canonical pathway, and it was proved that high *ATG7* expression level was associated with poor OS in patients with GC, and in this research,

ATG7 knockdown contributes to Tanshinone I-induced apoptosis in gastric cancer.[42] Examination about the relationship between autophagy and the endoplasmic reticulum (ER) revealed that ER-induced autophagy could drive cells into S-phase to inhibit apoptosis, moreover, when cells were infected with *ATG5* and *ATG7* overexpression adenovirus, the cleaved caspase-3 and cleaved caspase-12 expressions were markedly reduced.[43] Cerebral angiogenesis research showed that *ATG7* could upregulate expression of laminin-5, a major constituent of the vascular extracellular matrix, via NF-κB signaling and promote tube formation, supporting a role for *ATG7* in cell migration.[44] From these previous data, it appears that *ATG7* has the function to promote cell proliferation, suppress apoptosis, and drive blood vessel generation. Our PCR results show that downregulation of *ATG7* expression following VE-822 treatment could significantly increase cancer cell apoptosis, which is consistent with the results of previous studies.

Kaplan–Meier plotter results show that high *ATG12* correlates with poor OS in patients with GC. In the formation of autophagosome, *ATG12* has three roles in this process, it functions as a ubiquitin-like module through conjugating to *ATG5*, it forms an E3 complex with *ATG5* and *ATG16*, and it interacts with *ATG3*. Using a mouse model, it was previously demonstrated that *ATG12* depletion led mice to develop obesity at a faster rate than in controls.[45] In tumor cells, *ATG12* could lead to cell death in an autophagy-independent manner by regulating cancer metabolism and functioning as an oncogene capable of sustaining mitochondrial biogenesis. *ATG12* is involved in nutrient metabolism and mitochondrial respiration, and decreasing ATG12 expression levels, by sh*ATG12* infection, caused impaired metabolic activity and mitochondrial biogenesis in H1299 lung cancer and MDA-MB-231 breast cancer cells revealed by the declined Ki-67-marked cells, lower speed of colonies formation and the weaken expression level of mitochondrial-localizing proteins and the master regulator of mitochondria, PGC1-α[45] Therefore, *ATG12* functions as an oncogene in the development of GC tumors, which is supported by the results of this study.

ATG16L1, another key role of autophagosome formation, was reported has autophagy-independent anti-inflammatory activities.[46,47] However, genome wide association studies of *ATG16L1* variants indicate that they may be risk factors for Crohn's disease.[48,49] Previously, it was shown that miR-874 can downregulate

ATG16L1 mRNA levels resulting in autophagy inhibition and sensitizing the response of GC to chemotherapy.[50] These results are consistent with those presented in this study.

Compared with other autophagy-related proteins, *TECPR1,* which forms a complex with *ATG5-12* to promote autophagosome maturation, was the most sensitive biomarker for prostate cancer.[51] In our study, *TECPR1* overexpression correlated with poor OS in patients with GC patients, when the effective treatment VE-822 was applied, its level was downregulated (Figure 11).

Although there has already been much research about the role of autophagy in cancer, these biomarkers may exert adverse effects in different types of cancer. This comment can be demonstrated by the recent study of Li et al.[52] In the results of their study, MKL1 could promote the proliferation of gastric cancer cell by inhibiting the expression of *FOXP3* (the important regulator of Tregs) via miR-34a and the knockdown of *FOXP3* was likely to exacerbate this phenomenon which indicated that *FOXP3* was an oncogene in gastric cancer. However, another study of *FOXP3* in breast cancer revealed that *FOXP3* could activate P21, the inhibitor of cell cycle and therefore, retarded the proliferation of cancer cells and this certified the suppressive role of *FOXP3* in breast cancer.[53,54] Therefore, it is necessary to explore the special role of this kind of biomolecules in gastric cancer through a large amount of clinical samples to improve its overall survival. And the present study investigated the prognostic value of autophagy-related proteins for patients with GC and used in vitro experiments to verify above outcomes. These results presented here contribute to understanding of the molecular mechanism of GC development and progression and would be helpful in the development of treatments with improved efficacy.

Disclosure

The authors report no conflicts of interest in this work.

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