The expression characteristics and prognostic roles of autophagy-related genes in gastric cancer

Mengya Wang [Equal first author, 1, 2], Jingjing Jing [Equal first author, 1, 2], Hao Li [3], Jingwei Liu [2], Yuan Yuan [Corresp., 1, 2], Liping Sun [Corresp. 1, 2]

[1] Tumor Etiology and Screening Department of Cancer Institute, and Key Laboratory of Cancer Etiology and Prevention in Liaoning Education Department, the First Hospital of China Medical University, Shenyang, China

[2] Key Laboratory of GI Cancer Etiology and Prevention in Liaoning Province, the First Hospital of China Medical University, Shenyang, China

[3] Department of Clinical Laboratory, the First Hospital of China Medical University, Shenyang, China

Corresponding Authors: Yuan Yuan, Liping Sun
Email address: yyuansubmission@hotmail.com, lpsun@cmu.edu.cn

Background. Autophagy is an evolutionally highly conserved process, accompanied by the dynamic changes of various molecules, which is necessary for the orderly degradation and recycling of cellular components. The aim of the study was to identify the role of autophagy-related (*ATG*) genes in the occurrence and development of gastric cancer (GC). **Methods.** Data from Oncomine dataset was used for the differential expression analysis between cancer and normal tissues. The association of *ATG* genes expression with clinicopathologic indicators was evaluated by the Cancer Genome Atlas (TCGA) database and Gene Expression Omnibus (GEO) database. Moreover, using the TCGA datasets, the prognostic role of *ATG* genes was assessed. A nomogram was further built to assess the independent prognostic factors. **Results.** The expression of autophagy-related genes *AMBRA1*, *ATG4B*, *ATG7*, *ATG10*, *ATG12*, *ATG16L2*, *GABARAPL2*, *GABARAPL1*, *ULK4* and *WIPI2* showed differences between cancer and normal tissues. After verification, *ATG14* and *ATG4D* were significantly associated with TNM stage. *ATG9A*, *ATG2A*, and *ATG4D* were associated with T stage. *VMP1* and *ATG4A* were low-expressed in patients without lymph node metastasis. No gene in autophagy pathway was associated with M stage. Further multivariate analysis suggested that *ATG4D* and *MAP1LC3C* were independent prognostic factors for GC. The C-index of nomogram was 0.676 and the 95%CI was 0.628 to 0.724.

Conclusion. Our study provided a comprehensive illustration of *ATG* genes expression characteristics in GC. Abnormal expressions of the ubiquitin-like conjugated system in *ATG* genes plays a key role in the occurrence of GC. *ATG8/LC3* sub-system may play an important role in development and clinical outcome of GC. In the future, it is necessary to further elucidate the alterations of specific *ATG8/LC3* forms in order to provide insights for the discovery, diagnosis, or targeting for GC.

1 **The Expression Characteristics and Prognostic Roles of**

2 **autophagy-related genes in gastric cancer**

3 Mengya Wang[12]*, Jingjing Jing[12]*, Hao Li[3], Jingwei Liu[2], Yuan Yuan[12], Liping Sun[12]

4 [1] Tumor Etiology and Screening Department of Cancer Institute, and Key Laboratory of Cancer Etiology

5 and Prevention in Liaoning Education Department, the First Hospital of China Medical University,

6 Shenyang 110001, China

7 [2] Key Laboratory of GI Cancer Etiology and Prevention in Liaoning Province, the First Hospital of China

8 Medical University, Shenyang 110001, China

9 [3] Department of Clinical Laboratory, the First Hospital of China Medical University, Shenyang 110001,

10 China

11 * These authors contributed equally to this work and are considered to be co-first authors.

14 Corresponding Author:

15 Liping Sun[12]

16 No. 155, Nanjing North Street, Heping District, Shenyang City, Liaoning Province, 110001, China

17 Email address: lpsun@cmu.edu.cn

18 Yuan Yuan[12]

19 No. 155, Nanjing North Street, Heping District, Shenyang City, Liaoning Province, 110001, China

20 Email address: yuanyuan@cmu.edu.cn

Abstract

Background. Autophagy is an evolutionally highly conserved process, accompanied by the dynamic changes of various molecules, which is necessary for the orderly degradation and recycling of cellular components. The aim of the study was to identify the role of autophagy-related (*ATG*) genes in the occurrence and development of gastric cancer (GC).

Methods. Data from Oncomine dataset was used for the differential expression analysis between cancer and normal tissues. The association of *ATG* genes expression with clinicopathologic indicators was evaluated by the Cancer Genome Atlas (TCGA) database and Gene Expression Omnibus (GEO) database. Moreover, using the TCGA datasets, the prognostic role of *ATG* genes was assessed. A nomogram was further built to assess the independent prognostic factors.

Results. The expression of autophagy-related genes *AMBRA1*, *ATG4B*, *ATG7*, *ATG10*, *ATG12*, *ATG16L2*, *GABARAPL2*, *GABARAPL1*, *ULK4* and *WIPI2* showed differences between cancer and normal tissues. After verification, *ATG14* and *ATG4D* were significantly associated with TNM stage. *ATG9A*, *ATG2A*, and *ATG4D* were associated with T stage. *VMP1* and *ATG4A* were low-expressed in patients without lymph node metastasis. No gene in autophagy pathway was associated with M stage. Further multivariate analysis suggested that *ATG4D* and *MAP1LC3C* were independent prognostic factors for GC. The C-index of nomogram was 0.676 and the 95%CI was 0.628 to 0.724.

Conclusion. Our study provided a comprehensive illustration of *ATG* genes expression characteristics in GC. Abnormal expressions of the ubiquitin-like conjugated system in *ATG* genes plays a key role in the occurrence of GC. *ATG8/LC3* sub-system may play an important role in development and clinical outcome of GC. In the future, it is necessary to further elucidate the alterations of specific *ATG8/LC3* forms in order to provide insights for the discovery, diagnosis, or targeting for GC.

Key words: autophagy, gastric cancer, gene expression, TCGA, *ATG4*.

Introduction

Autophagy is an evolutionally highly conserved process, which is necessary for the orderly degradation and recycling of cellular components (Yang et al. 2020). In normal cells, autophagy keeps low-level constitutive function. Basal autophagy plays an important role in maintaining homeostatic control and elimination of unfavorable proteins. Its activity can be accelerated by a variety of cellular stressors including nutrient starvation, DNA damage, and organelle damage. Autophagy is closely related to the occurrence and treatment of tumors (Rahman et al. 2020).Recently, the paradoxical roles of autophagy in tumor suppression and tumor promotion

61   have been widely observed. As a physiological quality control process, autophagy exerts a
62   cytoprotective effect to suppress cancer development by removing damage that leads to aberrant
63   mutations. On the other hand, as cancer progresses, starving and oxidative stress situation can
64   active autophagy to fulfill the high metabolic need of cancer cells (Mathew et al. 2007).

65   The process of autophagy is accompanied by the dynamic changes of various molecules.
66   Identification of the autophagy-related biomarkers will contribute to improving diagnosis and
67   treatment of cancers. Autophagy is executed by a set of autophagy-related (*ATG*) genes, which
68   have been investigated extensively in yeast. Although the discovery of *ATG* genes greatly
69   advanced the understanding of autophagy, the function and mechanisms involved in *ATG* genes
70   need to be further explored in mammalian. Recently, several studies have investigated the
71   association of *ATG* genes and cancers. By activating ATG6-mediated autophagy, the down-
72   regulation of microRNA-30a increases the chemoresistance of osteosarcoma cells, thereby
73   inhibiting cell proliferation and invasion (Xu et al. 2016). Upregulation of UCA1 inhibits cell
74   proliferation, migration, invasion, and drug resistance via ATG7-mediated autophagy (Wu et al.
75   2019). The methyltransferase MGMT inhibits the expression of ATG4B, thereby inhibiting
76   autophagy and reducing the chemosensitivity of cisplatin in gastric cancer (GC) (Lei et al. 2020).
77   Moreover, comprehensive study of all *ATG* genes has been conducted in breast, head neck and
78   kidney carcinoma (Deng et al. 2018; Pei et al. 2018).

79   GC is the fourth most common cancer and the second leading cause of cancer death in the
80   world (Van Cutsem et al. 2016). The incidence is mainly related to diet, lifestyle, genetic
81   predisposition, family history, treatment and medical conditions, infections, demographic
82   characteristics, occupational exposures and ionizing radiation (Yusefi et al. 2018). Abnormal
83   expression of *ATG* gen*es* may lead to the dysregulation of autophagy and tumorigenesis. However,
84   the diagnostic and prognostic values of *ATG* genes have not been fully realized in GC. Since large-
85   scale expression data is available, it is feasible to display an overview of *ATG* genes from the
86   perspective of expression characteristics and prognostic role in GC. In the current study, we
87   performed systematic analysis by using available datasets of ONCOMINE and the Cancer Genome
88   Atlas (TCGA), in order to evaluate the differential expression of *ATG* genes and their associations
89   with clinicopathological parameters and prognosis of GC. Our data may provide a new
90   understanding of the autophagy-related mechanism in gastric carcinogenesis.

91

92   **Materials & Methods**
93   **Autophagy-related genes selection**

94   Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.kegg.jp/) is an online tool for

95   analysis of the gene function (Kanehisa et al. 2020). Reactome (https://reactome.org/) is a

96   bioinformatics resource for visualization, interpretation and analysis of pathways (Jassal et al.

97   2020). Using the two datasets, we selected the genes in autophagy pathways as *ATG* genes,

98   which composed four functional units including the *ULK* protein complex, *Beclin-1/PI3K*

99   complex, ubiquitin-like conjugation system and other genes (Mizushima et al. 2011). All the

100   isoforms of a gene were included, such as *ATG4A*, *ATG4B*, *ATG4C* and *ATG4D*. A total of 40

101   genes were selected. The Pathvisio (Version:3.3.0, https://www.pathvisio.org/) was used to

102   visualize the autophagy genes (Kutmon et al. 2015). which composed four functional units

103   including *ULK* complex, *PI3K* complex, ubiquitin-like conjugation system and other genes

104   (Table 1 and Fig. 1).

105   **Differential gene expression analysis by Oncomine**

106   By consulting the public data in Oncomine (https://www.oncomine.org/resource/login.html)

107   (Rhodes et al. 2004), a powerful online database with 715 sub datasets and 86,733 samples, we

108   established and logged in an oncomine account (haoli@cmu.edu.cn), and input all of 40 *ATG*

109   genes (gene symbols were listed in Table 1) to analyze their differential expression at

110   transcription level in GC and different Lauren types. Combination of P-value <0.05 and fold

111   change > 2 was identified as significant difference in gene expression.

112   **Correlation analysis of *ATG* genes expression and clinicopathological parameters from**

113   **TCGA and GEO datasets**

114   TCGA is a public database that contains the data of genomic expressions and the clinical features

115   in 33 types of cancers (Tomczak et al. 2015). The gene expression and clinicopathological

116   information of GC were downloaded from TCGA data portal

117   (https://portal.gdc.cancer.gov/projects/). R was performed to normalize the expression data. The

118   patients' TNM stage, T, N and M (Nagtegaal et al. 2020) were considered as the clinical

119   parameters.

120   **Verification of the differences of gene expression**

121   GSE62254 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62254) was a 300

122   samples microarray profile tested by the Asian Cancer Research Group (ACRG) (Cristescu et al.

123   2015). Using GSE62254, we verified the differences of gene expression identified from TCGA

124   datasets.

125   **Statistical analysis**

126   All statistical analyses were performed by R 3.14 (http://www.r-project.org/) and the package of

127   rms. Student's *t*-tests was used to analyze the differences between cancer samples and normal

128   tissues, of which the criterion is p-value <0.01 and fold change > 2.0. The association between

129   the *ATG* genes expressions and clinical features was accessed by Pearson X^2 test. The correlation

130   between *ATG* genes expressions and overall survival time was evaluated by Kaplan-Meier

131   method and compared by log-rank test. Univariate and multivariate Cox proportional hazard

132   regression models were used to recognize the independent prognostic factors. Based on the

133   multivariate Cox regression models, a nomogram was formulated together with all the

134   independent prognostic genes. The concordance index (C-index), which is similar to the area

135   under the receiver operating characteristic (ROC), was used to evaluate the nomogram. P<0.05

136   were considered significant difference.

137

138   **Results**

139   **Differential expression of *ATG* genes in GC**

140   By the Oncomine analysis, there were 10 genes of 40 *ATG* genes with significantly differential

141   expression between GC and normal samples, which were named as differentially expressed

142   genes (DEGs) (Fig. 2). Seven DEGs were belong to the ubiquitin-like conjugating system,

143   among them *ATG4B*, *ATG12* and *ATG16L2* were significantly up-regulated in GC, while

144   *ATG10*, *GABARAPL2* and *GABARAPL1* expressions were down-regulated in GC. As for *ATG7*,

145   the expression was uncertain. *ULK4*, belonging to the *ULK* complex, was found down-regulated

146   in GC. While *AMBRA1*, a member of the *PI3K* complex, was highly expressed in GC. As a

147   connection between *PI3K* complex, *ULK* complex and *ATG12* system, *WIPI2* showed higher

148   expression in cancer tissue.

149   Histological stratification analysis showed that *GABARAPL1* was down-regulated in all

150   types of GC compared with normal tissues, with fold change of -2.321 in intestinal gastric

151   adenocarcinoma, -2.287 fold in diffuse adenocarcinoma and -2.622 fold in mixed

152   adenocarcinoma (Cho Gastric, Cho,J.Y. 2011). Six DEGs showed significant differences in the

153   gastric mixed adenocarcinoma subgroup, among them *AMBRA1*, *ATG4B*, *ATG7* (probe

154   224025_s_at) and *ATG12* were up-regulated, while *GABARAPL1* and *ATG7* (probe 1569827_at)

155   were down-regulated. Four DEGs including *ATG10*, *ATG16L2*, *ULK4* and *GABARAPL1* showed

156   differences in diffuse gastric adenocarcinoma subgroup, while other four DEGs including *ATG7*

157   (probe 224025_s_at), *GABARAPL1*, *WIPI2* and *GABARAPL3* showed differences in gastric

158   intestinal type adenocarcinoma subgroup (DErrico Gastric, D'Errico, M. 2009) (Fig. 3A and 3B).

159   **Association between *ATG* genes expression and clinicopathologic variables of GC**

160   Data of 376 GC patients in TCGA were downloaded for the analysis. *ATG14*, *ULK3*, *ATG2B*,

161   *ATG12*, *ATG4C*, *ATG4D*, and *MAP1LC3A* showed significantly relationship with TNM stage.

162   After verification, *ATG14* and *ATG4D* were significantly associated with TNM stage (P=0.027,

163   0.048 respectively). *ATG9A* (P= 0.00083), *ATG2A* (P= 0.00417), and *ATG4D* (P= 0.00864) were

164   related with T stage. Low expression of *VMP1* and *ATG4A* suggested absence of lymph node

165   metastasis (P = 0.0018, 0.015, correspondingly). However, no gene in autophagy pathway was

166   observed to be associated with M stage (Table 2).

167   **Roles of *ATG* genes expression in the prediction of GC prognosis**

168   354 patients were included to analyze the overall survival of GC. The median value was used as

169   cut-off value to distinguish high expression and low expression of *ATG* genes. According to the

170   univariate survival analysis, *ATG4D*, *GABARAPL2* and *MAP1LC3C* were significantly

171   associated with the prognosis of GC. Moreover, the patients with low-expression of *ATG4D* or

172   high-expression of *GABARAPL2* and *MAP1LC3C* demonstrated longer survival time, and both

173   of the latter two genes belonged to *ATG8/LC3* system. Using the Cox's proportional hazards

174   model, we then performed the multivariate analysis adjusted by gender, age, TNM stage. *ATG4D*

175   and *MAP1LC3C* were identified as the independent prognostic factors, with adjusted hazard ratio

176   (HR) of 1.5727 (95%CI 1.1194-2.21) and 0.5767 (95%CI 0.4086-0.8138) separately (Fig. 4 and

177   Table 3). The summary of the correlation between *ATG* genes expression and TNM staging and

178   prognosis of GC was shown in Fig. 5.

179   **Joint prediction of the GC prognosis using *ATG4D* and *MAP1LC3C***

180   According to the expression of *ATG4D* and *MAP1LC3C* in GC, the gastric cancer patients were

181   divided into four groups: *ATG4D* high expression - *MAP1LC3C* high expression (HH), *ATG4D*

182   low expression - *MAP1LC3C* low expression (LL), *ATG4D* high expression - *MAP1LC3C* low

183   expression (HL) and *ATG4D* low expression - *MAP1LC3C* high expression (LH). A significant

184   difference was displayed among the four groups (p=0.0056, Fig. 6A).

185   Furtherly, to predict 1-year and 3-year survival rate, we built a nomogram by the

186   multivariate Cox regression models. After validation, the C-index was 0.676 and the 95%CI was

187   0.628 to 0.724. According to the total score after added with points identified on the point scale,

188   we found that the likelihood of 1-year and 3-year OS for individual patient could be reasonably

189   predicated by nomogram (Fig. 6B). As shown in Fig. 6C and 6D, the survival evaluated by the

190   Kaplan-Meier method was marked on the y-axes, the predicted survival estimated by nomogram

191   was observed on the x-axes, and the red lines represented the ideal reference line for which

192   predicted survival corresponds with actual survival. The plot for the probability of OS 1-year or

193   3-year showed optimal agreement between the prediction by nomogram and actual observation

194   for nomogram.

195

Discussion

196

197   Considering the vital function of *ATG* genes in autophagy, many studies have focused on

198   their role in cancers. To date, no researcher has elaborated an overview of the impact of *ATG*

199   genes on the development, progression, and prognosis of GC. In the current study, for the first

200   time, we investigated the expression profiling and the prognostic roles of whole members of

201   *ATG* genes in GC using multiple databases. Our results elucidated that abnormal expressions of

202   some key *ATG* genes were significantly associated with GC progression and outcome.

203   Firstly, 10 DEGs were identified between cancer and normal tissues, and 7 of these genes

204   were associated with ubiquitin-like conjugating system, which intimately involved in driving the

205   biogenesis of the autophagosomal membrane (Nakatogawa 2013). *ATG4B* (Liu et al. 2014), the

206   core autophagy protein in *ATG8/LC3* system, was found to be up-regulated in cancer tissue in

207   our study. It has been reported that *ATG4B* can promote the growth of colorectal cancer, while

208   silencing the expression of *ATG4B* can reduce the colony formation of cancer cells and inhibit

209   tumor growth (Liu et al. 2014; PF et al. 2018). The E1-like activating enzyme *ATG7* and the E2-

210   like activating enzyme *ATG10* also play a vital role in activating and transferring key proteins in

211   the sub-systems. In our study, expression of *ATG12* and *ATG7* showed up-regulation while

212   *ATG10* expression was down regulated in cancer tissues. Similarly, Cao, Q. H et al (Cao et al.

213   2016) analyzed 352 tissue microarrays containing cancer and paired adjacent normal tissues and

214   found that *ATG7*, *ATG12* were highly expressed in the GC tissues, and *ATG10* was weakly

215   expressed in GC. Probably because autophagy plays a specific function as a cancer suppressor or

216   tumor promoter, mainly depends on the environment, and its activity will change with the

217   development of the tumor (Amirfallah et al. 2019). As for *ATG* genes of other functional units of

218   ubiquitin-like conjugating system, some studies (Lebovitz et al. 2015; Su et al. 2019) found that

219   *GABARAPL1* transcripts were less abundant in breast, prostate, liver and non-small cell lung

220   cancers than matched normal controls, indicating that *GABARAPL1* may be a tumor suppressor.

221   While *ATG16L2* transcripts increased in kidney cancer. As a high risk gene, its high expression

222   is associated with poor prognosis (Wan et al. 2019). The high expression of Ambra1 is beneficial

223   to cell survival (Sun et al. 2018). Falasca compared 26 prostate adenocarcinoma and 12 normal

224   specimens by immunohistochemistry and observed that *AMBRA1* was highly expressed in

225   prostate cancer (Falasca et al. 2015). The expression trend of those genes was consistent with our

226   results in GC. The above results indicate that the ubiquitin-like conjugated system plays a key

227   role in the occurrence of GC, and its mechanism deserves further study.

228   It has been reported that autophagy was associated with the invasion, migration as well as

229   implantation metastasis of cancer. Therefore, we further analyzed the relationship between *ATG*

230 genes and GC TNM staging, and verified the differential genes using GSE62254 to improve the

231 accuracy. After verification, *ATG9A*, *ATG2A* and *ATG4D* were found to be associated with T

232 stage. Among these genes, *ATG9A* was previously reported to be associated with T stage in non-

233 metastatic renal cell carcinoma (Tang et al. 2013). *ATG4D* affects the biological behavior of

234 prostate cancer by regulating the activity of androgen receptor (Hu et al. 2020). Besides, all of

235 these significant differences were observed at early T stage, which suggested that autophagy may

236 play its role mainly at the early stage of GC progression. By analyzing the expression of *ATG*

237 genes both in TCGA and GSE62254, the results showed that *VMP1* and *ATG4A* were over-

238 expressed in patients with lymph node metastasis. Similarly, Yang et al found that the expression

239 of *ATG4A* was associated with lymph node metastasis in 110 GC patients (Yang et al. 2016).

240 *VMP1* was reported to promote Kras G12D-mediated pancreatic cancer initiation and facilitate

241 lymph node metastasis (Loncle et al. 2016). In addition, *ATG4D* and *ATG14* were observed to be

242 associated with overall TNM stage according to our analysis. *ATG14* was up-regulated while

243 *ATG4D* was down-regulated in GC of stage III-IV, which suggested that the two genes may have

244 the opposite effect in GC progression. It has been reported that the low expression of *ATG4D*

245 was associated with Colorectal Cancer of III stage (Gil et al. 2018). Interestingly, significant

246 relation was observed between *ATG4* isoforms and all the three clinicopathologic variables, that

247 *ATG4D* was associated with TNM and T stage, and *ATG4A* showed difference in N stage. As

248 *ATG4* activity is essential and highly specific to autophagy, it may be a prospective autophagy-

249 specific target for GC therapy.

250 Previous investigations have also indicated the predictive role of autophagy pathway genes

251 in prognosis of various types of cancers. Here we analyzed all the *ATG* genes using TCGA

252 dataset to assess their prognostic values in GC. *ATG4D* and *MAP1LC3C* were confirmed to be

253 statistically significant in multivariate survival analysis. The expression of *ATG4D* and

254 *MAP1LC3C* is low in colorectal cancer, and *ATG4D* is related to the poor prognosis of

255 pancreatic cancer (Hu et al. 2020). The high expression of *ATG4D* and the low expression of

256 *MAP1LC3C* may indicate the poor survival of gastric patients. Furthermore, we developed a

257 nomogram according to the joint expression of *ATG4D* and *MAP1LC3C* along with other

258 clinicopathological parameters. The group of HL showed poor survival while the group of LH

259 indicated favorable prognosis. In the internal validation set, the calibration plot showed that the

260 predicted 3-year and 5-year overall survival were in correspondence with the actual survival

261 estimated by the Kaplan-Meier method. *MAP1LC3C* is a member of the LC3 family of proteins

262 and a key structural component of the autophagosome that undergoes processing by members of

263 the *ATG4* family (Costa et al. 2016). These two functionally related genes together may have

264 synergistic effect in GC prognosis. For the first time, our study formulated an *ATG*-based
265 nomogram that could predict outcome of GC with a better accuracy.
266   On the basis of the above results, we found that *ATG4* and *ATG8*, members of *ATG8/LC3*
267 system, were associated with both the occurrence and prognosis of GC in our study. *ATG4* was
268 up-regulated in cancer and was associated with poor GC survival. The over-expression of *ATG8*
269 was observed in normal tissues and involved with favorable prognosis of GC. *ATG8/LC3* is
270 essential for autophagosome biogenesis and it also functions as an adaptor protein for selective
271 autophagy (Lee & Lee 2016). At the same time, it is also widely used as a marker of autophagic
272 vacuoles (OA et al. 2020).Dysregulation of *ATG8/LC3* proteins may contribute to pathogenic
273 effects during progression of autophagy-associated human diseases. Our results indicated that the
274 *ATG8/LC3* system may play an important role in development and clinical outcome of GC.
275 Elucidation of alterations in specific *ATG8/LC3* forms in GC could provide insights for the
276 discovery, diagnosis, or targeting of this high-mortality disease.
277   In conclusion, our study provided a comprehensive illustration of *ATG* genes expression
278 characteristics in GC. Abnormal expressions of *ATG* genes were observed to be significantly
279 involved in the whole process of GC occurrence, progression and prognosis. Specially, the *ULK*
280 system, such as *ATG4* family and *ATG8/LC3*, may serve as valuable biomarkers to indicate
281 gastric carcinogenesis and prognosis. Considering the underlying important roles of *ATG* genes
282 in gastric carcinogenesis and progression, future molecular experiments concerning the functions
283 and mechanisms of *ATG* genes may generate promising significance in GC development and
284 treatment.
285

Conclusions

287 Our study provided a comprehensive illustration of *ATG* genes expression characteristics in GC.
288 Abnormal expressions of the ubiquitin-like conjugated system in *ATG* genes plays a key role in
289 the occurrence of GC. *ATG8/LC3* sub-system may play an important role in development and
290 clinical outcome of GC. In the future, it is necessary to further elucidate the alterations of
291 specific *ATG8/LC3* forms in order to provide insights for the discovery, diagnosis, or targeting
292 for GC.
293

References

295 Amirfallah A, Arason A, Einarsson H, Gudmundsdottir ET, Freysteinsdottir ES, Olafsdottir KA,
296   Johannsson OT, Agnarsson BA, Barkardottir RB, and Reynisdottir I. 2019. High
297   expression of the vacuole membrane protein 1 (VMP1) is a potential marker of poor

298   prognosis in HER2 positive breast cancer. *PLoS One* 14:e0221413.
299   10.1371/journal.pone.0221413

300   Cao QH, Liu F, Yang ZL, Fu XH, Yang ZH, Liu Q, Wang L, Wan XB, and Fan XJ. 2016.
301   Prognostic value of autophagy related proteins ULK1, Beclin 1, ATG3, ATG5, ATG7,
302   ATG9, ATG10, ATG12, LC3B and p62/SQSTM1 in gastric cancer. *Am J Transl Res*
303   8:3831-3847.

304   Costa JR, Prak K, Aldous S, Gewinner CA, and Ketteler R. 2016. Autophagy gene expression
305   profiling identifies a defective microtubule-associated protein light chain 3A mutant in
306   cancer. *Oncotarget* 7:41203-41216. 10.18632/oncotarget.9754

307   Cristescu R, Lee J, Nebozhyn M, Kim KM, Ting JC, Wong SS, Liu J, Yue YG, Wang J, Yu K,
308   Ye XS, Do IG, Liu S, Gong L, Fu J, Jin JG, Choi MG, Sohn TS, Lee JH, Bae JM, Kim
309   ST, Park SH, Sohn I, Jung SH, Tan P, Chen R, Hardwick J, Kang WK, Ayers M,
310   Hongyue D, Reinhard C, Loboda A, Kim S, and Aggarwal A. 2015. Molecular analysis
311   of gastric cancer identifies subtypes associated with distinct clinical outcomes. *Nat Med*
312   21:449-456. 10.1038/nm.3850

313   Deng Q, Liang L, Liu Q, Duan W, Jiang Y, and Zhang L. 2018. Autophagy is a major
314   mechanism for the dual effects of curcumin on renal cell carcinoma cells. *Eur J*
315   *Pharmacol* 826:24-30. 10.1016/j.ejphar.2018.02.038

316   Falasca L, Torino F, Marconi M, Costantini M, Pompeo V, Sentinelli S, De Salvo L, Patrizio M,
317   Padula C, Gallucci M, Piacentini M, and Malorni W. 2015. AMBRA1 and SQSTM1
318   expression pattern in prostate cancer. *Apoptosis* 20:1577-1586. 10.1007/s10495-015-
319   1176-3

320   Gil J, Ramsey D, Pawlowski P, Szmida E, Leszczynski P, Bebenek M, and Sasiadek MM. 2018.
321   The Influence of Tumor Microenvironment on ATG4D Gene Expression in Colorectal
322   Cancer Patients. *Med Oncol* 35:159. 10.1007/s12032-018-1220-6

323   Hu D, Jiang L, Luo S, Zhao X, Hu H, Zhao G, and Tang W. 2020. Development of an
324   autophagy-related gene expression signature for prognosis prediction in prostate cancer
325   patients. *J Transl Med* 18:160. 10.1186/s12967-020-02323-x

326   Jassal B, Matthews L, Viteri G, Gong C, Lorente P, Fabregat A, Sidiropoulos K, Cook J,
327   Gillespie M, Haw R, Loney F, May B, Milacic M, Rothfels K, Sevilla C, Shamovsky V,
328   Shorser S, Varusai T, Weiser J, Wu G, Stein L, Hermjakob H, and D'Eustachio P. 2020.
329   The reactome pathway knowledgebase. *Nucleic Acids Res* 48:D498-d503.
330   10.1093/nar/gkz1031

331   Kanehisa M, Furumichi M, Sato Y, Ishiguro-Watanabe M, and Tanabe M. 2020. KEGG:
332   integrating viruses and cellular organisms. *Nucleic Acids Res*. 10.1093/nar/gkaa970

333   Kutmon M, van Iersel MP, Bohler A, Kelder T, Nunes N, Pico AR, and Evelo CT. 2015.
334   PathVisio 3: an extendable pathway analysis toolbox. *PLoS Comput Biol* 11:e1004085.
335   10.1371/journal.pcbi.1004085

336 Lebovitz CB, Robertson AG, Goya R, Jones SJ, Morin RD, Marra MA, and Gorski SM. 2015.
337   Cross-cancer profiling of molecular alterations within the human autophagy interaction
338   network. *Autophagy* 11:1668-1687. 10.1080/15548627.2015.1067362
339 Lee YK, and Lee JA. 2016. Role of the mammalian ATG8/LC3 family in autophagy: differential
340   and compensatory roles in the spatiotemporal regulation of autophagy. *BMB Rep* 49:424-
341   430.
342 Lei Y, Tang L, Hu J, Wang S, Liu Y, Yang M, Zhang J, and Tang B. 2020. Inhibition of
343   MGMT-mediated autophagy suppression decreases cisplatin chemosensitivity in gastric
344   cancer. *Biomed Pharmacother* 125:109896. 10.1016/j.biopha.2020.109896
345 Liu PF, Leung CM, Chang YH, Cheng JS, Chen JJ, Weng CJ, Tsai KW, Hsu CJ, Liu YC, Hsu
346   PC, Pan HW, and Shu CW. 2014. ATG4B promotes colorectal cancer growth
347   independent of autophagic flux. *Autophagy* 10:1454-1465. 10.4161/auto.29556
348 Loncle C, Molejon MI, Lac S, Tellechea JI, Lomberk G, Gramatica L, Fernandez Zapico MF,
349   Dusetti N, Urrutia R, and Iovanna JL. 2016. The pancreatitis-associated protein VMP1, a
350   key regulator of inducible autophagy, promotes Kras(G12D)-mediated pancreatic cancer
351   initiation. *Cell Death Dis* 7:e2295. 10.1038/cddis.2016.202
352 Mathew R, Karantza-Wadsworth V, and White E. 2007. Role of autophagy in cancer. *Nat Rev*
353   *Cancer* 7:961-967. 10.1038/nrc2254
354 Mizushima N, Yoshimori T, and Ohsumi Y. 2011. The role of Atg proteins in autophagosome
355   formation. *Annu Rev Cell Dev Biol* 27:107-132. 10.1146/annurev-cellbio-092910-154005
356 Nagtegaal ID, Odze RD, Klimstra D, Paradis V, Rugge M, Schirmacher P, Washington KM,
357   Carneiro F, and Cree IA. 2020. The 2019 WHO classification of tumours of the digestive
358   system. *Histopathology* 76:182-188. 10.1111/his.13975
359 Nakatogawa H. 2013. Two ubiquitin-like conjugation systems that mediate membrane formation
360   during autophagy. *Essays Biochem* 55:39-50. 10.1042/bse0550039
361 OA M, W J, SR G, CL H, DDH T, M P, DL D, AS G, I G, and Autophagy GGJ. 2020.
362   Transgenic expression of GFP-LC3 perturbs autophagy in exocrine pancreas and acute
363   pancreatitis responses in mice.1-14. 10.1080/15548627.2020.1715047
364 Pei L, Kong Y, Shao C, Yue X, Wang Z, and Zhang N. 2018. Heme oxygenase-1 induction
365   mediates chemoresistance of breast cancer cells to pharmorubicin by promoting
366   autophagy via PI3K/Akt pathway. *J Cell Mol Med*. 10.1111/jcmm.13800
367 PF L, KL T, CJ H, WL T, JS C, HW C, CW S, YG G, HH T, CH W, JC R, LW Y, and
368   Theranostics SCJ. 2018. Drug Repurposing Screening Identifies Tioconazole as an ATG4
369   Inhibitor that Suppresses Autophagy and Sensitizes Cancer Cells to Chemotherapy.
370   8:830-845. 10.7150/thno.22012
371 Rahman MA, Saha SK, Rahman MS, Uddin MJ, Uddin MS, Pang MG, Rhim H, and Cho SG.
372   2020. Molecular Insights Into Therapeutic Potential of Autophagy Modulation by Natural
373   Products for Cancer Stem Cells. *Front Cell Dev Biol* 8:283. 10.3389/fcell.2020.00283

374   Rhodes DR, Yu J, Shanker K, Deshpande N, Varambally R, Ghosh D, Barrette T, Pandey A, and
375   Chinnaiyan AM. 2004. ONCOMINE: a cancer microarray database and integrated data-
376   mining platform. *Neoplasia* 6:1-6.
377   Su B, Zhang L, Liu S, Chen X, and Zhang W. 2019. GABARAPL1 Promotes AR+ Prostate
378   Cancer Growth by Increasing FL-AR/AR-V Transcription Activity and Nuclear
379   Translocation. *Front Oncol* 9:1254. 10.3389/fonc.2019.01254
380   Sun WL, Wang L, Luo J, Zhu HW, and Cai ZW. 2018. Ambra1 modulates the sensitivity of
381   breast cancer cells to epirubicin by regulating autophagy via ATG12. *Cancer Sci*
382   109:3129-3138. 10.1111/cas.13743
383   Tang JY, Hsi E, Huang YC, Hsu NC, Chen YK, Chu PY, and Chai CY. 2013. ATG9A
384   overexpression is associated with disease recurrence and poor survival in patients with
385   oral squamous cell carcinoma. *Virchows Arch* 463:737-742. 10.1007/s00428-013-1482-5
386   Tomczak K, Czerwinska P, and Wiznerowicz M. 2015. The Cancer Genome Atlas (TCGA): an
387   immeasurable source of knowledge. *Contemp Oncol (Pozn)* 19:A68-77.
388   10.5114/wo.2014.47136
389   Van Cutsem E, Sagaert X, Topal B, Haustermans K, and Prenen H. 2016. Gastric cancer. *Lancet*
390   388:2654-2664. 10.1016/s0140-6736(16)30354-3
391   Wan B, Liu B, Yu G, Huang Y, and Lv C. 2019. Differentially expressed autophagy-related
392   genes are potential prognostic and diagnostic biomarkers in clear-cell renal cell
393   carcinoma. *Aging (Albany NY)* 11:9025-9042. 10.18632/aging.102368
394   Wu J, Li W, Ning J, Yu W, Rao T, and Cheng F. 2019. Long noncoding RNA UCA1 targets
395   miR-582-5p and contributes to the progression and drug resistance of bladder cancer cells
396   through ATG7-mediated autophagy inhibition. *Onco Targets Ther* 12:495-508.
397   10.2147/ott.S183940
398   Xu R, Liu S, Chen H, and Lao L. 2016. MicroRNA-30a downregulation contributes to
399   chemoresistance of osteosarcoma cells through activating Beclin-1-mediated autophagy.
400   *Oncol Rep* 35:1757-1763. 10.3892/or.2015.4497
401   Yang SW, Ping YF, Jiang YX, Luo X, Zhang X, Bian XW, and Yu PW. 2016. ATG4A promotes
402   tumor metastasis by inducing the epithelial-mesenchymal transition and stem-like
403   properties in gastric cells. *Oncotarget* 7:39279-39292. 10.18632/oncotarget.9827
404   Yang Y, Li X, Wang T, Guo Q, Xi T, and Zheng L. 2020. Emerging agents that target signaling
405   pathways in cancer stem cells. *J Hematol Oncol* 13:60. 10.1186/s13045-020-00901-6
406   Yusefi AR, Bagheri Lankarani K, Bastani P, Radinmanesh M, and Kavosi Z. 2018. Risk Factors
407   for Gastric Cancer: A Systematic Review. *Asian Pac J Cancer Prev* 19:591-603.
408   10.22034/apjcp.2018.19.3.591

409

Figure 1

Schematic of autophagy pathway.

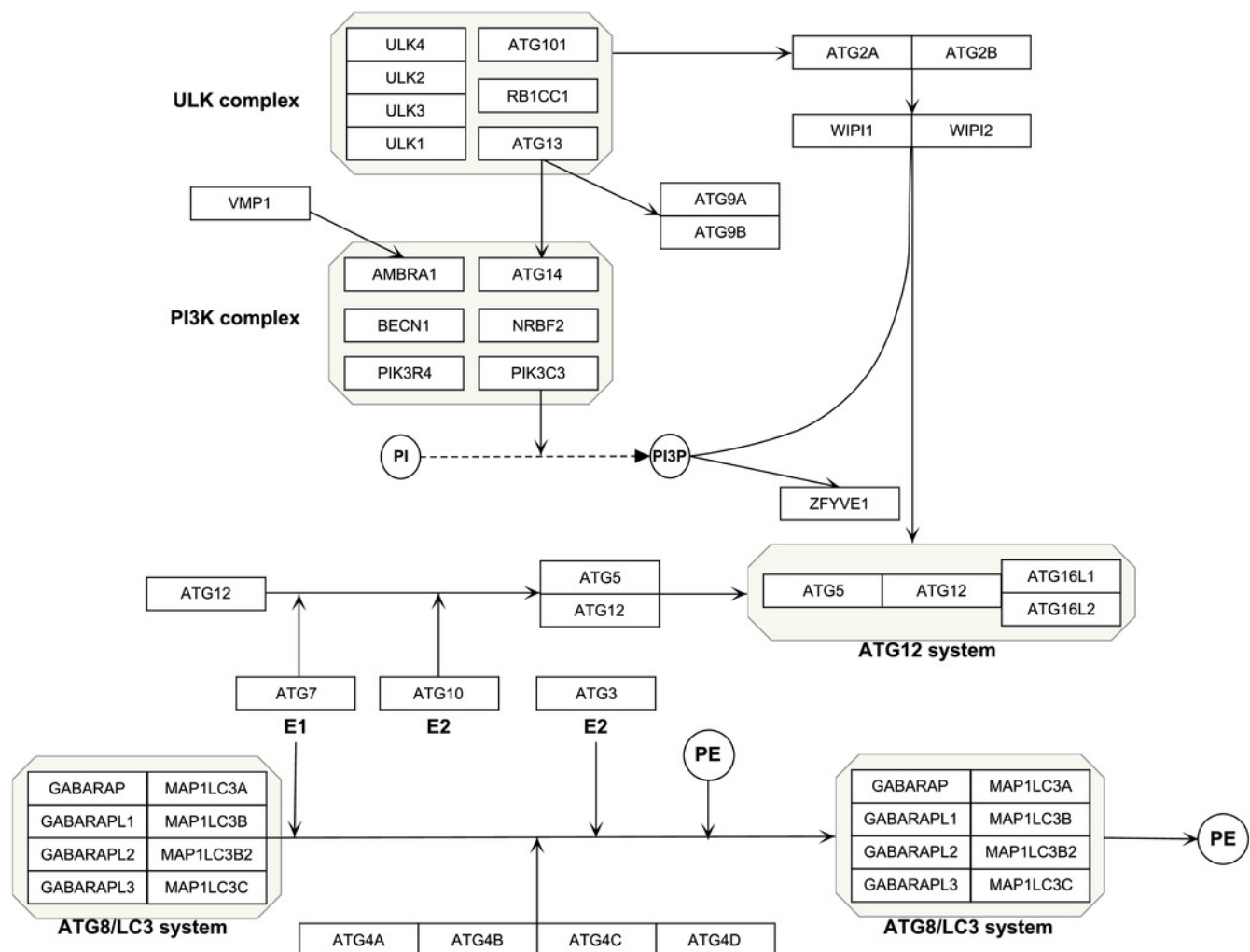



Figure 2

Different ATGs mRNA expression in different tumor types.

This graphic showed the numbers of datasets with statistically signifcant mRNA overexpression (red) or downexpression (blue) of the target gene (cancer vs. normal tissue). Cell color is determined by the best gene rank percentile for the analyses within the cell.

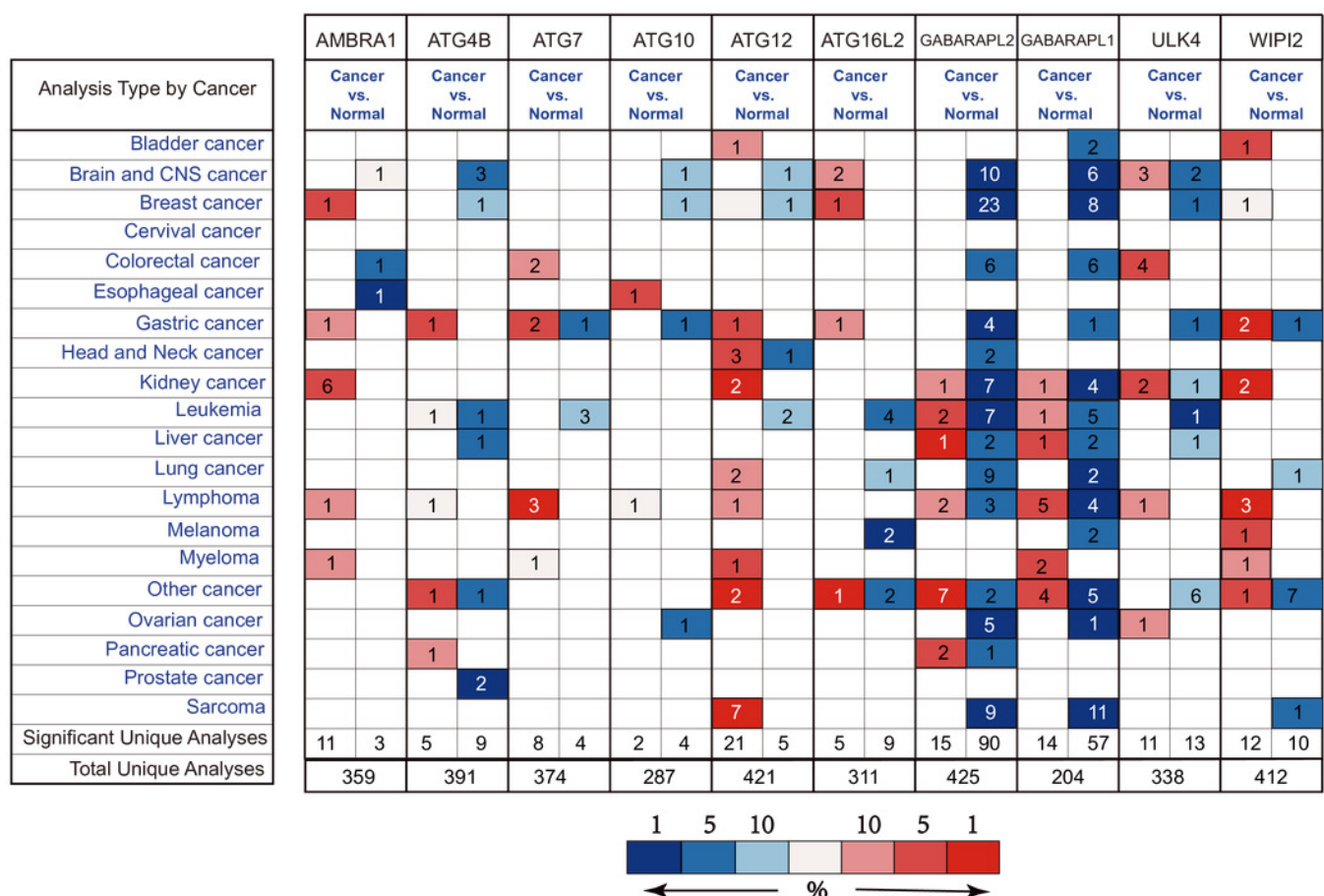



Figure 3

The detail information for the different ATGs.

(A)The detail information in the oncomine dataset. (B)The position of different ATGs in autophagy pathway. The blue color represents downexpression in cancer, while the red color represents overexpression in cancer. The gene with two different colors means two probe of the gene showed different expressions. The gradient color represents the gene's fold change.

A

gene	probe	Types of GC vs. gastric	Fold change	P value	Ref
AMBRA1	52731_at	Gastric Mixed Adenocarcinoma	2.218	0.0004	
ATG4B	212280__x_at	Gastric Mixed Adenocarcinoma	2.089	1E −06	
	224025_s_at	Gastric Mixed Adenocarcinoma	2.724	4E −05	
ATG7	224025_s_at	Gastric Intestinal Type Adenocarcinoma	2.288	5E −06	
	1569827_at	Gastric Mixed Adenocarcinoma	−3.115	9E −05	
ATG10	243477_at	Diffuse Gastric Adenocarcinoma	−2.267	0.005	DErrico Gastric
ATG12	204833_at	Gastric Mixed Adenocarcinoma	6.11	0.0001	
ATG16L2	229389_at	Diffuse Gastric Adenocarcinoma	2.634	0.004	
GABARAPL3	211458_s_at	Gastric Intestinal Type Adenocarcinoma	−2.461	1E −06	
ULK4	232206_at	Diffuse Gastric Adenocarcinoma	−2.151	0.004	
	202031_s_at	Gastric Intestinal Type Adenocarcinoma	2.28	6E −11	
WIPI2	202031_s_at	Gastric Cancer	2.17	3E −05	Wang Gastric
	1570033_at	Gastric Cancer	−2.865	0.005	
	LMN_215181	Gastric Intestinal Type Adenocarcinoma	−2.321	8E −07	
GABARAPL1	LMN_215181	Gastric Adenocarcinoma	−2.26	0.0002	Cho Gastric
	LMN_215181	Gastric Mixed Adenocarcinoma	−2.622	1E −05	
	LMN_215181	Diffuse Gastric Adenocarcinoma	−2.287	4E −06	

B

Figure 4

The prognostic value of mRNA level of independent prognostic factors.

(A)ATG4D. (B)MAP1LC3C.

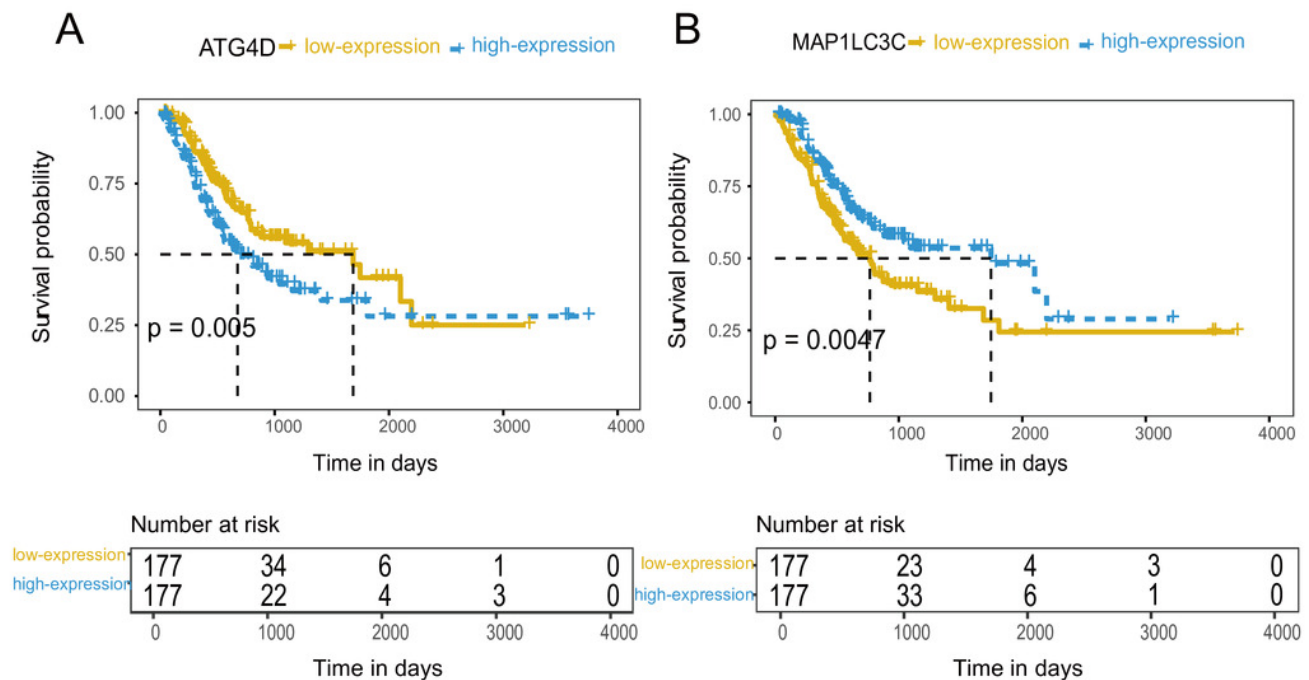



Figure 5

Summary of the correlation between *ATG* genes expression and TNM staging and prognosis.

The red frame represents genes with high significant expression, and the blue frame represents genes with low significant expression. "**" represents that gene expression has significant correlation with TNM staging or OS of GC.

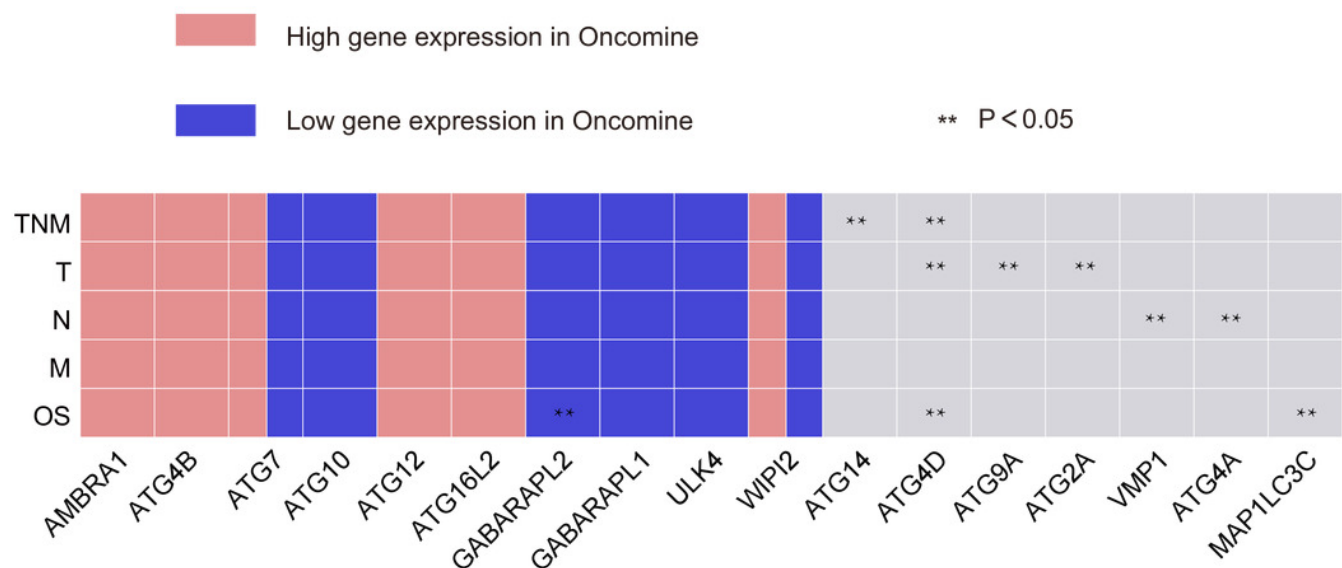
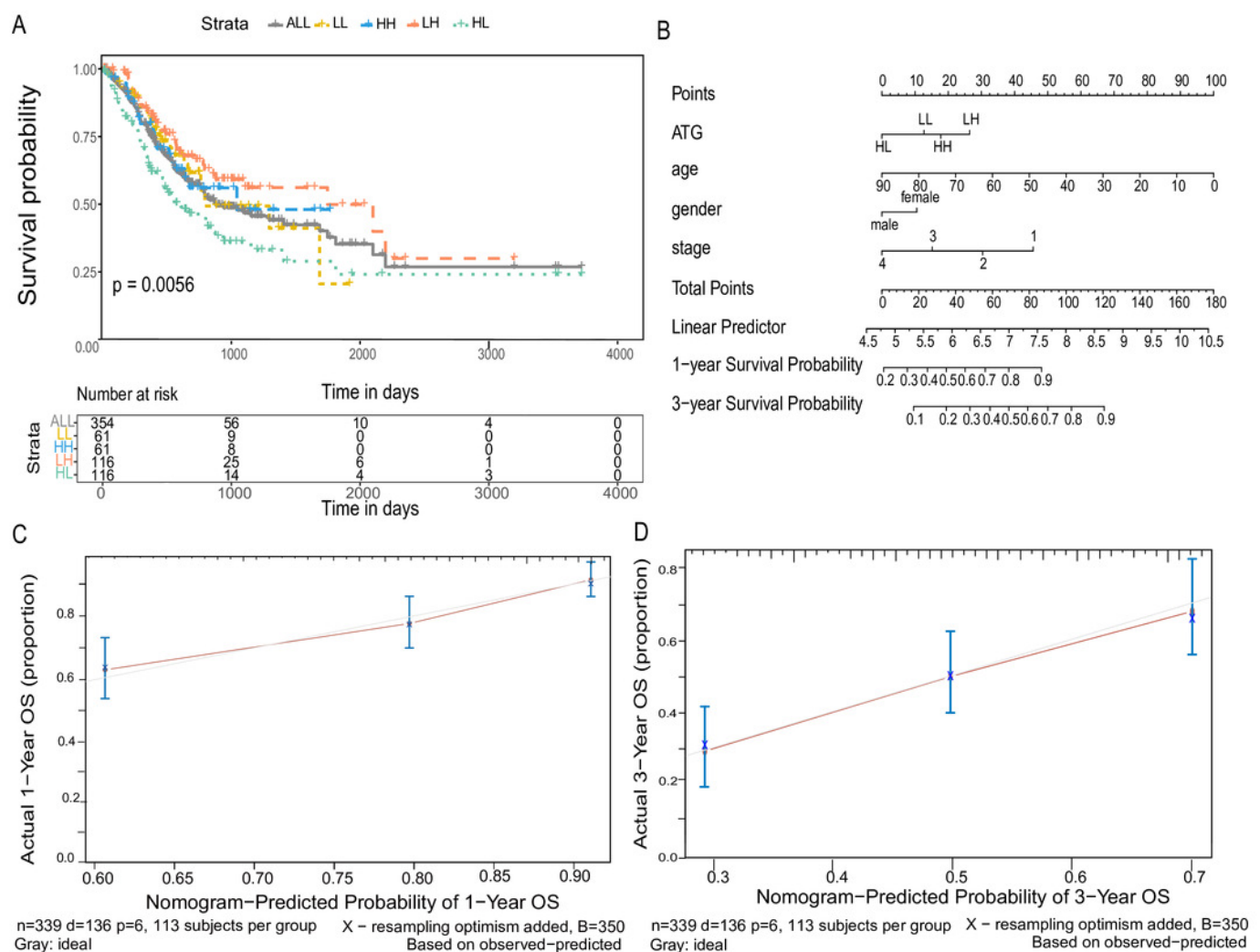



Figure 6

Joint predictive the patients prognosis using ATG4D and MAP1LC3C.

(A)The Kaplan-Meier plot for the four groups. (B)The nomogram for indicating the one- and three-year overall survival in patients with gastric cancer. (C)The calibration curve of nomogram for predicting overall survival at one year. (D)The calibration curve of nomogram for predicting overall survival at three year.

Description of autophagy related gene

	gene symbol	Aliases	function
ULK complex	ULK1/2/3/4	ATG1A/B/C/D	Acts upstream of PIK3C3 to regulate the formation of autophagophores
	ATG101	C12orf44	Stabilizes ATG13, protecting it from proteasomal degradation.
	ATG13	KIAA0652	essential for autophagosome formation
	RB1CC1	ATG17	direct interaction with Atg16L1
PI3K complex	BECN1	ATG6	Acts as core subunit of the PI3K complex
	PIK3R4	VPS15	Involved in regulation of degradative endocytic trafficking
	PIK3C3	VPS34	Catalytic subunit of the PI3K complex
	NRBF2	COPR	modulated ATG14 protein
	ATG14	ATG14L	Plays a role in autophagosome formation and MAP1LC3/LC3 conjugation to phosphatidylethanolamine
	AMBRA1	DCAF3	interacts with becn1
ubiquitin-like conjugating system	ATG12	APG12	Conjugation with ATG5
	ATG5	APG5	functions as an E1-like activating enzyme
	ATG16L1/L2	ATG16A/B	interacts with ATG12-ATG5 to mediate the conjugation of phosphatidylethanolamine (PE) to LC3
	ATG3	APG3	E2 conjugating enzyme
	ATG4A/B/C/D	APG4A/B/C/D	Cleaves the C-terminal amino acid of ATG8 family proteins to reveal a C-terminal glycine
	ATG7	APG7	E1-like activating enzyme
	ATG10	APG10	E2-like enzyme
	GABAR	ATG8A/B/C	Ubiquitin-like modifier

others	AP/L1/L2/L3	/D	
	MAP1LC3A/B/B2/C	ATG8E/F/G/J	Ubiquitin-like modifier
	WIPI1/2	ATG18A/B	Functions upstream of the ATG12-ATG5-ATG16L1 complex and LC3, and downstream of the ULK1 and PI3-kinase complexes
	ATG9A/B	APG9L1/L2	transmembrane protein
	ATG2A/B	/	Required for both autophagosome formation
	ZFYVE1	DFCP1	PI3P-binding FYVE-containing protein
	VMP1	EPG3, TANGO5, TMEM49	Plays a role in the initial stages of the autophagic process through its interaction with BECN1

The association between autophagy related gene and TNM stage

Gene symbol		TCGA			GSE62254		
		TNM					
		I-II	III-IV	*P*	I-II	III-IV	*P*
ATG14	low	96	80		72	76	
	high	71	105	0.00761	54	96	**0.02711**
ULK3	low	71	100		61	89	
	high	96	85	0.03	65	83	0.568
ATG2B	low	95	85		62	86	
	high	72	100	0.04032	64	86	0.8923
ATG12	low	96	83		58	91	
	high	71	102	0.018	68	81	0.241
ATG4C	low	94	84		65	83	
	high	73	101	0.041	61	89	0.57
ATG4D	low	70	100		55	95	
	high	97	85	0.022	71	77	**0.04822**
MAP1LC3A	low	71	105		65	83	
	high	96	80	0.0076	61	89	0.57
		T					
		T1T2	T3T4	P	T1T2	T3T4	P
WIPI1	low	41	147		101	49	
	high	58	121	0.022	87	63	0.0947
ATG9A	low	39	143		80	70	
	high	60	125	0.018	108	42	**0.00083**
ATG2B	low	42	145		95	85	
	high	57	123	0.047	93	57	0.092
ATG2A	low	40	141		82	68	

				P			P
	high	59	127	0.038	106	44	**0.00417**
ATG4D	low	39	141		83	67	
	high	60	127	0.025	105	45	**0.00864**
ATG7	low	40	146		90	60	
	high	59	122	0.017	98	52	0.3396

		N					
		N0	!N0	P	N0	!N0	P
PIK3R4	low	66	116		18	132	
	high	45	130	0.031	20	130	0.728
VMP1	low	65	116		28	122	
	high	46	130	0.046	10	140	**0.00178**
ATG12	low	66	113		16	134	
	high	45	133	0.018	22	128	0.2976
ATG4A	low	68	115		26	124	
	high	43	131	0.0111	12	138	**0.01509**

		M					
		M0	M1	P	M0	M1	P
ULK4	low	160	19		137	13	
	high	170	6	0.008	136	14	0.8401
MAP1LC3B	low	174	7		139	11	
	high	156	18	0.0171	134	16	0.3131

Prognosis analysis of autophagy related gene in TCGA datasets

	Univirable analysis		Multivanable analysis	
	HR(95CI)	*P*	HR(95CI)	*P*
ATG4D	1.602(1.153-2.225)	**0.00493**	1.5727(1.1194-2.21)	**0.009058**
GABARAPL2	0.6925(0.499-0.9609)	**0.0279**	0.7855(0.5597-1.102)	0.162447
MAP1LC3C	0.6242(0.4488-0.8682)	**0.00511**	0.5767(0.4086-0.8138)	**0.00173**